ASSOCIATION OF CHRONIC INFLAMMATION AND PERCEIVED STRESS WITH ABNORMAL FUNCTIONAL CONNECTIVITY IN BRAIN AREAS INVOLVED WITH INTEROCEPTION IN HEPATITIS C PATIENTS.

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ABSTRACT

Background: Sickness behavioral changes elicited by inflammation may become prolonged and dysfunctional in patients with chronic disease, such as chronic hepatitis C (CHC). Neuroimaging studies show that the basal ganglia and insula are sensitive to systemic inflammation.

Aim: To elucidate the clinical and neurobiological aspects of prolonged illnesses in patients with CHC.

Methods: Thirty-five CHC patients not treated with interferon-α or other antiviral therapy, and 30 control subjects matched for age and sex, were evaluated for perceived stress (perceived stress scale; PSS), depression (PHQ-9), fatigue and irritability through a visual analog scale (VAS), as well as serum levels of interleukin-6 (IL-6), prostaglandin E_2 (PGE_2) and oxidative stress markers. Functional MRI was performed, measuring resting-state functional connectivity using a region-of-interest (seed)-based approach focusing on the bilateral insula, subgenual anterior cingulate cortex and bilateral putamen. Between-group differences in functional connectivity patterns were assessed with two-sample t-tests, while the associations between symptoms, inflammatory markers and functional connectivity patterns were analyzed with multiple regression analyses.

Results: CHC patients had higher PSS, PHQ-9 and VAS scores for fatigue and irritability, as well as increased IL-6 levels, PGE_2 concentrations and antioxidant system activation compared to controls. PSS scores positively correlated with functional connectivity between the right anterior insula and right putamen, whereas PHQ-9 scores correlated with functional connectivity between most of the seeds and the right anterior insula. PGE_2 (positively) and IL-6 (negatively) correlated with functional connectivity between the right anterior insula and right caudate nucleus and between the right ventral putamen and right putamen/globus pallidus. PGE_2 and PSS scores accounted for 46% of the variance in functional connectivity between the anterior insula and putamen.

Conclusions: CHC patients exhibited increased perceived stress and depressive symptoms, which were associated with changes in inflammatory marker levels and in functional connectivity between the insula and putamen, areas involved in interoceptive integration, emotional awareness, and orientation of motivational state.

1. Introduction

Sickness behavior is a highly organized adaptive strategy to support the organism's defense against pathogens, and is characterized by changes in behavior, mood and cognition (Dantzer, 2001a; Garcia et al., 1955; Miller and Raison, 2016; Stieglitz, 2015).

The experience of "feeling sick" is common during acute infections or inflammatory responses to trauma (Hart, 1988; Miller and Raison, 2016). It clinically presents as a set of neurovegetative symptoms such as fatigue, anorexia, psychomotor retardation and increased sensitivity to pain, and is also associated with increased irritability, anhedonia, social responsiveness and increased stress sensitivity (Capuron and Miller, 2004; Dantzer et al., 2008; Maes et al., 2012). Animal and human studies suggest that soluble mediators, such as the pro-inflammatory cytokines interleukin- (IL-) 1, IL-6 and tumor necrosis factor-α (TNF-α), play a direct role in the development of sickness-related behaviors (Aubert et al., 1997; Avitsur et al., 1997; Dantzer, 2001b; Hart, 1988; Kent et al., 1996, 1992). Moreover, it has been observed that peripheral immunological activation may drive inflammation in the central nervous system, involving neurons, astrocytes and the microglia (Dantzer, 2009; Haroon et al., 2012; Miller et al., 2013).

Furthermore, neuroimaging studies have indicated that cortical and sub-cortical brain structures might play a relevant role in sickness behavior, identifying the insula, subgenual anterior cingulate cortex (sgACC) and basal ganglia, particularly the ventral striatum and substantia nigra (Harrison, 2017), as sensitive to peripheral inflammation. These studies involved inducing acute inflammation through the direct inoculation of endotoxins, such as the *Salmonella typhi* vaccine or lipopolysaccharide (LPS), or patients receiving treatment with the

pro-inflammatory cytokine interferon- (IFN-) α (Capuron et al., 2012; Eisenberger et al., 2011; Harrison et al., 2009; Udina et al., 2012). Situations in which the initial noxious stimulus cannot be removed could lead to prolonged and dysfunctional sickness behavior. Examples of this include chronic infections (i.e., human immunodeficiency virus (HIV) or hepatitis C virus (HCV) infections), auto-immune disorders (i.e., rheumatoid arthritis or inflammatory bowel disease) or chronic inflammatory conditions (i.e., cancer, diabetes or obesity), which have been often linked to an increased prevalence of depression (Liu et al., 2017). In this regard, increased perceived stress, fatigue, and irritability, which are also common in depressed patients (Chung et al., 2015; Farabaugh et al., 2004; Fava et al., 2010), have often been observed in chronic inflammatory conditions such as rheumatic diseases (Louati and Berenbaum, 2015), obesity (Capuron et al., 2016), cancer (Bower and Lamkin, 2013), and inflammatory bowel disease (Targownik et al., 2015).

Major depressive disorder (MDD) displays a phenomenological overlap with sickness behavior, and has been consistently associated with increased levels of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and acute-phase proteins (such as the C-reactive protein) (Haapakoski et al., 2015; Kohler et al., 2016). Moreover, brain structural and functional alterations have been identified in several neuroimaging studies on depression, mainly in the prefrontal-limbic-subcortical areas that are involved in emotional processing and awareness, similar to those involved during acute inflammatory challenges (Drevets et al., 2008; Feng et al., 2016; Harrison, 2017; Mulders et al., 2015; Savitz and Harrison, 2018; Savitz and Drevets, 2009). However, the type of symptoms and illness course differ between MDD and sickness behavior. Typically, MDD is considered a lifetime progressive disease, which differs from the acute and adaptive nature of sickness behavior (Freeman et al., 2017; Oriolo et al., 2018).

Moreover, depression can involve biological pathways that are different from those associated with acute pro-inflammatory cytokine stimulation, such as cell-mediated immune

activation, dysregulated anti-inflammatory mechanisms, neural sensitization to immune responses or auto-immunity processes (Dantzer et al., 2008). Importantly, the activation of oxidative and nitrosative stress (O&NS) pathways, resulting in increased levels of reactive oxygen and nitrogen species (ROS and RNS, respectively) that damage lipids, proteins and DNA, may be crucial in the chronic and progressive course of depression (Liu et al., 2015; Moylan et al., 2013).

Thus, as sickness behavior and depression share clinical phenomenology, inflammatory pathways and brain functional changes, it has been hypothesized that prolonged and dysregulated sickness behavior may contribute to the development of MDD in vulnerable patients (Capuron and Castanon, 2012; Rosenblat et al., 2014). Some studies in chronic hepatitis C (CHC) patients have tried to elucidate the neurobiological and neuroanatomical links between chronic inflammatory conditions and prolonged sickness behavior, excluding subjects with current severe mental illness and considering several ranges of neuropsychiatric symptoms such as depression, anxiety, fatigue or cognition (Aregay et al., 2018; Huckans et al., 2014; Loftis and Hauser, 2008). Depression is the leading cause of disability worldwide (World Health Organization, 2017), affecting more than 300 million people, a substantial proportion of whom do not respond adequately to current pharmacological therapies (Rush et al., 2006; Stotland, 2012); therefore, understanding the pathophysiological mechanisms linking inflammation to sickness and MDD seems to be crucial in developing new therapeutic targets (Udina et al., 2015, 2014). Moreover, CHC is a well-known systemic disease with a plethora of extrahepatic manifestations such as chronic kidney disease, mixed cryoglobulinemia, increased rates of insulin resistance, diabetes, and atherosclerosis, increased cardiovascular morbidity and neuropsychiatric symptoms, among others (Grignoli et al., 2015). Accordingly, the purpose of this study was to elucidate the clinical and neurobiological correlates of a prolonged sickness condition associated with chronic inflammation in a case-control study of patients with CHC

not treated with IFN-α or others antiviral therapies, and without MDD. We hypothesized that chronic low-grade inflammation secondary to CHC can induce alterations in brain connectivity in areas associated with interoceptive integration and awareness, emotional processing and orientation of motivational state, with such alterations correlating with some aspects of sickness behavior.

2. Materials and Methods

2.1 Participants

Fifty-one Caucasian outpatients aged between 18 and 55 years with CHC who were candidates for antiviral treatment, either with the pegylated IFN-α and ribavirin (RBV) combination or with the new direct-acting antivirals (DAA), were recruited between 2014 and 2016 at the Liver Units of two general university hospitals (Hospital Clínic and Hospital del Mar) in Barcelona. None of the patients had previously received anti-viral treatment (pegylated IFN-α or DAA). The exclusion criteria for the study were as follows: not fluent in Spanish language, the presence of other concomitant liver diseases, decompensated cirrhosis or hepatocarcinoma, HIV or HBV co-infection, any chronic disease or inflammatory condition (e.g., diabetes, asthma, obesity (body mass index ≥ 30) or cancer), auto-immune diseases (e.g., rheumatoid arthritis), any lifetime neurological disease or major psychiatric disorders (psychosis or bipolar disorder), any depressive or anxiety disorders until the preceding year, any drug or alcohol use disorder (except tobacco use) up until the preceding year, and the presence of metallic protheses or pacemakers. Patients were also excluded if they presented an uncontrolled medical condition or were receiving any anti-inflammatory treatment (e.g., corticoids, statins, non-steroidal anti-inflammatory drugs or antidepressant/anxiolytic drugs in the last six months). Moreover, 31 control participants without HCV infection, who were

matched for age, sex and laterality, were also recruited in the same time period at the Human Pharmacology unit, using the same exclusion criteria outlined above. Controls received a monetary reward to cover time and travel expenses.

After obtaining informed consent, all the participants underwent a detailed medical history check, routine laboratory tests and physical examinations to determine whether they met the inclusion criteria. Case patients were checked for HCV genotype, source of infection, viral load and grade of liver fibrosis (by means of a liver biopsy, indirect tests of fibrosis, ultrasound examination and/or transient elastography when available). Control participants were checked for anti-HCV antibodies, to ensure the absence of any HCV exposure. All subjects were interviewed by a senior psychiatrist using the Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998) to assess for current or past psychiatric disorders. Nine patients were excluded due to the presence of current psychiatric disorders. Five patients dropped out as they did not consent to the laboratory and fMRI assessments. Finally, two cases were excluded from the analyses due to excessive head motion during fMRI acquisition. One control subject was excluded due to non-optimal data acquisition. A final sample of 65 participants, 35 patients with CHC and 30 controls, were studied.

Clinical history and sociodemographic variables were collected for all the participants. The institutional review boards approved the study protocol (CEIC of Hospital Clínic and Hospital del Mar), which followed the tenets of the Declaration of Helsinki. All the participants were recruited after providing proper written informed consent.

2.2 Behavioral assessment

The validated Spanish version of Patient Health Questionnaire 9 (PHQ-9) (Diez-Quevedo et al., 2001) was used to evaluate the subthreshold symptoms of depression. PHQ is

a brief instrument that covers a wide range of psychopathology and is used to diagnose specific disorders, with the items corresponding to the symptom criteria for each disorder as outlined in the DSM-IV-TR (Kroenke et al., 2010a). Furthermore, PHQ has been validated across a variety of medical conditions in primary care settings, including CHC patients (Navinés et al., 2012; Spitzer et al., 1999). PHQ-9 has nine items with four response options ("not at all", "several days", "more than half the days" and "nearly every day") rated from 0 to 3. It can be used as a continuous measure, with scores ranging from 0 to 27 and the cut-off points of 5, 10, 15 and 20 representing mild, moderate, moderately severe and severe levels of depressive symptoms. Patients reporting "more than half the days" for 5 or more of the 9 items of PHQ-9 and presenting a depressed mood or anhedonia were considered to have MDD (Kroenke et al., 2010b). Those reporting "more than half the days" in the past two weeks for two, three or four of the items were considerd to have another depressive disorder (Navinés et al., 2012).

The intensity of fatigue and irritability was assessed through a visual analog scale (VAS-f and VAS-i) (Folstein and Luria, 1973) which is a visual tool in which the patient is asked to place an arrow on a line that ranges from 0 to 100 mm from left to right (0 = no fatigue or no irritability and 100 = severe fatigue or extreme irritability). VAS is a well-validated scale that can be used to determine illness severity and can be rapidly self-administered (Killgore, 1999). We used irritability and fatigue scores as they reflect the components of sickness behavior and depression that are not exhaustively covered by PHQ-9.

The perceived stress scale (PSS) is easy to use and provides valuable additional information about the relationship between perceived stress and pathology (Cohen, 1983). It includes 14 items scored on a 5-point Likert scale (0-4) with the total score ranges from 0 to 56, and can be administered in a few minutes (Remor, 2006). It has been used in different studies (He et al., 2014; Nagano et al., 2004; Vere et al., 2009) addressing stress in patients with

liver diseases, as stress has been linked to the initiation, course and outcome of liver disease (Vere et al., 2009).

2.3 Biological measurements

Blood samples (10 ml of venous blood) for measuring serum concentrations of inflammatory markers were obtained at the same day as the behavioral assessment was performed, and no more than 5 days before the image acquisition. Samples were collected at 09.00 in the morning after overnight fasting and were centrifuged (10 min, 1,000 *g* at 4°C) after clotting and sera were stored at -80°C until analysis.

Enzyme-linked immunosorbent assays (ELISA) were used to identify and quantify the immunological biomarkers. IL-6 was quantified using the Human IL-6 High sensitivity ELISA kit (Diaclone®, Item 950.035.192). No dilution was performed, and the chromatography absorption peak was at 450 nm. The results are shown as pg/ml and the assay had a sensitivity of 0.81 pg/ml and an overall intra-assay coefficient of 4.4% (Cassidy et al., 2002; Mukhopadhyay et al., 2016; Pemberton et al., 2009). The inflammatory prostaglandin PGE_2 was quantified using the PGE_2 ELISA kit - Monoclonal (Cayman® Chemical, Item 514010). Samples were diluted 1:40 in ELISA Buffer and the chromatography absorption peak was at 412 nm. The results are shown as pg/ml and the assay had a detection limit of 15 pg/ml and an overall intra-assay coefficient of 8.8% (Lyons et al., 2014). The anti-inflammatory prostaglandin 15-deoxy-Δ-^{12,14}-prostaglandin J_2 (15d-PGJ_2) was quantified using the 15d-PGJ_2 ELISA kit - Monoclonal (ENZO®, Item ADI-900-023). Samples were diluted 1:4 in ELISA Buffer and the chromatography absorption peak was at 405 nm. The results are shown as pg/ml and the assay had a sensitivity of 36.8 pg/ml and an overall intra-assay coefficient of 6.2% (Wang et al., 2011). Enzymatic colorimetric assays were used to identify and quantify the

oxidative stress biomarkers. Superoxide dismutase (SOD) activity was quantified using the DetectX® Colorimetric Activity kit (Arbor Assays, Item K028-H1). Samples were diluted 1:10 in Assay Buffer and the chromatography absorption peak was at 450 nm. The results are shown as units per ml (U/ml), one SOD unit being the amount of enzyme required to inhibit the 50% reduction of superoxide radicals. The sensitivity of the assay was 0.044 U/ml and the overall intra-assay coefficient was 9.6% (MacDowell et al., 2016). Catalase (CAT) activity was quantified using the DetectX® Colorimetric Activity kit (Arbor Assays, Item K033-H1). Samples were diluted 1:20 in Assay Buffer and the chromatography absorption peak was at 560 nm. The results are shown as units per ml (U/ml), one CAT unit being the amount of enzyme required to degrade 1 μM of hydrogen peroxide per minute at 25ºC at a pH of 7.0. Sensitivity was determined as 0.052 U/ml and the overall intra-assay coefficient was 4.1% (Ruiz-Ojeda et al., 2016). Gluthatione peroxidase (GPx) activity was quantified using the Glutathione Peroxidase Assay kit (Cayman Chemical, Item 703102). Samples were diluted 1:4 in Sample Buffer and the chromatography absorption peak was at 340 nm. The results are shown as "activity of GPx" (nmol/min/ml), one GPx unit being the amount of enzyme required to oxidize 1 nM of NADPH into $NADP^+$ per minute at 25ºC. The intra-assay coefficient of variation was 5.7% and the dynamic range 50-344 nmol/min/ml (Ceballos-Picot et al., 1992). As an index of peroxidation of lipid components, malondialdehyde (MDA) levels were quantified without dilution using the TBARS Assay Kit (Cayman Chemical, Item 10009055), which is based on the reaction between thiobarbituric acid (TBA) and MDA. This produces the MDA-TBA complex (which is referred to as thiobarbituric acid reactive substances, TBARS), which presents a chromatography absorption peak at 530-540 nm. The results are shown as μM for the entire sample. The intra-assay coefficient of variation was 5.5% and the dynamic range 0-50 μM (Joshi et al., 2018).

Spectrophotometric analysis was conducted using the ELISA spectrophotometer Synergy 2 (BioTek®, USA) and the Gen5 Data Analysis Software (BioTek®, USA). Statistical analysis was carried out using the statistical software GraphPad Prism 6 (GraphPad Software, Inc., USA).

2.4 Functional magnetic resonance imaging and connectivity analysis

2.4.1 Image acquisition

Resting-state functional magnetic resonance (fMRI) connectivity explores the correlation and integration of brain activity between brain regions regardless of their anatomical connection. The connectivity is assessed by measuring the blood oxygenation level-dependent (BOLD) time series of activations in different brain regions in subjects at resting state (that is, no task is being performed) (Dennis and Thompson, 2014). In our study, images were obtained using a 1.5T Signa Excite system (General Electric, Milwaukee, WI, USA) equipped with an eight-channel phased-array head coil and single-shot echo planar imaging (EPI) software. The functional sequence consisted of gradient recalled acquisition in the steady state under the following parameters: time of repetition (TR), 2,000 ms; time of echo (TE), 50 ms; pulse angle, 90º; field of view (FOV), 24 cm; 64 x 64 pixel matrix; slice thickness, 4 mm plus an interslice gap of 1.5 mm. Twenty-two interleaved slices were acquired parallel to the anterior-posterior commissure line covering the whole brain. A 6-minute continuous resting-state scan was performed on each participant. Participants were instructed to relax, stay awake and lie still without moving, while keeping their eyes closed throughout. This scan generated 180 whole-brain EPI volumes. The first four (additional) images in each run were discarded to allow magnetization to reach equilibrium.

2.4.2 Image processing

Imaging data were processed in a Microsoft Windows platform using the Statistical Parametric Mapping software (SPM8; Wellcome Department of Imaging Neuroscience, London, UK; http://www.fil.ion.ucl.ac.uk/spm/) running on MATLAB (MathWorks Inc., Natick, MA, USA). Image preprocessing involved motion correction, spatial normalization and smoothing using a Gaussian filter (full-width at half-maximum, 8 mm). Functional images were normalized to the standard SPM EPI template and resliced to a 2-mm isotropic resolution in Montreal Neurological Institute (MNI) space. A high-pass filter set at 128 seconds was used to remove low-frequency drifts of less than aproximately 0.008 Hz. All image sequences were inspected for potential acquisition and normalization artifacts.

2.4.3 Control of potential head motion effects

To control for the effects of head motion, the following procedures were adopted. Conventional SPM time series alignment to the first image volume was undertaken in each participant and 12 motion-related regressors and estimates of global brain signal fluctuations were included as confounding variables in our first-level (single-subject) analyses. Furthermore, within-subject, censoring-based MRI signal artifact removal (scrubbing) (Power et al., 2014) was used to discard motion-affected volumes. For each participant, interframe motion measurements (head position variations in each brain volume compared to the previous volume) served as an index of data quality to flag volumes of poor quality across the run. At points with interframe motions > 0.2 mm, we discarded the corresponding volume, the volume immediately preceding it and the following two volumes. Finally, potential motion effects were removed using a summary measurement for each participant (mean interframe motion across the fMRI run) as a covariate in the second-level (group) analyses in SPM (Pujol et al., 2014a).

2.4.4 Functional connectivity analysis

Resting-state functional connectivity analysis can be performed in several ways, including seed-based, independent component analysis-based and/or cluster-based methods. In our study, it was assessed using a region-of-interest (seed)-based approach, as detailed previously (Harrison et al., 2013; Pujol et al., 2014b). In this approach, a brain region ("seed") of interest is selected and the time course of activation in that seed is extracted. Brain regions with strong positive correlations with the seed are defined as functionally coupled (Dennis and Thompson, 2014). We based our analysis on brain regions reported to be associated with systemic inflammation and mood changes in previous neuroimaging studies (Felger et al., 2015; Hanken et al., 2014; Labrenz et al., 2016; Seminowicz et al., 2004). Our *a priori* primary region of interest was the insula, which has an important role in interoceptive and emotional awareness, particularly in its anterior part (Craig, 2009). Selected secondary regions representative of our network of interest were the subgenual anterior cingulate cortex (sgACC), considered crucial in emotional processing and mood regulation, and the putamen, which together with the caudate nucleus forms the striatum, the main input structure of the basal ganglia presenting one of the highest metabolic activities in the brain (Wichmann and De Long, 2013). Two maps were obtained using ventral and dorsal striatal seeds to comprehensively assess its functional connectivity, as it is made up of distinct functional subdivisions. Relevantly, activity changes (e.g., glucose metabolism and functional connectivity) in both the insula and the anterior cingulate cortex have been associated with inflammatory markers (Hanken et al., 2014; Hannestad et al., 2012). Thus, a total of four functional connectivity MRI maps were generated using peak coordinates taken from previous studies that were converted into MNI in mm and located bilaterally at the anterior insula (x = ± 38, y = 25, z = 5), sgACC (x = 8, y = 17, z = -9), and ventral (x = ±20, y = 12, z = -3) and dorsal (x = ± 28, y = 1, z = 3) putamen.

For all the locations, seeds were defined as 3.5-mm radial spheres (sampling approximately 25 voxels) using the MarsBaR region-of-interest toolbox in MNI stereotaxic space (Brett et al., 2002). To generate the seed maps, the signal time course of a selected seed region was calculated as the average signal of the voxels included in the seed at each time point and was used as a regressor to be correlated with the signal time course of every voxel in the brain. The obtained voxel-wise regression coefficients served to build first-level SPM contrast images. This process was performed for each subject and seed separately. To remove potential sources of physiological noise, we derived estimates for white matter, CSF, global brain signal fluctuations and 12 motion-related regressors to be included as confounding ("nuisance") variables alongside the variables of interest in each individual (first-level) SPM analysis.

The resulting first-level contrast images for each participant were then included in second-level (group) random-effects analyses. One-sample t-statistic maps were generated to obtain the functional connectivity maps for each group, while two-sample t-tests were performed to map between-group differences for the contrasts: CHC patients > controls and CHC patients < controls. In addition, whole-brain voxel-wise analyses in SPM were performed to map the correlation between resting-state functional connectivity measurements in our regions of interest and inflammatory markers (i.e., PGE_2, IL-6 and 15d-PGJ_2 as independent regressors) and behavioral outcomes (i.e., PSS, PHQ-9, fatigue and irritability scores as independent regressors) in participants with CHC. To assess the influence of sickness behavior symptoms on the relationship between inflammation and functional connectivity, the correlation maps were re-estimated after covarying for the patients' clinical scores.

Finally, a multiple regression analysis was performed to assess the combined contribution of inflammatory markers and behavior to functional connectivity measurements in the patient group. Functional connectivity measurements were included as the dependent variable and the potential predictors were serum PGE_2 levels and PSS scores.

2.4.5 Thresholding criteria

To control for multiple comparisons within seed-based analyses, results were considered to be significant with clusters above 2.2 ml (277 voxels) at a height threshold of $p < 0.005$, which satisfied the family-wise error (FWE) rate of $P_{FWE} < 0.05$ at the cluster level according to Monte Carlo simulations. Resting-state fMRI data were also adjusted for multiple testing (accounting for seven variables) using Bonferroni correction (significant cluster size ≥ 3.2 ml).

2.5 Data and statistical analyses

Characteristics of the study sample were summarized using the mean and standard deviation (SD) for continuous variables and percentages for categorical variables. Sociodemographic features of the participants were compared between the groups using two-sample t-tests for continuous variables and chi-square tests for categorical variables. Behavioral assessment scores (PSS, PHQ-9, and VAS scores) were compared between the groups using multivariate analysis, controlling for age, sex and tobacco use, to evaluate possible interactions of these factors.

A Shapiro-Wilk test of normality was performed to determine the distribution of the biological marker variables (IL-6, PGE_2, 15d-PGJ_2, TBARS, GPx, SOD and CAT). Measurements that had > 3 SD above or below the mean were considered outliers and excluded from the analyses. Biomarker serum levels were log transformed if they were not normally distributed. Univariate analysis for independent samples was conducted (t-test) to compare values between the groups. Multivariate analyses controlling for age, sex and tobacco use were also performed.

Statistical analyses were undertaken with SPSS version 23.0. The tests of significance were two-tailed, with the degree of significance set at $p < 0.05$.

3. Results

3.1 Characteristics of the study participants

The study sample comprised 35 patients with CHC and 30 control subjects without HCV infection matched for sex, age and laterality. About two-thirds of the participants were male (66.1%) and the mean age of the study sample was 40.2 years (SD = 9.4; range 18-52) (**Table 1**). Genotype 1 was the most common HCV genotype (80%), whereas the route of HCV infection could not be ascertained in most of the cases (74.3%). The median of HCV RNA (viral load) was $1.5x10^6$ IE/mL (range=$3.0x10^5$-$6.3\ x10^6$). Only two patients (5.7%) had advanced of fibrosis and compensated cirrhosis (inclusion criteria for antiviral treatment; see **Table 1**). Furthermore, CHC patients more actively used tobacco than control subjects ($\chi^2 = 5.737$, $p = 0.017$).

In the whole sample, 21.5% of the individuals had a past history of depression/anxiety disorders up until one year before the start of the study. Among the CHC patients, 25.7% had a past history of psychiatric disorders, whereas 16.7% of the control sample had a past history of depression/anxiety. No other differences were found between the groups.

3.2 Biological markers

As illustrated in **Table 2**, CHC patients showed higher serum levels of the pro-inflammatory cytokines IL-6 and PGE_2 compared to control subjects, which was statistically

significant ($t = -4.352$, $p < 0.001$ and $t = -4.228$, $p < 0.001$, respectively). Regarding anti-inflammatory markers, significant differences were observed in the 15d-PGJ_2 serum levels ($t = -4.805$, $p < 0.001$), with CHC patients showing lower levels. The antioxidant enzymatic system was activated in CHC patients compared to controls, as highlighted by the increased serum levels of SOD ($t = -2.474$, $p = 0.016$) and CAT ($t = -4.328$, $p < 0.001$). GPx levels were not significantly increased in CHC patients ($t = 0.208$, $p = 0.836$). In line with these results, the serum levels of MDA-TBARS, a final product of lipid peroxidation and an indicator of oxidative stress, were lower in CHC patients than in control subjects ($t = -2.201$, $p = 0.032$), demonstrating the correct functioning of the antioxidant system.

When controlling for age, sex and active tobacco use, the differences in the biological marker serum levels between the groups remained significant after covariance (IL-6: $F = 15.379$, $p < 0.001$; PGE_2: $F = 16.506$, $p < 0.001$; 15d-PGJ_2: $F = 21.779$, $p < 0.001$; MDA-TBARS: $F = 5.764$, $p = 0.020$; SOD: $F = 4.582$, $p = 0.036$; CAT: $F = 18.344$, $p < 0.001$).

3.3 Behavioral assessment: PHQ-9, VAS-f, VAS-i and PSS scores

Total PHQ-9 scores were significantly higher in CHC patients than in healthy controls ($t = -2.914$, $p = 0.005$). As expected, the mean values observed could not be considered clinically relevant (see **Table 2**). After controlling for age, sex and tobacco use, this difference remained significant ($F = 5.883$, $p = 0.018$). Interestingly, the first item of PHQ-9, which evaluates anhedonia ("little interest or pleasure in doing things"), was also significantly higher in CHC patients ($t = -2.029$, $p = 0.047$). After categorical diagnosis using the PHQ-9 questionnaire, MDD was observed in 1 CHC patient; however, the clinical interview (MINI) was negative for current MDD. The difference with the control group was not statistically significant ($\chi^2 = 3.653$, $p = 0.118$).

Significant differences were observed in both the irritability (VAS-i) and fatigue (VAS-f) scores ($t = -3.484$, $p = 0.001$ and $t = -2.652$, $p = 0.01$, respectively), with the mean scores in CHC patients (**Table 2**) being in line with those previously reported (Udina et al., 2012). Finally, PSS scores were significantly increased in CHC patients compared to healthy controls ($t = -3.528$, $p = 0.002$). When controlled for age, sex and tobacco use, differences in the VAS-f, VAS-i and PSS total scores between the groups remained significant ($F = 8.374$, $p = 0.005$; $F = 4.793$, $p = 0.032$ and $F = 6.408$, $p = 0.014$, respectively), highlighting the effects of CHC on psychometric alterations.

3.4 Association between biological markers and clinical outcomes

The association between inflammation and clinical outcome related to sickness behavior was not confirmed, as no significant linear associations were found between the clinical scores and biological markers in CHC patients. Nevertheless, when considering the whole sample, significant positive correlations were observed between PGE_2 serum levels and the PHQ-9 total score ($r = 0.298$, $p = 0.019$), and between PGE_2 levels and PSS scores ($r = 0.245$, $p = 0.055$), whereas negative correlations were found between 15d-PGJ_2 and the VAS-i score ($r = -0.255$, $p = 0.042$) and between 15d-PGJ_2 and the VAS-f score ($r = -0.294$, $p = 0.018$). See **Table S1** for details.

3.5 Functional connectivity analysis and differences between the groups

Within-group maps. One-sample (group) seed maps corresponded to well-defined functional connectivity networks in both CHC patients and controls. Positive correlations were found between our regions of interest and cortical frontal areas (e.g., insula and operculum,

lateral prefrontal cortex, ACC, supplementary motor area) and ventral brain structures. Negative correlations were found with the seed regions mostly involved the medial prefrontal cortex, parietal areas (e.g., angular gyri, precuneus), occipital cortices and the cerebellum. **Figures S1 to S4** in the Supplementary Material illustrate the within-group functional connectivity maps. No substantial hemispheric differences were noted for any of the maps.

Between-group differences. A comparison of functional connectivity between groups identified few differences, as illustrated in **Figure 1**. Specifically, compared to control subjects in the contrast CHC < controls, patients showed a significant increase in the negative correlation (more anticorrelation) between the left dorsal putamen and left angular gyrus, and between the sgACC and the fusiform gyrus. Conversely, in the contrast CHC > controls, patients showed a significant increase in the positive correlation between the right ventral putamen and left frontal operculum, as well as a reduction in the negative correlation (lower anticorrelation) between the sgACC and precuneus. No significant differences were observed when controlling for tobacco use, as shown in **Table 3.**

3.6 Functional connectivity analysis and correlation with biological markers

In CHC patients, serum levels of PGE_2 showed a significant positive correlation with functional connectivity measurements between the right anterior insula and regions in the basal ganglia and related structures (more PGE_2 associated with more connectivity). Positive correlations were also observed with functional connectivity between the right putamen seeds and adjacent regions in the basal ganglia (see **Table S2**). **Figure 2** illustrates the main findings. No significant positive associations were observed for the left hemisphere seeds.

Regression analysis with IL-6 serum levels showed a significant negative correlation with functional connectivity measurements in the basal ganglia (more IL-6 associated with less

connectivity) in both the insula and the dorsal and ventral putamen seed maps only for the right hemisphere (**Figure 3** and **Table S3**). Of note, functional connectivity between the anterior insula and the right caudate nucleus, as well as between the ventral putamen and the right putamen/globus pallidus (**Figure 3**, top and bottom rows), was positively associated with increasing PGE_2 levels and negatively associated with increasing IL-6 levels. **Figure 4** shows the scatter plots illustrating these correlations.

Finally, no significant correlations were found between the serum levels of the anti-inflammatory marker 15d-PGJ_2 and functional connectivity of our regions of interest.

After adjusting for clinical variables, the associations between functional connectivity and the inflammatory markers stayed generally consistent in terms of direction and statistical significance (see **Table S2** and **S3**).

3.7 Functional connectivity analysis and correlation with clinical outcomes

In the whole-brain analysis, the severity of perceived stress positively correlated with functional connectivity between the right anterior insula and the right putamen (more perceived stress being associated with more connectivity). Putamen connectivity maps reciprocally confirmed the specificity of the association, showing a positive correlation of PSS scores with functional connectivity between the dorsal putamen and the left and right insulae (**Figure 5** and **Table S4**).

Depressive symptoms showed a significant positive correlation with functional connectivity between most of our regions of interest and the insula (higher PHQ-9 score, more connectivity; see **Table S5**). **Figure 5** illustrates the pattern of correlations and shows the extent to which the identified associations overlap with the results of PSS score analysis. Of note, the

largest changes were identified at the anterior insula, predominantly in the right hemisphere. Interestingly, this effect was more specific when only the first item of the PHQ-9 scale, which evaluates anhedonia, was considered. That is, anhedonia scores positively correlated with functional connectivity between the putamen (dorsal and ventral, left and right) and the right anterior insula (see **Figure S5**).

Fatigue and irritability scores (independently) provided similar results, also demonstrating a significant positive correlation with functional connectivity (higher scores being associated with stronger connectivity) between the dorsal putamen and the left insula in the case of fatigue, and between the dorsal putamen and bilateral insula in the case of irritability scores (see **Tables S6, S7**).

A similar pattern of correlations was obtained after including inflammatory markers as covariates in the analyses (**Table S4-S7**).

3.8 Multiple regression analysis

Overall, results indicated that the inflammatory markers PGE_2 and IL-6 were associated with functional connectivity changes between the insular cortices and structures in the basal ganglia and within the basal ganglia. Symptoms of sickness behavior, in turn, were associated with functional connectivity changes in regions that partially overlapped with the changes associated with the inflammatory markers (e.g., **Figure 2, Figure 5** and Tables in Supplementary Material). A multiple regression analysis including measures from both inflammatory markers and clinical outcomes showed that PGE_2 and PSS scores accounted for significant unique variance in the functional connectivity between the anterior insula and putamen (**Figure 6**). In a stepwise approach, (1) increased PGE_2 serum levels and (2) increased

PSS scores were entered into the equation, accounting for 46% of the variance in functional connectivity measurements (adjusted R square = 0.42).

4. Discussion

Results from this study indicate that increased inflammation, as reflected by increased IL-6 and PGE_2 serum levels, and greater perceived stress and subclinical depressive symptoms in patients with CHC are associated with abnormal functional connectivity in brain regions associated with interoceptive awareness (**see Figure S6**).

We observed that patients with CHC perceived more stress and reported greater levels of irritability and fatigue than control subjects, as expected for patients with chronic disease. Moreover, the PHQ-9 scores reflected the increased subclinical depressive symptoms in CHC patients, particularly anhedonia and a reduced interest in doing things. This subtle difference was particularly suggestive, as a diagnosis of MDD was an exclusion criterion in this study. Symptoms of fatigue, irritability and anhedonia have been widely described as part of sickness behavior (Dantzer, 2009). These may persist in chronic inflammatory conditions without reaching greater clinical relevance and are the most common complaints of CHC patients (D'Mello and Swain, 2014; Huckans et al., 2014; Yarlott et al., 2017).

As expected, CHC patients showed increased serum levels of inflammatory mediators, namely IL-6 and PGE_2, as well as reduced levels of the anti-inflammatory 15d-PGJ_2. These findings, in line with previous studies (Aregay et al., 2018; Senzolo et al., 2011; Shah et al., 2015; Waris and Siddiqui, 2005), demonstrated the increased inflammatory activity in patients with CHC. IL-6 is a highly versatile pro-inflammatory cytokine with pleiotropic effects, which is secreted in response to environmental stress factors, such as infections or obesity (Castanon et al., 2014; Tanaka et al., 2014), and contributes to the development of chronic inflammatory

illnesses (Baran et al., 2018). Furthermore, IL-6 is involved in several physiological functions in the central nervous system, such as neuron homeostasis. Thus, its chronic dysregulation may lead to various diseases (Rothaug et al., 2016; Spooren et al., 2011). Similarly, PGE_2 has been linked to the transition to and maintenance of chronic inflammation (Leonard, 2018; Narumiya, 2009) by promoting inflammation through inducing the expression of pro-inflammatory cytokines and suppressing Th2 cell differentiation and the anti-inflammatory system (Leonard, 2018). PGE_2 regulates sickness following systemic inflammation and is associated with increased body temperature, reduced food intake and changes in cognitive functions such as learning and memory (Poon et al., 2015). Inhibition of PGE_2 synthesis in mice has been reported to reduce the sickness behavior induced by LPS treatment (de Paiva et al., 2010). In line with these results, our finding of reduced levels of the anti-inflammatory 15d-PGJ_2 in CHC patients was expected, as this prostanoid is known to exert anti-inflammatory effects via its nuclear peroxisome proliferator-activated receptor-γ (PPARγ) (García-Bueno et al., 2008). This imbalance between cyclooxygenase-produced pro- and anti-inflammatory mediators has been described in experimental models as well as in patients with psychiatric disorders (García-Álvarez et al., 2018; García-Bueno et al., 2014; Leza et al., 2015).

In addition to the increased inflammation observed in CHC patients, our results highlighted the absence of oxidative stress in these subjects, which was not expected. It has been demonstrated that chronic inflammation may induce excessive production of ROS and RNS, which can cause nitro-oxidative damage to proteins, lipids or nucleic acids. The resulting O&NS can cause mitochondrial dysfunction, glial activation, neuroinflammation and apoptosis in the central nervous system, and has been associated with several neuropsychiatric conditions (Linqvist, 2017). The brain is particularly vulnerable to oxidative damage due to its high oxygen use and relatively weak antioxidant defences (Ng et al., 2008). For example, increased levels of polyunsaturated lipid oxidation, namely MDA-TBARS levels, have been reported in MDD

patients (Lopresti et al., 2014; Palta et al., 2014), chronic and recurrent depression, as well as aging (Maurya and Rizvi, 2010). Nevertheless, the intrinsic antioxidant enzymatic system (i.e., SOD, CAT and GPx) may be activated in certain conditions to maintain ROS/RNS concentrations at desirable levels and to prevent O&NS (Sousa et al., 2016). In our study, increased SOD and CAT activities were reflected by decreased levels of MDA-TBARS in CHC patients. MDA accumulation and no clear disruptions in the antioxidant systems in CHC patients indicate that the antioxidant system in CHC patients in our study was functioning and still able to manage ROS/RNS production. This is noteworthy, as O&NS may be crucial in the development of psychiatric illnesses, which was an exclusion criterion in our study. Further longitudinal studies are needed to determine whether these changes might be used as status biomarkers in this particular clinical setting.

Our results demonstrated that differences in sub-syndromic clinical symptomatology and inflammation between the groups were reflected by brain functional changes in areas involved in interoceptive awareness, psychomotor functions and affective processing. The chronic inflammatory disruption that characterizes CHC may contribute to the pathogenesis of neuropsychiatric symptoms, as cytokines may interact with several neurobiological pathways involved in psychiatric disorders (Furtado and Katzman, 2015; Oriolo et al., 2018; Yarlott et al., 2017). Moreover, several studies have postulated that the brain may be a minor replication site for HCV (Fishman et al., 2008; Laskus et al., 2005), which can cross the blood brain barrier (BBB) and enter the central nervous system through infected monocytes (Thomas et al., 1999). HCV can interact with the microglia, inducing its activation by increasing the production of pro-inflammatory mediators. Interestingly, differences in functional connectivity between CHC patients and healthy controls were found in basal ganglia and limbic structures, which are sensitive to peripheral inflammation and associated with symptoms of sickness behavior. Importantly, in the literature similar results were reported for psychiatric conditions. Decreased

functional connectivity between the sgACC and the precuneus was also observed in patients with MDD compared to healthy controls (Connolly et al., 2013; Ho et al., 2014), whereas increased functional connectivity between ventral putamen and frontal operculum was found in subjects with a high risk of psychosis (Dandash et al., 2014). Another intriguing result was the positive correlation between increased PGE_2 serum levels and increased functional connectivity of the insula with the dorsal putamen. The same brain areas were associated with perceived stress, as the insula to dorsal putamen connectivity positively correlated with PSS scores. Due to its lipid composition, PGE_2 can directly enter the brain parenchyma, spreading through the BBB and mainly interacting with the signalling receptors EP2 and EP3 on neurons to modulate neurotransmission (Furuyashiki and Narumiya, 2011). Interestingly, these receptors are mostly expressed in brain areas implicated in emotional and behavioral control, as PGE_2 has been reported to be involved in the mediation of behavioral response to circulating cytokines (Zhang and Rivest, 1999). By contrast, IL-6 in CHC patients negatively correlated with the functional connectivity between the dorsal and ventral putamen and the caudate nucleus. These same brain areas were associated with subclinical depressive symptoms, as the dorsal and ventral putamen connectivity positively correlated with PHQ-9 scores. Although these results may appear contradictory, animal studies indicate that IL-6 in the brain may contribute to the expression of brain cytokines in response to immune stimuli (Dantzer et al., 2008), resulting in several effects in the central nervous system that can be both detrimental and advantageous. Importantly, the effects of IL-6 partly depend on diverse factors, such as the presence of other cytokines or growth factors in the environment, the brain region involved and the physiological state of the tissue. Moreover, low or high IL-6 concentrations can exert opposite effects (Gadient and Otten, 1997; Spooren et al., 2011), mediating both neuroprotective and neurotoxic microglial responses (Eskes et al., 2002; Krady et al., 2008). It should be noted that PGE_2 and IL-6 are

pro-inflammatory mediators that are also produced by the microglia and neurons, their secretion being modulated by the direct neuropathogenic effects of HCV (Wilkinson et al., 2010).

Taken together, our findings demonstrate how changes in peripheral inflammation can influence insula and basal ganglia connectivity, illustrating how changes in internal bodily states can disturb neural representations, emotional states and executive functions. This is in line with current theories that postulate that emotional feeling states may arise through the perception of bodily signals, given that interoceptive and emotional processes share similar neural substrates (Critchley, 2005; Damasio, 1994; Quadt et al., 2018). The insular cortex is believed to represent and integrate interoceptive signals, such as inflammatory markers, providing the basis of interoceptive and emotional awareness, that is, the experiential side of sickness behavior (Craig, 2009). Studies using acute inflammatory challenges (Harrison, 2017) have demonstrated that subjective experiences of inflammation-associated symptoms derive from interoceptive signals converging on the insula (Harrison et al., 2009). Indeed, structural and functional changes in the posterior, mid or anterior insula have been associated with subjective feelings of malaise and fatigue following inflammation (Bushara et al., 2001; Farrer et al., 2003; Klein et al., 2007). Moreover, several studies have reported a posterior-to-mid-to-anterior pattern of integration of interoceptive information (Craig, 2003). For example, activation of the posterior insula is linked to the objective intensity of heat pain, whereas anterior insular activation is associated with subjective pain evaluation (Kong et al., 2006). The patients in our study experienced increased subjective stress, which may be modulated partly by the effects of PGE_2 on the insular cortex. However, results from the multiple regression analyses indicated that increased PGE_2 serum levels and increased PSS scores independently accounted for 46% of the variance in functional connectivity measurements. The basal ganglia consists of subcortical structures involved in the integration and coordination of executive functions, reward, emotions and mood, with a specific relevance for adaptive shaping and action

selection (Grace, 2012; Wichmann and De Long, 2013). Specifically, the dorsal putamen has been associated with the control of habitual behaviors (Redgrave et al., 2010; Wunderlich et al., 2012) and is believed to modulate the balance between goal-directed and habitual action control together with the insula (Hong et al., 2015). We observed that disruption of the dorsal putamen-insula interaction correlated with increased subjective perceived stress and reduced interest in doing things in CHC patients, which may be part of the modulation of the goal-directed versus habitual action control. Moreover, several neuroimaging studies have suggested that disrupted connectivity between the basal ganglia and putamen elicits behavioral changes following inflammatory challenges (Brydon et al., 2008; Felger and Miller, 2012). For example, PET studies revealed increased glucose metabolism in the putamen following IFN-alpha administration, which correlated with increased fatigue (Capuron et al., 2007; Juengling et al., 2000). Furthermore, an fMRI study revealed increased substantia nigra activity after administering the typhoid vaccine, which correlated with increased IL-6 peripheral blood concentrations and psychomotor retardation (Brydon et al., 2008). Interestingly, in a recent meta-analysis of neuroimaging and spectroscopic studies on patients with CHC performed by our group (Oriolo et al., 2018), increased choline/creatine ratios, glutamine plus glutamate and creatine levels were observed in the basal ganglia of CHC patients compared to healthy controls, indicating chronic metabolic changes in the basal ganglia induced by CHC.

Our results provide valuable information on the brain areas involved in perceived stress, fatigue and subclinical depressive symptoms during chronic inflammation, highlighting the crucial role of interoception in coordinating prolonged sickness behavior. Since affective and emotional alterations characterize most psychiatric illnesses (Limanowski and Blankenburg, 2013), they may constitute the link between interoception and mental disorders (Quadt et al., 2018). However, our study had several limitations. First, the cross-sectional design of this study reduced the possibilities of inference. The absence of longitudinal assessment precluded

predictive analysis, which could have strengthened the validity of our hypothesis. Moreover, a larger sample size would have increased the statistical power of this study. Second, we analysed only a few types of inflammatory markers. Analysing other markers such as CRP or TNF-α could have increased the reliability of our results. However, CRP might be a less reliable marker as several reports have found reduced CRP levels in CHC patients that may be due to auto-antibody activities (Sjöwall et al., 2012) or interferences by IL-6 on CRP synthesis (Shah et al., 2015). Third, we did not know the time between HCV infection and diagnosis, making it difficult to ascertain the duration of CHC. In general, the relationship between chronic inflammation and psychiatric symptoms is less easy to identify because patients who have such medical conditions are examined at different stages of their disease process (Dantzer, 2009). Moreover, it is even more difficult to determine the beginning of the disease in CHC patients as the source of infection is often unknown. This may result in much higher inter-individual variability. Finally, as mentioned before, HCV may directly affect the central nervous system (Oriolo et al., 2018; Yarlott et al., 2017), making it difficult to disentangle the effects of chronic inflammation *per se* versus the direct effects of the virus on the brain.

5. Conclusions

Patients with CHC infection exhibited increased perceived stress and subthreshold depressive symptoms, as well as higher levels of inflammatory markers, compared to control subjects. These subtle clinical and inflammatory differences were reflected by functional connectivity changes in brain areas involved in interoceptive awareness, psychomotor functions and emotional processing. Our findings provide evidence that chronic inflammation may induce prolonged activation of interoceptive pathways, which in turn may promote long-standing maladaptive neurobiological and behavioral impairments implicated in the pathophysiology of depression (Miller et al., 2009; Savitz and Harrison, 2018). As we hypothesized, chronic

inflammation together with the possible direct effects of HCV on the central nervous system, may account for the disruption in the connectivity between the insula and dorsal putamen, regions that provide cortical representation of internal state of the body, including changes in peripheral inflammation. In this sense, HCV seems to prime the brain and may account for a chronic sickness condition that is reflected by increased subjective stress perception and/or subclinical depressive symptoms (such as anhedonia), which may derive from aberrant interoceptive processing. This may represent a trigger for psychiatric illnesses in vulnerable patients or be a vulnerability factor itself, inducing a cascade of neurobiological pathways linked to mental disorders, such as oxidative and nitrosative stress. However, longitudinal studies are needed to disentangle the intricate interactions between the immune system, HCV neurotropism and the brain. The study of patients before and after a sustained viral response would be crucial in identifying the metabolic and functional changes specifically associated with HCV infection, which may also provide new information on the pathophysiology of neuropsychiatric symptoms and the identification of novel therapeutic targets.

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Conflicts of interest to declare

RMS, GO, LBH, RN, DMH, MC,DG, JC, LC, and JP: none.

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Table 1. Characteristics of the study participants.

	Whole sample **N = 65** **Mean (SD)/N (%)**	**CHC patients** **N = 35** **Mean (SD)/N (%)**	**Controls** **N = 30** **Mean (SD)/N (%)**
Age, Mean (SD)	40.2 (± 9.4)	41.1 (± 9.6)	39.2 (± 9.1)
Female (%)	22 (33.9)	12 (34.3)	10 (33.3)
Marital status	46 (72.1)	26 (74.2)	20 (66.7)
Educational level			
Middle school or less	9 (13.8)	7 (20.0)	2 (6.7)
Professional diploma or undergraduate degree	28 (43.1)	15 (42.9)	13 (43.3)

Graduate degree	28 (43.1)	13 (37.1)	15 (50.0)
Job status			
Student	3 (4.6)	2 (5.7)	1 (3.3)
Active	55 (84.6)	28 (80.0)	27 (90.0)
Unemployed/retired	7 (10.8)	5 (14.3)	2 (6.7)
Depression/Anxiety (> 1 year before)			
Depressive disorders	9 (13.8)	6 (17.1)	3 (10.0)
Anxiety disorders	9 (13.8)	7 (20.0)	2 (6.7)
Family history of psychiatric disorders	27 (41.5)	15 (42.9)	12 (40.0)
Current tobacco use	18 (27.7)	14 (40.0) *	4 (13.3)
HCV genotype			
Gen 1		28 (80.0)	
Gen 2		2 (5.7)	
Gen 3		1 (2.9)	
Gen 4		4 (11.4)	
Source of HCV infection			
Transfusion		3 (8.6)	
Parenteral drug use		3 (8.6)	
Surgery		3 (8.6)	
Unknown		26 (74.3)	
HCV RNA IE/mL median (range)		$1.5x10^6$ ($3.0\ x10^5$-$6.3x10^6$)	
Compensated cirrhosis		2 (5.7)	

Comparisons were performed between groups of CHC patients and healthy controls. Analyses were performed using two-sample t-tests for continuous variables and chi-square tests for categorical variables.
Abbreviations: **CHC,** chronic hepatitis C; **HVC,** hepatitis C virus; **SD,** standard deviation; **SUD,** substance use disorder.
*$p < 0.05$

Table 2. Clinical outcomes and serum concentrations of biological markers.

Clinical outcomes	**CHC patients N = 35 Mean (± SD)**	**Controls N = 30 Mean (± SD)**
PHQ-9		
Total score	3.6 (±3.9) *	1.4 (±1.9)
Anhedonia ("Little interest or pleasure in doing things")	0.46 (±0.7) *	0.17 (±0.4)

VAS		
Irritability	3.4 (±2.1) **	1.8 (±1.5)
Fatigue	3.6 (±2.2) *	2.2 (±1.9)
PSS		
Total score	19.5 (±11.1) **	12.6 (±5.5)
Pro-inflammatory markers		
IL-6, pg/ml	1.90 (±0.88) ***	1.15 (±0.72)
PGE_2, pg/ml	1627.90 (±995.83)[a] ***	1050.73 (±1432.67)[b]
Anti-inflammatory markers		
15d-PGJ_2, pg/ml	38904.08 (±54935.06)[a] ***	232669.17 (±359036.46)
Oxidative stress markers		
MDA-TBARS, μM	12.70 (±4.27) *	16.07 (±6.17)
Anti-oxidant activity markers		
GPx, nmol/min/ml	80.56 (±13.61)	82.91 (±19.85)
SOD, U/ml	0.74 (±0.23) **	0.59 (±0.20)
CAT, U/ml	52.64 (±23.60) ***	27.63 (±16.71)

Comparisons were performed between groups of CHC patients and healthy controls. Analyses were performed using two-sample t-tests for continuous variables, which were log transformed in the case of the serum concentrations of biological markers. Analysis of covariance was further conducted, controlling for age, sex and tobacco use.

Abbreviations: **15d-PGJ_2**, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; **CAT**, catalase; **CHC,** chronic hepatitis C; **GPx**, gluthatione peroxidase; **IL-,** interleukin-; **MDA-TBARS,** malondialdehyde-thiobarbituric acid reactive substances; **PGE_2**, prostaglandin E_2; **PHQ,** physical health questionnaire; **PSS,** perceived stress scale; **SD,** standard deviation; **SOD,** superoxide dismutase; **VAS,** visual analog scale.

*p < 0.05
**p < 0.01
***p < 0.001

[a] Missing data in 1 patient
[b] Missing data in 2 patients
[c] Missing data in 5 patients

Table 3. Between-group differences in functional connectivity maps.

	CHC patients < Controls			
	Cluster size (ml)	*x y z*	*t*	*Adj. t*
Left Dorsal Putamen map				
Angular gyrus	4.3	-54 -56 34	3.8	3.3
Subgenual ACC map				

Fusiform gyrus	2.7	42 -42 -28	4.4	4.4
	CHC patients > Controls			
Right Ventral Putamen map				
Frontal operculum	3.0	-56 32 -6	4.3	4.4
Subgenual ACC map				
Precuneus	2.6	-6 -52 52	3.9	4.0

x y z, coordinates (mm) given in Montreal Neurological Institute (MNI) space. Statistics at cluster-level corrected threshold $P_{FWE}<0.05$ estimated using Monte Carlo simulations. *Abbreviations:* CHC = chronic hepatitis C; ACC = anterior cingulate cortex. *Adj.t* = Model adjusted for tobacco use.

Figures

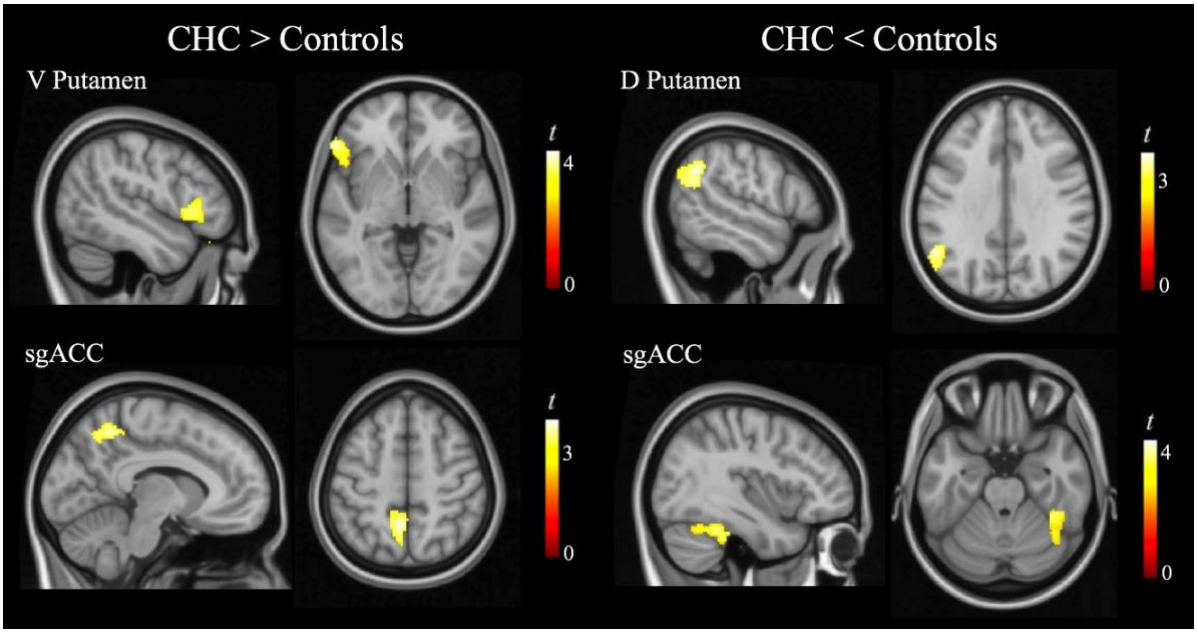

Figure 1. Between-group differences in functional connectivity. CHC, chronic hepatitis C patients; V, ventral; D, dorsal; ACC, anterior cingulate cortex. The right hemisphere corresponds to the right side of axial and coronal views.

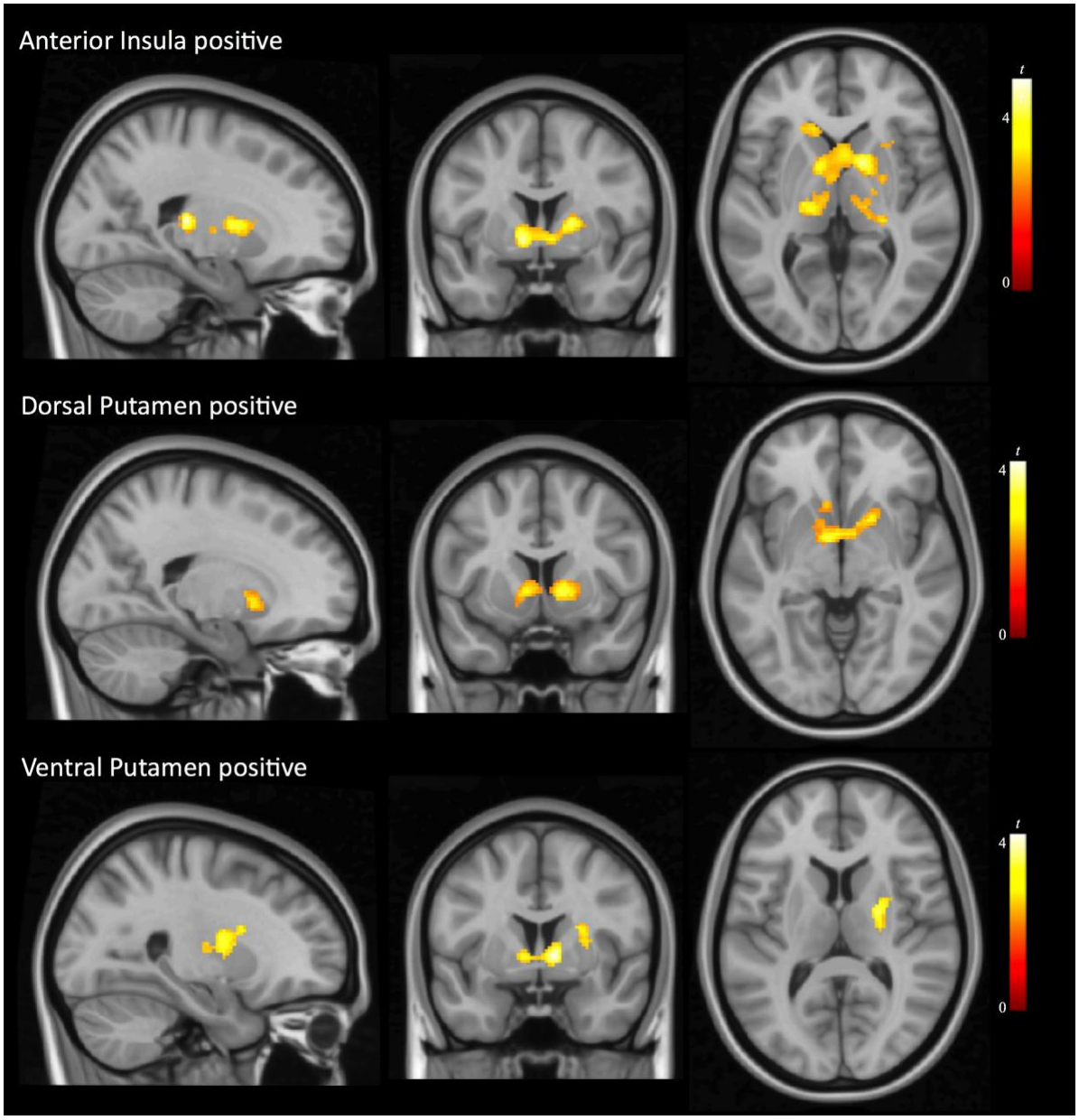

Figure 2. Representative correlation analysis for the pro-inflammatory marker prostaglandin E_2 (PGE_2) in chronic hepatitis C (CHC) patients. *Top panel:* positive correlation of PGE_2 serum levels with functional connectivity between the anterior insula and the basal ganglia and thalamus. *Middle panel:* positive correlation of PGE_2 serum levels with connectivity between the dorsal putamen and ventral basal ganglia. *Bottom panel:* positive correlation of PGE_2 serum levels with connectivity between the ventral putamen and basal ganglia. The right hemisphere corresponds to the right side of the axial and coronal views.

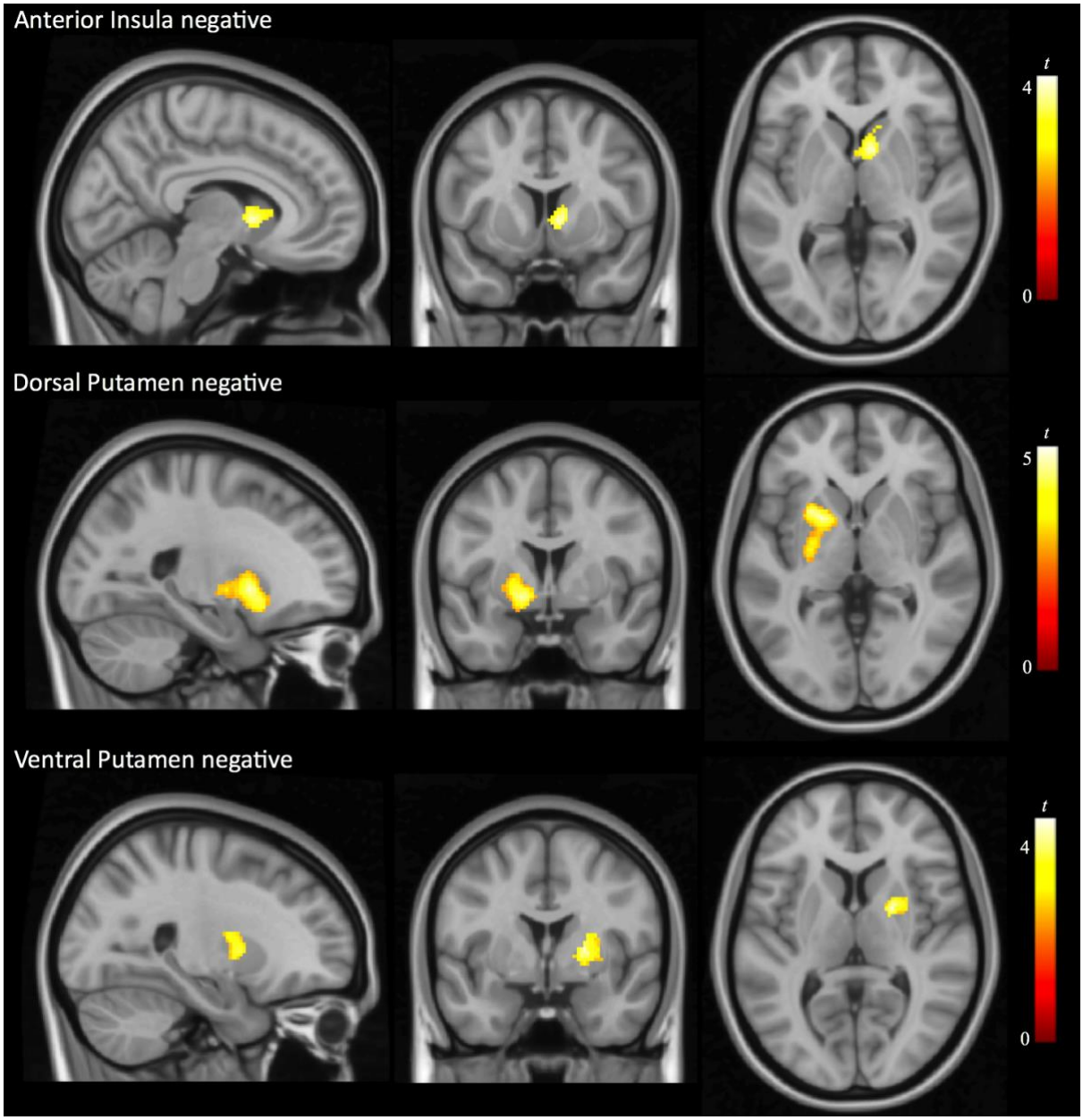

Figure 3. Representative correlation analysis for the pro-inflammatory cytokine interleukin-6 (IL-6) in chronic hepatitis C (CHC) patients. *Top panel:* negative correlation of IL-6 serum levels with functional connectivity between the anterior insula and the right caudate nucleus. *Middle panel:* negative correlation of IL-6 serum levels with connectivity between the dorsal putamen and the left putamen/globus pallidus. *Bottom panel:* negative correlation of IL-6 serum levels with connectivity between the ventral putamen and the right

putamen/globus pallidus. The right hemisphere corresponds to the right side of the axial and coronal views.

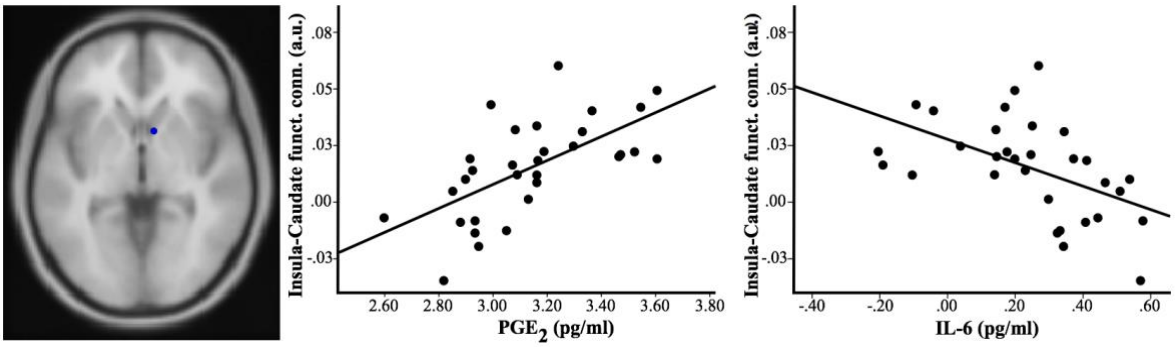

Figure 4. Plots of correlation analysis for the pro-inflammatory cytokines prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6) in chronic hepatitis C (CHC) patients. Functional connectivity between the anterior insula (seed map) and the right ventral striatum (caudate nucleus; blue dot in the left panel at MNI coordinates x = 8, y = 8, z = -2) was positively associated with prostaglandin E_2 (PGE_2; R = 0.628, p = 0.00015) and negatively associated with interleukin-6 (IL-6; R = -0.513, p = 0.003). See also Figures 1 and 2.

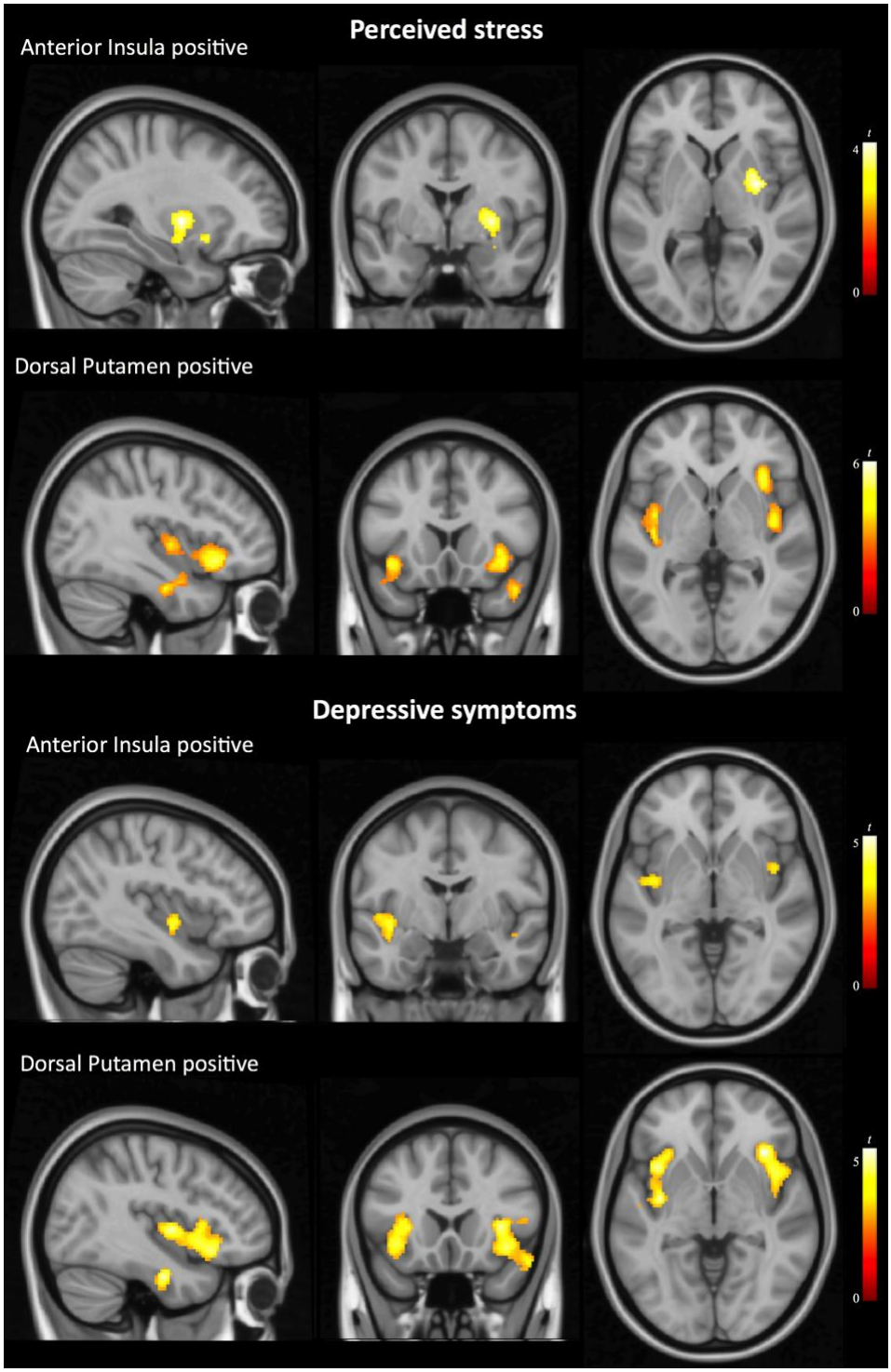

Figure 5. Representative correlations between functional connectivity measurements and clinical variables. *Top panels:* positive correlations of perceived stress scale (PSS) scores with functional connectivity between the anterior insula and the right putamen and between the dorsal putamen and bilateral insulae. *Bottom panels:* positive correlations of depressive symptoms [measured with Patient Health Questionnaire-9 (PHQ-9) scores] with functional

connectivity between both the anterior insula and dorsal putamen and the bilateral insulae. The right hemisphere corresponds to the right side of the axial and coronal views.

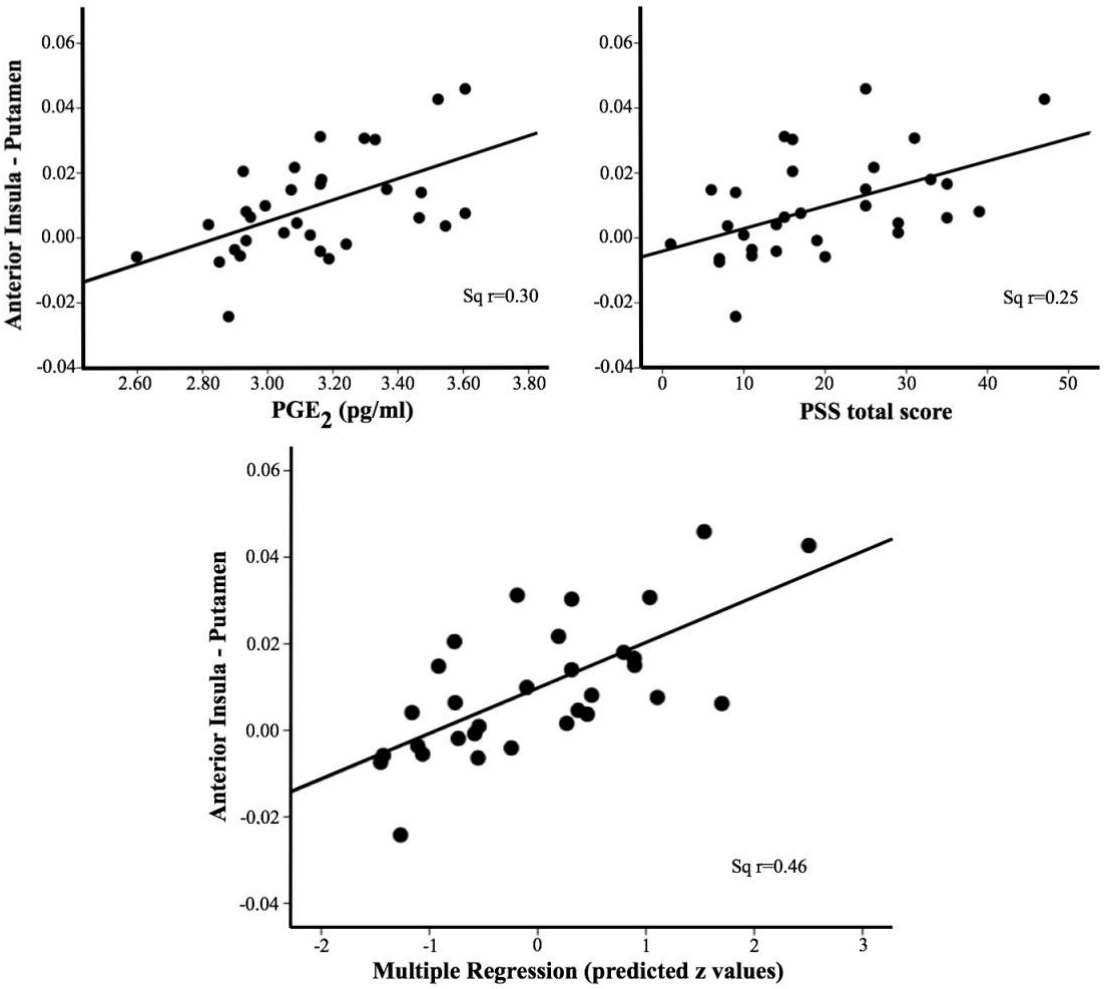

Figure 6. Plots of the correlations between functional connectivity measurements and biological and clinical variables. Functional connectivity values (y axis) indicate the correlation between the right insula (seed region) and the functionally connected region in the right putamen. *Top panel:* positive correlations for prostaglandin E_2 (PGE_2; R = 0.300) and for perceived stress scale (PSS) scores (R = 0.250). *Bottom panel:* increased PGE_2 serum levels and increased PSS scores were entered into the equation in the multiple regression analysis,

accounting for 46% of the variance in functional connectivity measurements (adjusted $R = 0.42$). All correlations were significant at $p < 0.008$.