

# Accepted Manuscript

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PII: S0889-1591(15)00429-8  
DOI: <http://dx.doi.org/10.1016/j.bbi.2015.08.005>  
Reference: YBRBI 2678

To appear in: *Brain, Behavior, and Immunity*

Received Date: 4 May 2015  
Revised Date: 4 August 2015  
Accepted Date: 4 August 2015

Please cite this article as: Krishnadas, R., Nicol, A., Sassarini, J., Puri, N., Burden, A.D., Leman, J., Combet, E., Pimlott, S., Hadley, D., McInnes, I.B., Cavanagh, J., Circulating Tumour Necrosis Factor is highly correlated with brainstem serotonin transporter availability in humans, *Brain, Behavior, and Immunity* (2015), doi: <http://dx.doi.org/10.1016/j.bbi.2015.08.005>

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**Title page: Circulating Tumour Necrosis Factor is highly correlated with brainstem serotonin transporter availability in humans**

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## Abstract

Preclinical studies demonstrate that pro-inflammatory cytokines increase serotonin transporter availability and function, leading to depressive symptoms in rodent models. Herein we investigate associations between circulating inflammatory markers and brainstem serotonin transporter (5-HTT) availability in humans. We hypothesised that higher circulating inflammatory cytokine concentrations, particularly of tumour necrosis factor (TNF- $\alpha$ ), would be associated with greater 5-HTT availability, and that TNF- $\alpha$  inhibition with etanercept (sTNFR:Fc) would in turn reduce 5-HTT availability. In 13 neurologically healthy adult women, plasma TNF- $\alpha$  correlated significantly with 5-HTT availability ( $\rho=0.6$ ;  $p=0.03$ ) determined by [ $^{123}\text{I}$ ] -beta-CIT SPECT scanning. This association was replicated in an independent sample of 12 patients with psoriasis/psoriatic arthritis ( $\rho=0.76$ ;  $p=0.003$ ). Indirect effects analysis, showed that there was a significant overlap in the variance explained by 5-HTT availability and TNF- $\alpha$  concentrations on BDI scores. Treatment with etanercept for 6-8 weeks was associated with a significant reduction in 5-HTT availability ( $Z= 2.09$  ;  $p=0.03$ ;  $r=0.6$ ) consistent with a functional link. Our findings confirm an association between TNF- $\alpha$  and 5-HTT in both the basal physiological and pathological condition. Modulation of both TNF- $\alpha$  and 5-HTT by etanercept indicate the presence of a mechanistic pathway whereby circulating inflammatory cytokines are related to central nervous system substrates underlying major depression.

## Introduction

Studies over the last few decades have found a strong link between inflammatory processes and psychiatric illnesses, particularly major depressive disorder (MDD). At least three meta-analyses have confirmed the presence of greater circulating inflammatory marker levels in major depressive disorder (Dowlati et al., 2010; Howren et al., 2009; Liu et al., 2012). Greater circulating inflammatory marker levels have been found to predict the development of MDD in longitudinal studies (Valkanova et al., 2013). Among medically ill, those with inflammatory diseases are at greater risk of developing a major depressive illness (Kurd et al., 2010).

While the above findings are essentially correlational, a causal link has been proposed by findings that patients treated with cytokines for illnesses like hepatitis C, are at greater risk of developing a major depressive illness (Loftis et al., 2013; Myint et al., 2009). Conversely, biological anti-inflammatory agents, that target proinflammatory cytokines in a highly specific manner, induce an antidepressant effect in patients, independent of improvement in the inflammatory condition (Feldmann and Maini, 2001, 2003, 2010; Feldmann et al., 2010; Tying et al., 2006). Given these clinical observations, it is proposed that the immune system and inflammation may play a role in the aetiopathogenesis of MDD (Dantzer et al., 2008; Kelley et al., 2003; Krishnadas and Cavanagh, 2012; Raison et al., 2006; Smith, 1991; Stein et al., 1991).

When exploring the pathophysiological underpinnings of depression, the monoamine neurotransmitter systems remain a central and translationally relevant biological substrate (Schildkraut, 1965).

Among the monoamines, the serotonergic system modulates emotional regulation, reward and punishment processing, behavioural inhibition, delay discounting and has been implicated in the aetiopathogenesis of MDD (Cools et al., 2008; Massart et al., 2012; Schildkraut, 1965) [see (Albert and Benkelfat, 2013; Albert et al., 2012) for a special issue on an update on the serotonin hypothesis in the neurobiology of depression]. The mesencephalic rostral raphe nuclei (including the median and dorsal raphe) account for 85% of all serotonergic neurons in the brain. They innervate the cerebral cortex and the limbic forebrain, including the hypothalamus, hippocampus and amygdala and receive descending projections from the limbic forebrain and ascending projections from the periaqueductal grey matter (Descarries et al., 2010). The serotonin transporter (5-HTT) plays an essential role in serotonin neurotransmission, by regulating synaptic serotonin levels. They are not only present in the projection areas on the neuronal presynaptic cell membrane, but also on cell bodies within the raphe nuclei distributed near the midline of the brainstem (Canli and Lesch, 2007; Descarries et al., 2010; Hornung, 2003; Maximino, 2012). In vitro studies confirm a significant correlation between 5-HTT levels and tissue concentrations of serotonin, suggesting that the former is a proxy marker for serotonin in healthy brain (Dewar et al., 1991). Depressive symptoms in humans have been associated with increased availability/function of 5-HTT, pointing to a reduction in synaptic serotonin levels (Meyer, 2008; Savitz and Drevets,

2013). While the monoamine hypothesis by itself is far from complete in explaining the pathophysiology underlying depression, a majority of available antidepressants inhibit 5-HTT at therapeutic doses and promote an increase in synaptic serotonin (Meyer, 2012).

The serotonergic neurotransmitter system is a prime candidate to explore the link between inflammation and the aetiopathogenesis of MDD. This potential link is supported by findings from preclinical studies in rodent models that directly link proinflammatory cytokines with brain 5-HTT availability through activation of MAPK pathways (Baganz and Blakely, 2013; Couch et al., 2013; Katafuchi et al., 2006; Malynn et al., 2013; Morikawa et al., 1998; Ramamoorthy et al., 1995; Samuvel, 2005; Tsao et al., 2008). There are number of pathways through which circulating inflammatory markers can signal the brain including active transport across the blood-brain barrier (BBB); second messenger signals from the endothelial lining of BBB; through afferent vagal pathways; passage of cytokines through 'leaky' regions in the BBB (Capuron and Miller, 2011). Of particular theoretical relevance is that, in humans, the raphe nuclei (dorsal) are located close to the cerebral aqueduct making it particularly vulnerable to inflammatory signalling molecules present in the cerebral spinal fluid (CSF) (Howerton et al., 2014). They are also highly connected with humoral sensing circumventricular organs like the subfornical organ, which have fenestrated capillaries that allow exposure to large molecules like inflammatory cytokines, and form part of the key viscerosensory paths in the brain (Critchley and Harrison, 2013; Wallacelind, 1986).

No human studies have examined the association between circulating inflammatory markers and brainstem 5-HTT availability. Herein, we addressed two key questions. Firstly, is there a relationship between circulating inflammatory markers and 5-HTT in humans? And secondly, will highly specific cytokine inhibition result in changes in 5-HTT availability? Thus, we explored the association between circulating inflammatory markers and brainstem 5-HTT availability in a cohort of neurologically healthy volunteers and replicated our finding in a cohort of patients with psoriasis / psoriatic arthritis. We also ascertained whether administration of etanercept (TNF receptor:Fc fusion protein) would be associated with changes in serotonin transporter availability. In keeping with preclinical studies, we hypothesised that greater pro-inflammatory cytokine concentrations would be associated with greater 5-HTT availability in the brain, and that treatment with etanercept would be associated with a reduction in 5-HTT availability.

## **Materials and Methods**

### **Study design**

The study was approved by the West of Scotland Research ethics committee and the Administration of Radioactive Substances Advisory Committee. All participants gave written informed consent. The study was conducted on two independent groups of subjects

**Healthy subjects:**

Firstly, we explored the association between, circulating inflammatory markers and brainstem 5-HTT availability in a group of healthy adult subjects (Figure 1a). Circulating inflammatory marker concentrations and 5-HTT availability data were obtained from thirteen healthy menopausal women who were non-smokers, non-hypertensive, non-diabetic and not taking any drugs which could affect vascular function, and not being prescribed antidepressant medications or hormone replacement treatment. Data from this sample exploring a different hypothesis has been published previously as part of another study (Sassarini et al., 2014).

**Psoriasis/ psoriatic arthritis subjects:**

We then replicated this association in a group of patients with psoriasis/psoriatic arthritis. Fifteen subjects diagnosed with psoriasis/psoriatic arthritis aged 30-65 years, were recruited into the study. Three patients dropped out - one withdrew consent, and the other two had incidental findings on the MRI. Therefore, data from 12 individuals were analysed. All patients met disease activity criteria set by NICE pertaining to the use of Etanercept for psoriasis and psoriatic arthritis, and had been previously treated with a disease modifying agent (methotrexate) for at least 6-8 weeks. Those who incidentally fulfilled the criteria for MDD were included in the study. However, those with a history of antidepressant intake in the previous 3 months; history of cerebrovascular disease, documented head trauma or neurological disorders, lifetime history of DSM Axis I psychiatric diagnoses other than depression (measured using SCID), alcohol and/or substance misuse, pregnancy, other connective tissue or



systemic inflammatory disease were excluded from participation. None of the patients fulfilled a SCID diagnosis for a DSM IV Axis 1 psychiatric disorder, and none of them were started on an antidepressant during the study.

Finally, in order to examine if highly specific TNF inhibition would alter 5-HTT availability, we compared 5-HTT availability, before and after treatment with etanercept for 6 - 8 weeks (Figure 1b). Etanercept is a recombinant human TNF- $\alpha$  receptor fusion protein with 934 aminoacids and weighs 150kDa (Strober et al., 2008; Tan et al., 2007). It binds to TNF- $\alpha$ , thereby blocking its interaction with cell-surface receptors (Feldmann and Maini, 2001, 2010). Etanercept is licensed for use in adults with active psoriasis/ psoriatic arthritis (Fantuzzi et al., 2008). The administration of etanercept provided a molecular scalpel to interrogate the functional implications of the elevated TNF levels (and consequent inflammation) present in patients with psoriasis / psoriatic arthritis. The required sample size was calculated based on a pilot study by Cavanagh et al (Cavanagh et al., 2010). In order to demonstrate a reduction in 5-HTT availability with an effect size of  $d=1.2$ , with 80% power, with an alpha of 0.05, the total sample size required was 10 subjects.

#### **Inflammatory markers**

The levels of 5 circulating inflammatory markers (from the meta-analyses of inflammatory markers in depression) were measured from plasma (Dowlati et al., 2010; Howren et al., 2009; Liu et al., 2012) extracted on the same day as the SPECT scans, just before the administration of the ligand. Blood (10 mL) was drawn into BD Vacutainer System tubes (BD Diagnostics,

Perianalytical Systems, Oxford) containing ethylenediamine tetraacetic acid (EDTA) anticoagulant and centrifuged immediately at 3000rpm for 10 min.

Plasma was extracted, and frozen at  $-80^{\circ}$  C before analysis. Cytokines were measured in duplicates using sandwich immunoassays, with all samples from the same volunteer on the same plate. They included TNF- $\alpha$ , IL-1b and IL-6 (Ready-set-go ELISAs, eBioscience, UK); sIL-2R (Platinum ELISA, eBioscience, UK) and hsCRP (ELISA, Invitron, UK). The sensitivity, intra- and inter-assay coefficient of variations, are shown in table 2 (supplemental materials). Two out of 13 healthy subjects had non-detectable levels of plasma TNF- $\alpha$ . For non-detectable values, we imputed the data using half the limit of detection (LOD/2) as suggested by Hornung et al (Hornung and Reed, 1990). Since most of the data was missing (11 out of 13), we did not analyse the relationship between IL6 and 5-HTT in healthy subjects.

#### **Beck's depression inventory (BDI II) (Beck et al., 1996)**

Beck's depression inventory II is a 21 item self-rated Likert-scale questionnaire that measures depressive symptoms over the past 2 weeks. Each question is scored on a scale of 0 to 3 yielding a total score ranging from 0 to 63, with higher scores indicating greater depressive symptoms. The scale was administered on the same day as the SPECT scans.

#### **[ $^{123}$ I] -beta-CIT SPECT measurement of 5-HTT availability**

All participants underwent a [ $^{123}$ I] -beta-CIT SPECT scan to ascertain brainstem 5-HTT availability. Those with psoriasis/arthritis underwent the

scan at least 14 days before and again 6 - 8 weeks after the commencement of weekly etanercept 50mg administered subcutaneously.

The SPECT imaging protocol has been validated and described in detail in the past (Cavanagh et al., 2006). Briefly, brain SPECT imaging was performed with a dedicated state of the art, 12 headed Neurofocus 900 SPECT scanner (spatial resolution 7mm full width at half maximum with a line source in air; NeuroPhysics, Shirley, Massachusetts), which acquires sequential single transaxial brain sections (see figure 4). Taking advantage of its pharmacokinetic properties, brainstem and diencephalon [ $^{123}\text{I}$ ]-beta-CIT uptake at 3 -4 hours was used to quantify 5-HTT availability as proposed by de Win et al (de Win et al., 2005b). Details of acquisition and [ $^{123}\text{I}$ ]-beta-CIT are given in the supplemental material.

#### **Radioligand [ $^{123}\text{I}$ ]-beta-CIT preparation for the study**

[ $^{123}\text{I}$ ]-beta-CIT was prepared via electrophilic iododestannylation of the corresponding tributylstannyl precursor (Baldwin et al., 1993). Briefly, reagents were added to carrier free  $\text{Na}^{123}\text{I}$  (370-740 MBq) in approximately 10-20  $\mu\text{l}$  of 0.05 M NaOH, in the following order: 30  $\mu\text{g}$  tributylstannyl precursor in 300  $\mu\text{l}$  glacial acetic acid followed by 10% V/V peracetic acid. The reaction proceeded for 20 minutes at room temperature. 500  $\mu\text{l}$  of NaOH was then added. The mixture was purified by reverse-phase HPLC and the solvent removed by rotary evaporation. The [ $^{123}\text{I}$ ]-beta-CIT was formulated as 150 MBq in 5 ml  $\leq 6\%$  ethanol in isotonic citrate acetate buffer and filtered through a 0.22  $\mu\text{m}$  filter. The [ $^{123}\text{I}$ ]-beta-CIT was produced with

an isolated radiochemical yield of  $69.6 \pm 8.9$  % (n = 29). Pyrogenicity tests and sterility tests were performed.

### SPECT Imaging

The SPECT imaging protocol has been validated and described in detail in the past (Cavanagh et al., 2006). [ $^{123}\text{I}$ ] -beta-CIT binds with high affinity in vitro to both dopamine transporter (DAT) and 5-HTT. In vivo studies in both humans and nonhuman primates have shown that [ $^{123}\text{I}$ ] -beta-CIT accumulates in two distinct brain regions. In the striatum, where the density of DAT is much higher than that of 5-HTT, [ $^{123}\text{I}$ ] -beta-CIT binding mainly reflects DAT density, whereas in the brainstem and diencephalon binding seems to be specific for 5-HTT. The kinetics of [ $^{123}\text{I}$ ] -beta-CIT binding differs markedly between the DAT-rich and 5-HTT-rich regions. The slow uptake in the striatum, which reaches a peak after 20-30 hours, is in contrast to the faster kinetics seen in the brainstem and the diencephalon, where peak activity is attained after 2-4 hours after administration. In this study, early imaging of 5-HTT regions was performed to minimize any possible effect of DAT on the 5-HTT measurement because DAT uptake is proportionally lower at 3 hours (de Win et al., 2005a).

Brain SPECT imaging was performed with a dedicated state of the art, 12 headed Neurofocus 900 SPECT scanner (spatial resolution 7mm full width at half maximum with a line source in air; NeuroPhysics, Shirley, Massachusetts), which acquires sequential single transaxial brain sections. Up to 25 axial sections 6 mm apart were scanned and the energy window (140-178 keV) was placed symmetrically around the  $^{123}\text{I}$  gamma energy of

159 keV. A linear attenuation correction was applied, based on an automatically detected ellipse matching the outer head surface. As proposed by de Win et al, subjects were scanned starting 3 - 4 hours after IV administration of [ $^{123}\text{I}$ ] -beta-CIT in order to establish uptake in 5-HTT rich areas (de Win et al., 2005a). To minimize thyroid uptake of radioactive iodine, 120mg of potassium iodide was administered orally to each subject at least one hour prior to [ $^{123}\text{I}$ ] -beta-CIT injection. Scanning time was approximately 50 min per scan.

### Regions of Interest analysis

Region of interest (ROI) analysis was carried out by an investigator (AN) blinded to the subject's clinical and demographic history. A standard ROI template was constructed with the aid of two image templates: (1) the standard magnetic resonance imaging template known as ICBM152, which is based on 152 normal MRI scans and is available from the Statistical Parametric Mapping web site of the Functional Imaging Laboratory (<http://www.fil.ion.ucl.uk/spm/>); and (2) an in-house cerebral perfusion template based on 32 normal SPECT scans of cerebral perfusion.

The ROI template consisted of manually drawn regions representing the brain stem/diencephalon ROI (referred to as the brain stem ROI below for brevity), and a reference ROI. The brain stem ROI (23 cm<sup>3</sup>) was drawn on seven axial sections 5mm apart and comprised the thalamus-hypothalamus, mid-brain, and pons. In order to see if the [ $^{123}\text{I}$ ] -beta-CIT uptake is specific to 5-HTT and not to DAT we estimated [ $^{123}\text{I}$ ] -beta-CIT uptake in the striatum. The striatum ROI (7.6 cm<sup>3</sup> on each side) was drawn on three axial

sections 5 mm apart and encompassed the head of caudate and putamen in both hemispheres. The reference ROI (35 cm<sup>3</sup>) was drawn on three axial sections 5 mm apart in the medial and lateral occipital lobe bilaterally. The occipital region was chosen to represent nonspecific and non-displaceable [<sup>123</sup>I]-beta-CIT uptake (i.e., signal not associated with binding to transporters) because it has a negligible density of serotonin transporters. The [<sup>123</sup>I]-beta-CIT uptake in each ROI was expressed as mean counts per pixel, and the specific uptake of [<sup>123</sup>I]-beta-CIT was calculated as:

$$\text{Beta CIT uptake in the brainstem ROI} - \text{BetaCIT uptake in the occipital ROI}$$

Binding potential quantifies the equilibrium concentration of specific binding as a ratio to a reference concentration. As defined by Innis et al,  $BP_{ND}$  refers to the ratio at equilibrium of specifically bound radioligand to that of non-displaceable radioligand in tissue (Innis et al., 2007).  $BP_{ND}$  for 5-HTT was then defined as the ratio of specific uptake to uptake in the occipital reference region using the formula:

$$BP_{ND} = \frac{\text{BetaCIT uptake in the brainstem ROI} - \text{BetaCIT uptake in the occipital ROI}}{\text{BetaCIT uptake in the occipital ROI}}$$

Under equilibrium conditions the binding ratio is proportional to transporter binding potential ( $BP_{ND}$ ). Provided transporter affinity and nonspecific binding are invariant across subjects, the ratio is then a measure of transporter availability.

#### Statistical analysis

Given the small sample size, non-parametric tests were performed wherever possible in IBM SPSS Statistics version 19 (IBM Corp, 2011). Differences between two independent groups were tested using Mann Whitney U Test. Differences between dependent variables were tested using Wilcoxon signed-rank test. Spearman's correlation was used to explore the relationship between inflammatory markers and 5-HTT.

To explore the relationship between TNF- $\alpha$ , 5-HTT (BP<sub>ND</sub>) and BDI scores, we conducted an "indirect effects" analysis with TNF- $\alpha$  levels as independent variable, 5-HTT as the "indirect variable" and BDI scores as dependent variable. For this analysis, we combined the data from the healthy controls and the patient population. Details of the calculation are in the supplemental material. Briefly, we aimed to examine if a proportion of variance in 5-HTT could explain the relationship between TNF- $\alpha$  levels and BDI scores. "This analysis differs from multiple regression which estimates the proportion of variance in the dependent variable accounted for by each of several independent predictor variables while allowing for the variance accounted for by the other predictors in the model." (Palaniyappan and Liddle, 2012) This analysis is similar to classic mediation analysis. While mediation analysis typically assume causal interactions, it should be noted that causal assumptions are not made here as the cytokine, 5-HTT and BDI were measured simultaneously. We therefore use the term "indirect effects analysis" as recommended by Preacher and Hayes (Preacher and Hayes, 2008). The indirect effects analysis partitions the variance explained by the independent into i) a part that is independent of the indirect variable, and ii) a part that is accounted for via the indirect variable. We used the

bootstrap method of Preacher and Hayes to estimate the bias-corrected 95% confidence interval (CI) of the "indirect effect" based on 20,000 bootstrap samples using an SPSS macro (indirect)(Preacher and Hayes, 2008). This analysis requires no assumption regarding the underlying distributions since the statistical significance level is determined non-parametrically. All p values mentioned are two-tailed.



## Results

### **Circulating TNF- $\alpha$ concentrations correlated with brainstem 5-HTT availability in healthy adults**

This analysis was conducted on data from the thirteen healthy volunteers (mean age = 57 years). As age correlated with 5-HTT availability ( $\rho=-0.52$ ;  $p=0.08$ ), 5-HTT availability was corrected for age in order to maximise the variance explained by TNF- $\alpha$ . Plasma TNF- $\alpha$  showed a significant positive correlation with brainstem 5-HTT availability (corrected for age) ( $n=13$ ; Spearman's  $\rho=0.6$ ;  $p=0.03$ ) (figure 2.a). None of the other inflammatory markers showed a statistically significant correlation with 5HTT availability (figures 2 b-d).

### **Circulating TNF- $\alpha$ concentrations correlated with brainstem 5-HTT availability in psoriasis/psoriatic arthritis**

This analysis was conducted on data from twelve patients [6 males and 6 females (mean age = 47.58; s.d.=8.8)] with psoriasis/psoriatic arthritis. The 5-HTT availability was corrected for age, sex and diagnosis (psoriasis/psoriatic arthritis). Plasma TNF- $\alpha$  showed a significant positive correlation with 5-HTT availability ( $n=12$ ; Spearman's  $\rho=0.76$ ;  $p=0.004$ ) (figure 2.e). None of the other circulating inflammatory markers showed a statistically significant correlation with 5-HTT availability (figures 2 f - i).

Although brainstem [ $^{123}\text{I}$ ] -beta-CIT uptake at 3-4 hours has been attributed to 5-HTT availability, it has been proposed [ $^{123}\text{I}$ ] -beta-CIT uptake in the narrow brain regions that span between the midbrain periaqueductal area and substantia nigra may also represent DAT uptake (Laruelle et al., 1993). We therefore examined if the association was specific to 5-HTT. We used

striatal [ $^{123}\text{I}$ ] -beta-CIT uptake at 24 hours as a measure of DAT specific binding from 11 participants from the psoriasis group. We found some non-significant correlation between 5-HTT and DAT ( $\rho=0.39$ ;  $p=0.26$ ). However, there was no correlation between TNF- $\alpha$  and striatal DAT availability ( $\rho=-0.042$ ;  $p=0.9$ ). In addition, the correlation between TNF- $\alpha$  and 5-HTT improved after controlling for DAT availability ( $\rho=0.92$ ;  $p<0.001$ ), suggesting that the correlation between TNF- $\alpha$  and midbrain [ $^{123}\text{I}$ ] -beta-CIT binding index may be 5-HTT specific.

#### **TNF- $\alpha$ has an indirect effect on depression scores, through brainstem 5-HTT availability**

The indirect effects analysis examined if the variance explained by the 5-HTT availability and TNF concentrations on BDI scores overlapped. Figure 3 shows that 5-HTT availability had a significant indirect effect on the relationship between circulating TNF- $\alpha$  and BDI scores (Indirect effect:  $\beta=0.37$ ;  $SE=0.24$ ; 95% CI = 0.02 to 1.16). In other words, there was a significant overlap in the variance explained by 5-HTT and TNF concentrations on BDI.

#### **Specific TNF- $\alpha$ inhibition was associated with a reduction in 5-HTT availability**

Psoriasis/ psoriatic arthritis participants received etanercept for an average 6.58 weeks (s.d. = 0.79). There was no significant difference in the dose of [ $^{123}\text{I}$ ] -beta-CIT at baseline (average dose = 150.08 MBq) and post-treatment scans (average dose = 148.5 MBq) ( $Z=-0.92$ ;  $p=0.36$ ). A Wilcoxon Signed-ranks test indicated that 5-HTT availability decreased following etanercept

treatment ( $Z= 2.09$  ;  $p=0.03$ ;  $r=0.6$ ) (Figure 4) consistent with a functionally meaningful relationship between TNF- $\alpha$ , or its downstream effector pathways and 5-HTT expression in humans. As expected, hsCRP ( $Z=2.59$ ;  $p=0.009$ ;  $r=0.75$ ) and IL6 ( $Z=2.36$ ;  $p=0.02$ ;  $r=0.68$ ) showed a significant decrease with treatment. As previously reported, TNF levels increased following treatment with etanercept ( $Z=2.70$ ;  $p=0.007$ ;  $r=0.78$ ), reflecting an increase in bound TNF to the fusion protein that is detected by the ELISA (Bhatia and Kast, 2007; Kotyla et al., 2015). BDI scores also showed a reduction following treatment with etanercept ( $Z=2.2$ ;  $p=0.03$ ;  $r=0.63$ ) (Table 1). We found no association between change in CRP levels and change in BDI ( $\rho=-0.15$ ;  $p=0.6$ ) and change in 5-HTT ( $\rho=0.014$ ;  $p=0.9$ ).

## Discussion

We examined the relationship between circulating inflammatory markers and brainstem 5-HTT availability - a biological substrate of mood regulation. For the first time in two independent human samples, we have established an association between circulating TNF- $\alpha$  and 5-HTT availability (brainstem [ $^{123}$ I] -beta-CIT uptake). Circulating TNF- $\alpha$  showed a strong positive correlation with 5-HTT availability in both a basal physiologic state, with low circulating cytokine concentrations and a pathological state with high circulating cytokines concentrations. We found no association between TNF- $\alpha$  and DAT availability (striatal [ $^{123}$ I] -beta-CIT uptake). In addition, controlling for any striatal uptake (representative of DAT availability) increased the correlation between TNF- $\alpha$  and brainstem uptake, suggesting that the association is driven by 5-HTT and not DAT availability.

We chose a sample of patients with psoriasis for two reasons. Firstly, this is a condition where inflammation is fundamental in its pathophysiology and depression is highly prevalent (Coimbra et al., 2012; Gladman et al., 2005; Hayes and Koo, 2010; Kurd et al., 2010; Nestle et al., 2009; Schmitt and Ford, 2007). Secondly, this population gave us the ideal opportunity to explore the effect of TNF antagonism on 5-HTT. Earlier research in this population has shown that TNF antagonism was associated with an antidepressant effect that was independent of disease improvement (Gelfand et al., 2008; Krishnan et al., 2007; Langley et al., 2010; Tying et al., 2006). Consistent with previous studies, TNF- $\alpha$  explained only around 3% of and brain stem 5-HTT availability explained only around 7% variance in depression severity (Gryglewski et al., 2014; Valkanova et al., 2013).

However the relationship between TNF- $\alpha$  and BDI scores was explained at least in part by an indirect effect through 5-HTT availability, suggesting that our findings are behaviourally relevant (Valkanova et al., 2013). Crucially, we replicated our previous findings that highly specific TNF- $\alpha$  inhibition was associated with a significant reduction in brainstem 5-HTT availability (Cavanagh et al., 2010). Since etanercept does not cross BBB it is likely that modulation of circulating inflammatory markers was responsible for the changes (Krishnadas, 2010). While it is possible that the reduction in 5-HTT availability was the result of improvement in clinical symptoms of psoriasis/arthritis, reduction in CRP - a marker of clinical inflammatory activity - was not associated with the reduction in 5-HTT (Beygi et al., 2013; Coimbra et al., 2009; Strober et al., 2008). In addition, clinical response rates to etanercept are around 50% and peak at 4 to 6 months following therapy. We examined our participants 6-8 weeks following treatment with etanercept. Our findings are consistent with clinical studies that have shown a hedonic effect of etanercept that precedes clinical improvement of psoriasis (Tyring et al., 2006).

Our findings are in keeping with preclinical studies that have shown an association between TNF- $\alpha$  and 5-HTT. TNF- $\alpha$  treatment is associated with increases in MAPK-dependent 5-HTT uptake capacity, maximum uptake velocity and mRNA expression (Malynn et al., 2013; Mossner et al., 1998; Zhu et al., 2006). These changes have been associated with behaviour consistent with 'sickness behaviour' in rodent models (Zhu et al., 2010). [<sup>123</sup>I]-beta-CIT uptake does not differentiate between changes 5-HTT affinity to its ligands, 5-HTT density on the cell surface, or serotonergic neuronal

numbers. While it could be argued that at least some of 5-HTT activity is dependent on its cell-surface density, there clearly exist mechanisms by which synaptic serotonin is controlled by mechanisms independent of transporter numbers. In this context, it should be noted that [ $^{123}\text{I}$ ]-beta-CIT uptake also does not differentiate glial and neuronal 5-HTT availability. In contrast to previous studies, we did not find any correlation between other circulating inflammatory markers and brainstem 5-HTT availability (Ramamoorthy et al., 1995). Since we did not measure central cytokine concentrations, it is not possible to infer if circulating TNF levels reflects central levels. While it is known that TNF is produced in the central nervous system (CNS) by astrocytes, microglia and certain neurons, CSF levels of TNF have been found to be significantly lower than plasma levels (Ellison et al., 2005; Lampa et al., 2012). At physiological conditions, the presence of soluble and cell bound TNF- $\alpha$  in the brain, makes it difficult to estimate CSF TNF- $\alpha$  levels reliably. Interestingly, in our sample, circulating TNF- $\alpha$  level did not correlate significantly with other pro-inflammatory cytokines suggesting TNF- $\alpha$  explain significant variance in 5-HTT that is independent of other cytokines (Supplemental tables 3 & 4). Many factors will regulate their measurable concentrations of cytokine in serum or plasma including synthesis rate, renal clearance, presence of soluble neutralising receptors etc. Thus we would not have expected direct correlations between cytokine measures. IL-6 levels correlated with to CRP ( $\rho=0.68$ ;  $p=0.01$ ) given the rather direct role it has in driving hepatocyte acute phase reactant synthesis. However, it is unclear if this lack of correlation between inflammatory markers was reflected centrally. Previous research has shown

that relationship between TNF and other cytokines differ in the periphery and the CNS (Skelly et al., 2013). In addition, we restricted our analysis to those cytokines that have shown significant elevation in depressive illnesses. Previous studies have found a significant association between other inflammatory markers (that we did not explore), including IFN  $\alpha$ ,  $\gamma$  and 5-HTT in animal models (Katafuchi et al., 2006; Morikawa et al., 1998; Tsao et al., 2008).

So, can circulating inflammation have an effect centrally? Circulating inflammatory markers signal the brain through neuronal and humoral pathways. Essentially, inflammatory mediators activate neuronal pathways, including vagal afferents, spinal afferents and humoral pathways including sensory circumventricular organs. These interoceptive signals are integrated in the 'viscero-sensory hubs' within the brainstem including the raphe nuclei, with further relay to higher subcortical and cortical centres, evoking sickness behaviour including fatigue, malaise etc (Brydon et al., 2008; Critchley and Harrison, 2013; Harrison et al., 2009b; Strike et al., 2004). Brainstem raphe nuclei are highly connected with circumventricular organs and their proximity to the cerebral aqueduct make them vulnerable to inflammatory insults (Hornung, 2003; Howerton et al., 2014). Modulating peripheral inflammation has been known to increase expression of CNS cytokine mRNA in rodents, activate CNS microglia (measured using a translocator protein (TSPO) PET imaging ligand, [ $^{11}\text{C}$ ]-PBR28) in live primates, and modulate glucose metabolism and BOLD response in regions associated with mood regulation in humans (Hannestad et al., 2012a; Hannestad et al., 2012b; Harrison et al., 2009a; Skelly et al., 2013). While

raised circulating inflammatory markers have been found to be associated with depression, two studies that have explored the relationship between depression and microglial activation (measured using PET imaging of the TSPO) in humans have found conflicting results (Hannestad et al., 2013; Setiawan et al., 2015). Interestingly, both studies found no relationship between circulating inflammatory markers and TSPO binding suggesting that circulating inflammatory markers may not reflect central processes and to some extent, may be independent of microglial processes.

Our findings add to a handful of other studies that have demonstrated an association between systemic inflammation and molecular substrates of behaviour, in particular the dopaminergic system in primates and humans (Capuron et al., 2012; Felger et al., 2013). Serotonergic systems interact highly with dopaminergic systems it is likely that these systems interact and there are a number of intermediate steps that link circulating TNF to brainstem 5-HTT availability.

The reduction in brainstem 5HTT availability following specific TNF inhibition, was at best, modest (average reduction - 9% and 36% in the best case). The minimal effective dose for SSRIs produces a 5-HTT occupancy of around 70 to 80% measured using SPECT (Preskorn, 2012). A recent meta-analysis of antidepressant effects of anti-inflammatory agents found an effect size similar to those of conventional antidepressant agents (Kohler et al., 2014). While we found a significant reduction in BDI scores, it is unlikely that all of the variance in reduction in depression scores is mediated fully through changes in 5-HTT availability. Indeed, Raison et al noted that antidepressant effect was present in those who have not responded to



conventional antidepressants which block 5-HTT activity (Raison et al., 2013).

Our study had several limitations. We have assumed that the changes in the brain are a direct result of changes in circulating inflammatory markers. However, the relationship between serotonin and inflammation is thought to be bidirectional. In other words, central serotonin can have an effect on circulating inflammatory markers. For example, 5HT<sub>2A</sub> receptor activation has been known to suppress TNF- $\alpha$  induced inflammation with extraordinary potency (Yu et al., 2008). In addition, anecdotal evidence suggest that antidepressants that increase serotonergic neurotransmission have anti-inflammatory and analgesic effects. Due to ethical concerns, we did not have a control subject group, when examining the effect of etanercept on 5-HTT availability and the participants were not blind to the intervention. It should be noted that all patients were treated with a disease modifying agent (DMARD) prior to treatment with etanercept, and the pre-treatment level of 5-HTT is reflective of this. Our data suggest that specific TNF inhibition is associated with further reduction in 5-HTT availability. Our sample size was small, but grounded on power calculation based on our pilot study (Cavanagh et al., 2010). However, we may have been underpowered to detect correlations between other circulating inflammatory markers and 5-HTT availability. We were not powered enough to explore the effects of a large number of covariates in the model. Nevertheless, our findings with these small numbers reflect non-trivial effects. Vitally, we have replicated our findings in two independent samples, indicating the robustness of our findings.

We did not compare the 5-HTT and cytokine levels between healthy subjects and psoriasis patients, as this was not the primary aim of the study.

The median cytokine levels were higher in the psoriasis population in general. Age, gender, circadian rhythm, food intake, exercise and stress can explain the variance in cytokine levels in health. While we controlled for the effect of age and gender and plasma was extracted at the same time of day, using the same protocol, our healthy group was older and also was comprised of menopausal women. Menopausal status has been associated with increase in cytokine levels (Pfeilschifter et al., 2002). This may account for the variance in cytokines in this population.

We used SPECT to measure 5-HTT availability, due to ease of access to [<sup>123</sup>I]-beta-CIT and access to the 12-detector dedicated head SPECT unit (Neurofocus 900) equipped with high-resolution collimator with an in-slice and z-axis resolution of 7 mm full-width half-maximum. Although more selective and superior ligands for 5-HTT are available, the uptake of [<sup>123</sup>I]-beta-CIT in the brainstem at 4 hours is a validated measure of 5-HTT availability (de Win et al., 2005b).

In summary, we have shown a significant association between circulating TNF- $\alpha$  and central 5-HTT availability in two independent samples. Greater circulating TNF- $\alpha$  was associated with greater 5-HTT. Treatment with Etanercept was associated with a reduction in 5-HTT levels. Our findings give some evidence of the relationship between how inflammatory markers may directly affect substrates of the brain responsible for mood regulation. We provide additional evidence as to how these therapeutic agents may be useful in depression.

**Acknowledgements:** Dr Rajeev Krishnadas was supported by Theresa and Mortimer Sackler foundation and a Wellcome Trust travel award to attend the EBPS Immunopsychiatry workshop at Brighton in 2014. We would like to acknowledge the Scottish mental health research network (SMHRN) research assistant Seonaid Cleare, nurses Anne Thorrat and May Gallagher.

**Conflict of interest:** The SPECT scans done on the psoriasis/psoriatic arthritis patients were funded by research support from Pfizer. Pfizer was not involved in the study design, collection, analysis of data and decision to publish. The authors declare no other conflicts of interest pertaining to this study.

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Table 1: Details of demographic and clinical measures

	Healthy volunteers median (interquartile range)	Pre-treatment median (interquartile range)	Post- treatment median (interquartile range)	Z*	p* (FDR correct ed p)	r
Age (mean/s.d.)	57 (5.22)	47.58 (8.88)				
Gender n of females(%)	13 (100)	6(50%)				
5-HTT	1.05(0.2)	1.10(0.2)	1.00(0.3)	-2.09	.03 (0.04)	0.60
TNF (pg/ml)	3.8 (3.65)	12.75(83.56)	248.87(273.34)	-2.70	.007 (0.02)	0.77
IL1B (pg/ml)	3.2 (5.35)	3.39(5.52)	7.29(9.51)	-1.37	.16 (0.18)	0.39
sIL2R (pg/ml)	2.3 (1.15)	4.6(2.23)	4.7(1.2)	-0.05	0.9 (0.9)	0.01
CRP (mg/ml)	1.78 (2.17)	3.90(9.2)	3.26(5.57)	-2.59	.009 (0.02)	0.75
IL6 (pg/ml)	not available	2.84(5.41)	1.79(2.17)	-2.36	.01 (0.02)	0.68
BDI	8 (5)	10 (11.75)	5 (6.75)	-2.2	.02 (0.03)	0.63

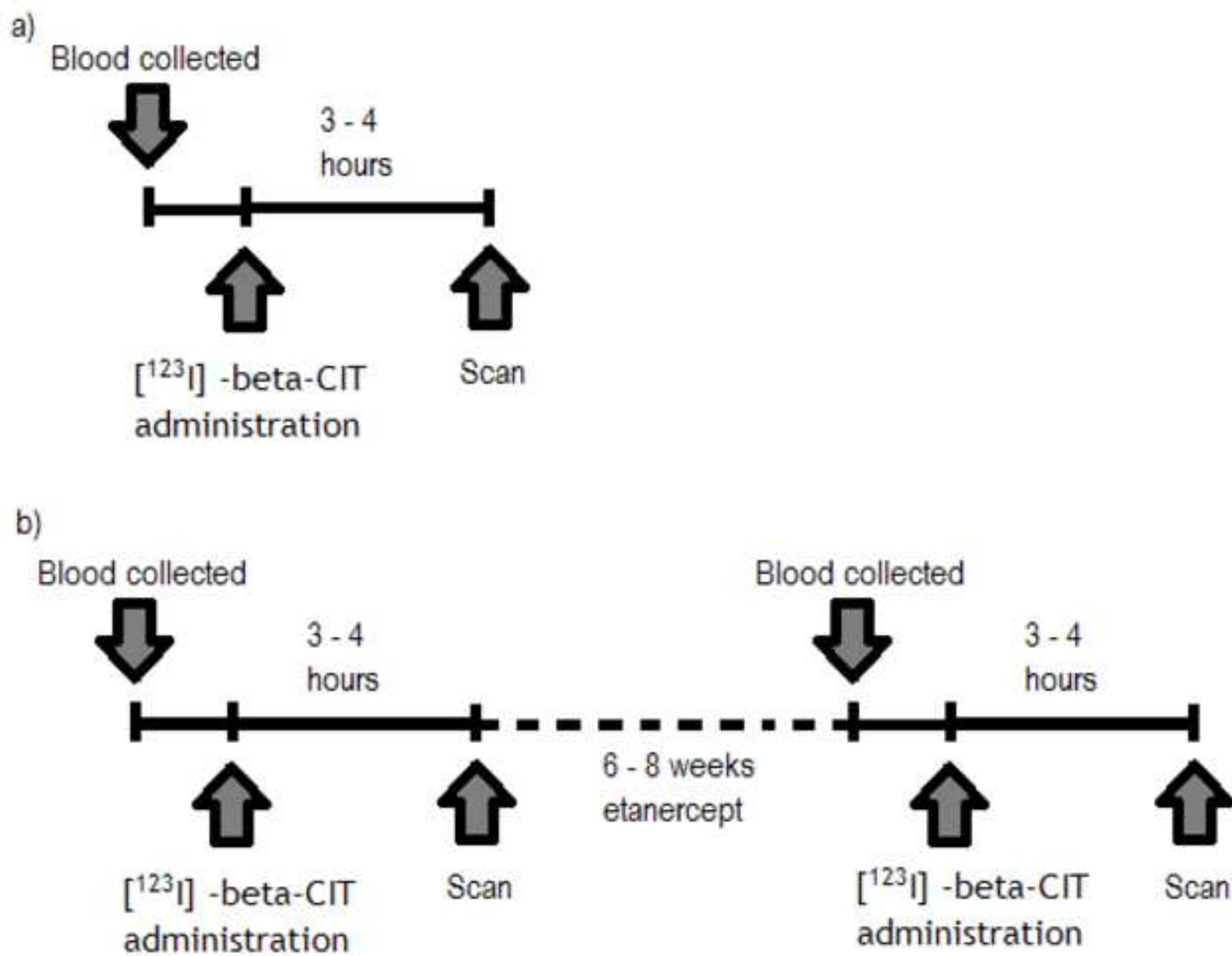
\*Wilcoxon Signed-ranks test exploring the difference between variables pre and post etanercept; r = effect size calculated as  $Z/\sqrt{n}$ ; FDR - False discovery rate corrected probability for 7 comparisons.

**Figure 1:** Study design. a) healthy subjects ; b) psoriasis/psoriatic arthritis subjects

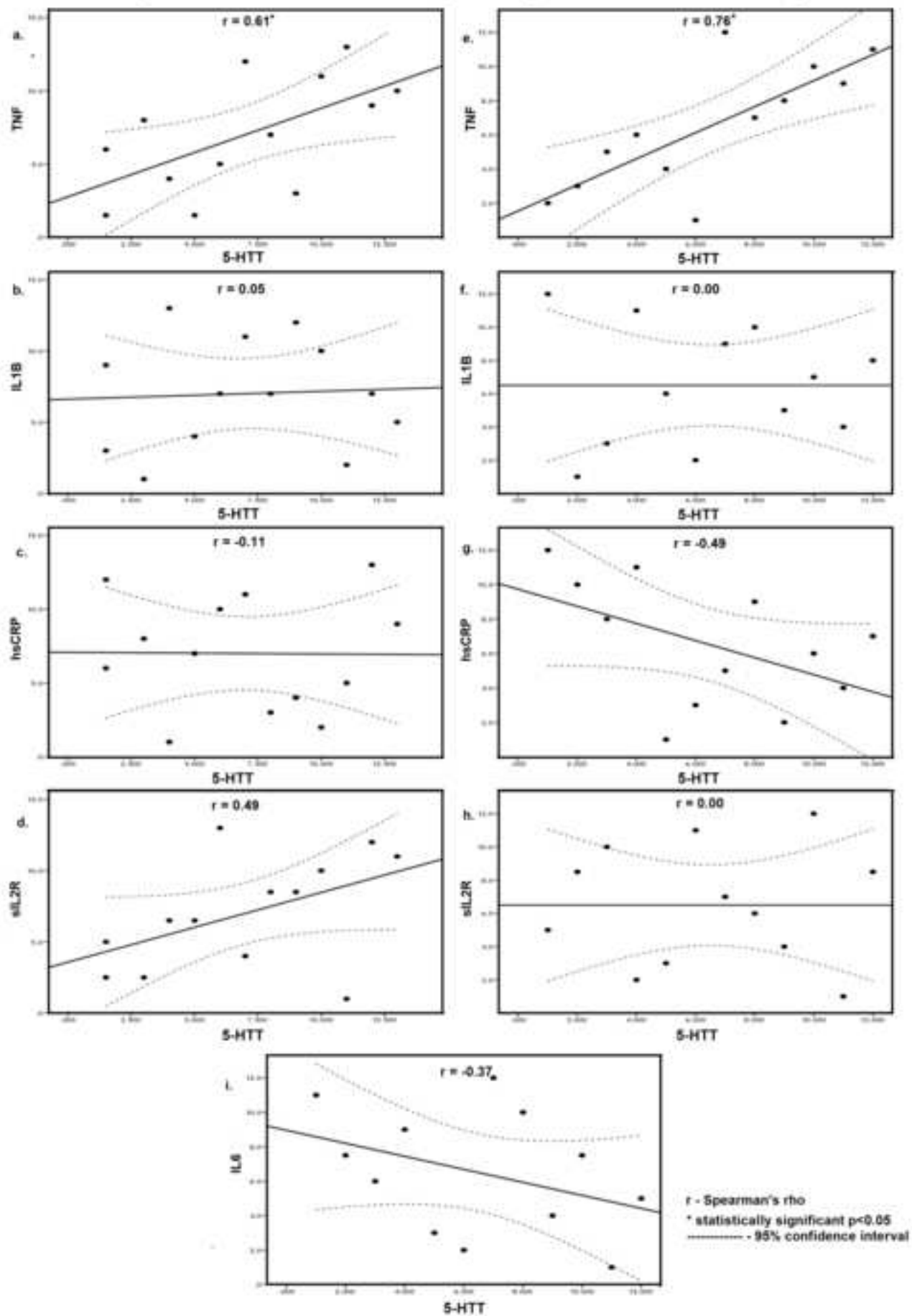
**Figure 2:** Scatterplot showing the spearman's rank correlation between circulating inflammatory markers and brainstem 5-HTT availability. Scatterplot a,b,c and d show the relationship in healthy individuals. Only the relationship between TNF and 5-HTT in reached statistical significance ( $\rho=0.61$ ;  $p=0.03$ ). 5HTT was corrected for age; . Scatterplot e, f, g, h and i show the association between circulating inflammatory markers and brainstem 5-HTT in psoriasis/psoriatic arthritis. Only the relationship between TNF- $\alpha$  and 5-HTT reached statistical significance ( $\rho=0.76$ ;  $p=0.004$ ). 5HTT corrected for age, sex and diagnosis. Dotted lines depict 95% confidence intervals.

**Figure 3:** Indirect effects analysis shows that 5-HTT mediates the association between TNF- $\alpha$  and BDI scores in the whole sample ( $n=25$ ). The mediation analysis partitions the total variance (total effect - c) explained by the predictor into a part that is independent of the mediating variable (direct effect - c'), and a part that is accounted for via the mediating variable (indirect effect - a\*b). a represents the 'a' path and b represents the 'b' path.

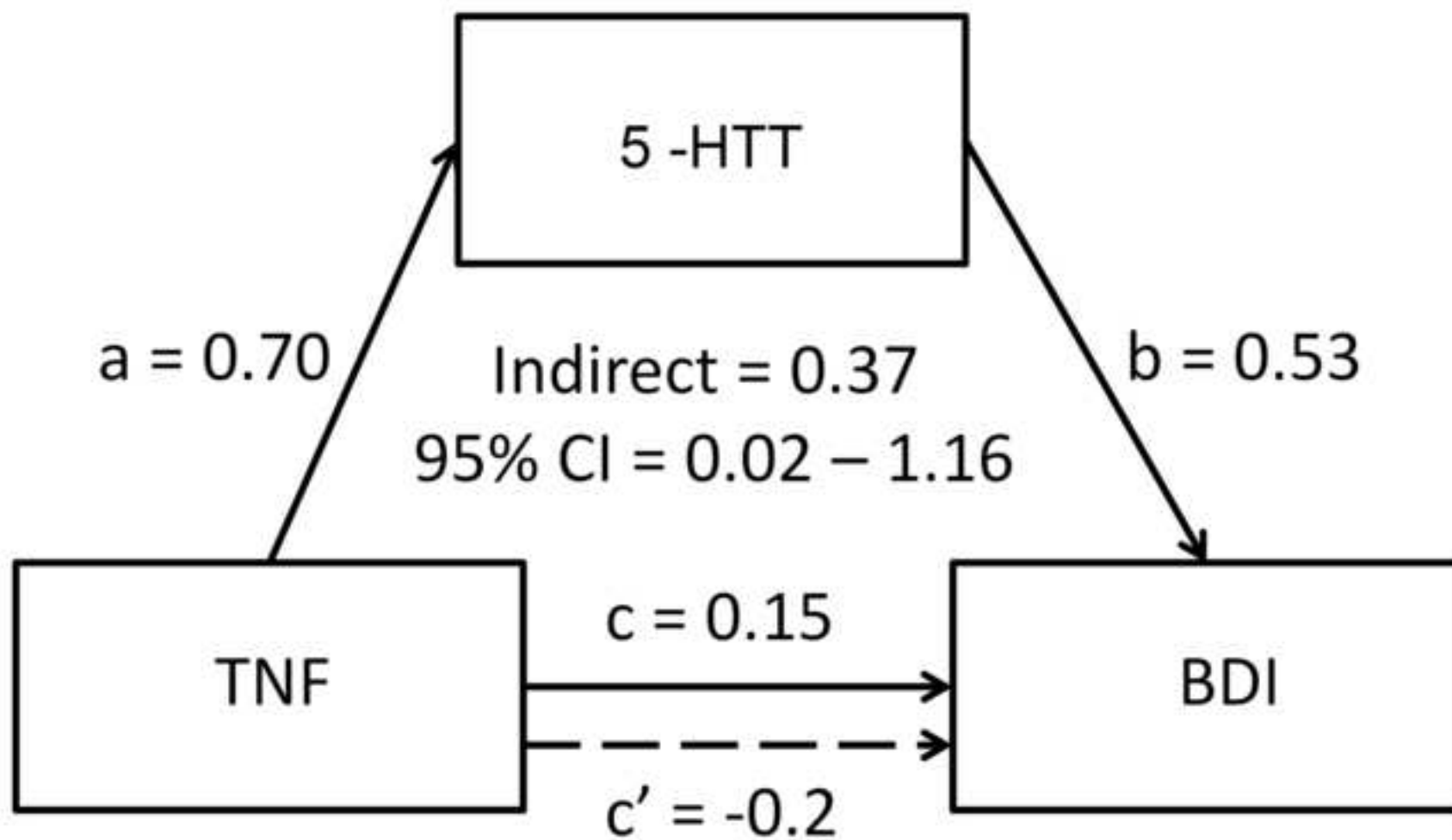
**Figure 4:** A representative scan (sagittal image) of a patient showing [ $^{123}$ I] - beta-CIT uptake at 4 hours in the brainstem (arrows) - before (top row) and after (bottom row) treatment with Etanercept.

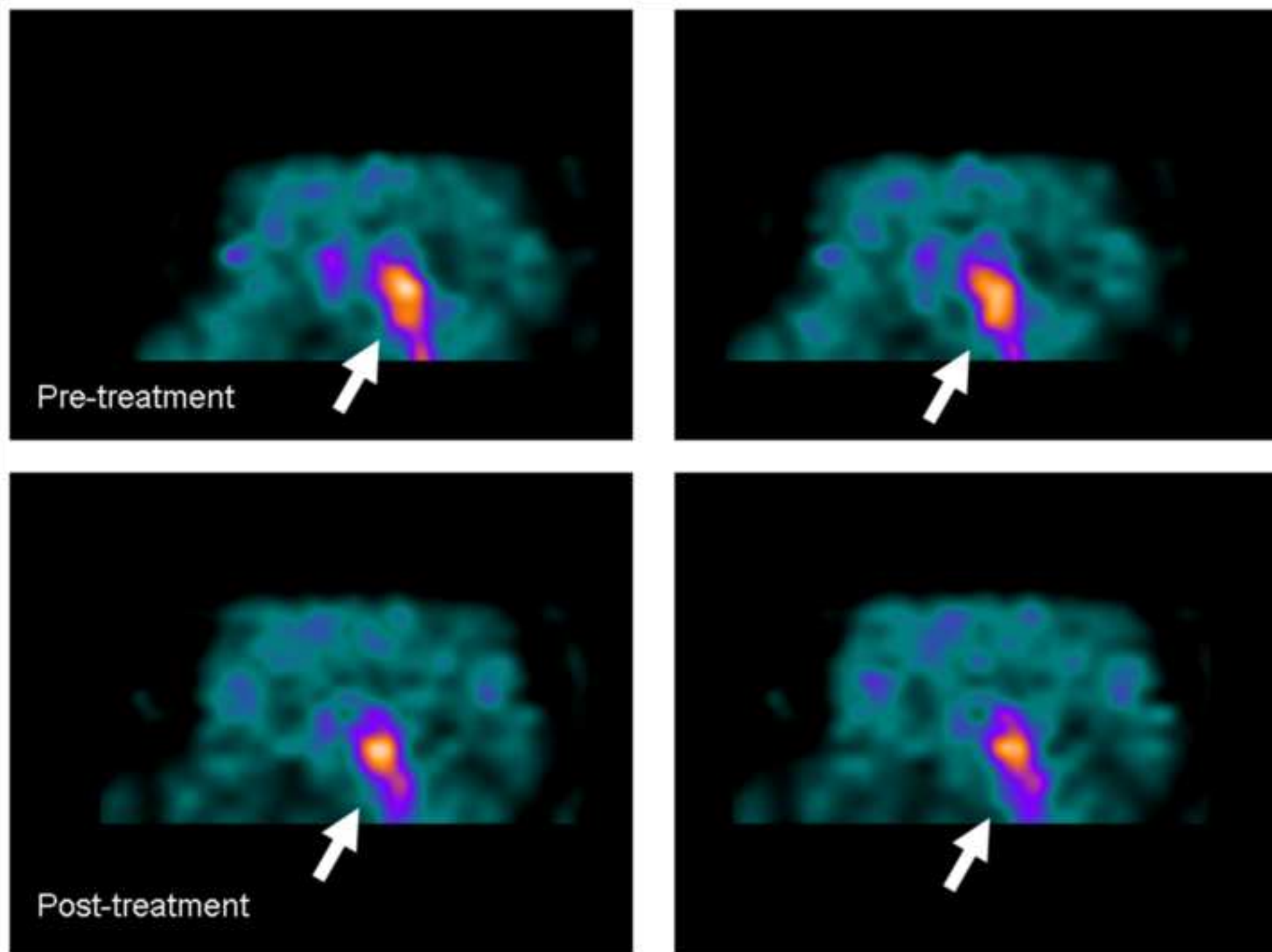


Spearman's rank correlation between circulating inflammatory markers and midbrain 5-HTT availability









## Highlights

- Preclinical studies have found that cytokines affect 5HTT availability.
- In humans, circulating TNF- $\alpha$  correlated with brainstem 5HTT availability.
- Specific TNF antagonism was associated with reduction in 5HTT availability.

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