Anxiety is associated with higher levels of global DNA methylation and altered expression of epigenetic and interleukin-6 genes

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Objectives **Anxiety is associated with elevated levels of the inflammatory cytokine interleukin-6 (IL-6) and an increased risk for diseases with an inflammatory aetiology. In cancer, higher levels of IL-6 have been associated with increased expression of the epigenetic enzymes *DNMT1* and Enhancer of Zeste Homolog 2 (*EZH2*). However, the relationship between IL-6 and DNA methyltransferases (DNMTs) and *EZH2* expression has not previously been examined in anxious individuals.**

Methods **Global DNA methylation levels were measured using the Methylflash Methylated DNA Quantification Kit and gene expression levels of the *DNMT* and *EZH2* genes in anxious ($n = 25$) and nonanxious individuals ($n = 22$) were compared using quantitative real-time PCR. Specifically, we investigated whether global DNA methylation or aberrant expression of these genes was correlated with *IL-6* mRNA and protein serum levels in anxious individuals.**

Results **Anxious participants had significantly higher levels of global DNA methylation compared with controls ($P = 0.001$). There were no differences in the mean mRNA expression levels of the *DNMT1/3A/3B*, *EZH2* and *IL-6* genes in anxious individuals compared with controls. However, the expression of *DNMT1/3A*, *EZH2* and *IL-6* genes increases with increasing Hospital Anxiety and Depression Scale-Anxiety scores in the anxious cohort only. Interestingly, *IL-6* gene expression was correlated strongly with *DNMT1/3A/3B* and *EZH2* expression, highlighting a potential relationship between *IL-6* and important epigenetic regulatory enzymes.**

Conclusion **This study provides novel insight into the relationship between anxiety, epigenetics and *IL-6*. Moreover, our findings support the hypothesis that changes in DNA methylation profiles may contribute to the biology of anxiety. *Psychiatr Genet* 25:71–78**

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Keywords: anxiety, DNA methylation, DNA methyltransferase, inflammation, interleukin-6

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Introduction

Anxiety is associated with an increased risk for many diseases with an inflammatory aetiology including cardiovascular and autoimmune diseases and some forms of cancer (Kiecolt-Glaser *et al.*, 2002). However, the mechanisms by which anxiety might promote these diseases are not well understood. Prolonged upregulation of proinflammatory cytokines is thought to contribute to chronic inflammation in anxious individuals, which may mediate their increased risk for inflammation-related diseases (Kiecolt-Glaser *et al.*, 2002). Previously, our group has shown that anxious individuals have significantly higher serum levels of the systemic proinflammatory cytokine, interleukin-6 (IL-6), compared with nonanxious individuals (O'Donovan *et al.*, 2010). In turn, high levels of IL-6 increase the risk for cardiovascular and autoimmune diseases as well as cancer (Kiecolt-Glaser *et al.*, 2003).

Epigenetics, defined as the mechanisms that initiate and maintain heritable patterns of gene expression without altering the sequence of the genome (Holliday, 1987), may play an important role in the pathology of psychiatric disorders (Poulter *et al.*, 2008; Zhubi *et al.*, 2009) and inflammatory-related diseases, including cancer (Baylin, 2005; Wilson, 2008). The epigenetic mark DNA methylation involves the addition of methyl groups to the 5′-position of cytosine rings [5-methylcytosine (5-mC)] at CpG dinucleotides. DNA methylation is orchestrated by several DNA methyltransferases (DNMTs – DNMT1, DNMT3A, DNMT3B, DNMT3L) in combination with polycomb group proteins (Viré *et al.*, 2006). DNMT1 is a maintenance enzyme that binds methyl groups to hemimethylated DNA during DNA replication

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(Smith *et al.*, 1992). DNMT3A and 3B are de-novo DNMT enzymes that establish methylation patterns during embryonic development and genomic imprinting (Chédin, 2011). DNMT3L interacts with and enhances DNMT3A/3B methyltransferase activity *in vivo* (Chen *et al.*, 2005). Interestingly, the polycomb group protein, Enhancer of Zeste Homolog 2 (EZH2), is known to interact with DNMTs and is required for DNA methylation of EZH2-target promoters (Viré *et al.*, 2006). Thus, DNMTs and EZH2 are essential epigenetic regulatory enzymes involved in the heritable repression of gene activity.

Higher levels of IL-6 are associated with increased expression and activity of DNMT1 and EZH2 in cancer (Croonquist and Van Ness, 2005; Foran *et al.*, 2010). Interestingly, IL-6-dependent miRNAs that regulate *DNMT1* gene expression levels in malignant cholangiocytes have recently been identified (Braconi *et al.*, 2010). Therefore, regulation of miRNAs may represent a mechanism by which IL-6 induces the expression of *DNMT1*. Conversely, epigenetic modifications such as DNA methylation are excellent biological candidates for the molecular arbitration of the immune-dysfunction phenotype associated with anxiety. DNA methylation can target genes for transcriptional silencing (Baylin, 2005) and is heavily influenced by environmental triggers such as diet, smoking and traumatic life events (Uddin *et al.*, 2010; Nandakumar *et al.*, 2011; Scoccianti *et al.*, 2011). In this way aberrant DNA methylation is a hallmark of a number of human diseases (Robertson, 2005). Recently, immune-related genes were identified as targets of aberrant DNA methylation in post-traumatic stress disorder, supporting the hypothesis that DNA methylation contributes to the biology of anxiety-related disorders (Uddin *et al.*, 2010).

Given the importance that epigenetic regulatory genes have in the establishment and maintenance of DNA methylation patterns, it is likely that changes in their expression contribute to the molecular aetiology of neuropsychiatric disorders including anxiety. Moreover, altered epigenetic regulatory gene expression has been observed in patients with neuropsychiatric disorders (depression and schizophrenia) and has been found to be associated with increased anxiety levels in a mouse model of Rett syndrome (Poulter *et al.*, 2008; Adachi *et al.*, 2009; Zhubi *et al.*, 2009). However, the relationship between the cytokines expressed, such as IL-6, in response to anxiety and *DNMTs/EZH2* expression and global DNA methylation has not previously been investigated. Thus, we profiled (i) the mRNA expression levels of *DNMT1*, *DNMT3A*, *DNMT3B*, *DNMT3L* and *EZH2*, and (ii) the global methylation changes in a cohort of anxious and nonanxious individuals and associated the findings with the mRNA and protein serum IL-6 levels in our patient cohorts.

Methods

Clinical sample collection

Participants were selected from an anxiety cohort described previously (O'Donovan *et al.*, 2010). Briefly, participants experiencing high or low levels of psychological distress were recruited from a large suburban area in Dublin, Ireland. The sample cohort included 27 participants with Hospital Anxiety and Depression Scale-Anxiety (HADS-A) scores in the clinical range (Zigmond and Snaith, 1983; Bjelland *et al.*, 2002; anxious group, scores > 8) and 29 age-matched participants with low scores on the HADS-A (nonanxious group, scores < 8). Exclusion criteria were chronic illness, current or acute illness within the previous 2 weeks, alcoholism, use of medication, anaesthesia in the previous 3 months and night shift work in the previous 2 weeks. The HADS measure (a widely used and well-validated measure of nonsomatic symptoms of anxiety and depression) and the self-reported physical health measures used in this study have been described in detail previously (O'Donovan *et al.*, 2010). HADS-D scores and serum IL-6 levels had been previously measured for each participant (O'Donovan *et al.*, 2010). Blood samples were collected between 7:30 and 9:30 a.m. Participants fasted and abstained from smoking and caffeine from midnight, and avoided alcohol and exercise for 24 h before their appointment. The study was approved by the Vincent's Healthcare Group Ethics and Medical Research Committee.

Clinical biological sample collection

Twenty-five participants with HADS-A scores in the clinical range (anxious group) and 22 participants with low HADS-A scores (nonanxious group) had DNA/RNA of sufficient quantity and quality to carry out gene expression and global methylation analyses. Participants' blood samples (15 ml) were collected in sterile heparinized tubes and processed immediately. Whole-blood was overlaid onto a Ficoll-Paque layer and centrifuged for 30 min at 400*g*. Peripheral blood mononuclear cells (PBMCs) were removed from the interface, stained using trypan blue and counted using a haemocytometer. Cells were subsequently transferred into liquid nitrogen for long-term storage. Genomic DNA and total RNA were extracted from the PBMCs using an AllPrep DNA/RNA Mini Kit (Qiagen Ltd, West Sussex, UK). Concentration (absorbance at 260 nm) and purity (ratio of absorbance at 260 nm to absorbance at 280 nm) of the DNA and RNA were measured using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Delaware, Texas, USA). DNA and RNA samples with an A260 : A280 ratio between 1.7 and 2.0, and 1.9 and 2.1, respectively, were selected for methylation and expression analyses.

Gene selection

DNMT1, *DNMT3A*, *DNMT3B*, *DMNT3L* and *EZH2* were chosen on the basis of the following criteria: (i) their known epigenetic regulation functionality (defined by the Gene-Ontology database, *http://www.geneontology.org/*),

(ii) evidence of their altered expression in anxiety or other associated psychiatric disorders (Poulter *et al.*, 2008; Zhubi *et al.*, 2009) and (iii) evidence of their direct/indirect regulation by IL-6 in inflammation-related diseases (Croonquist and Van Ness, 2005; Foran *et al.*, 2010). In addition, *IL-6* mRNA expression was also determined.

Gene expression analysis

Differential gene expression between anxious and nonanxious individuals was determined by quantitative real-time PCR using the comparative C_T method. All reagents were supplied by Applied Biosystems (Carlsbad, California, USA) unless otherwise stated. cDNAs were synthesized from total RNA (120 ng) using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, California, USA). Custom-made TaqMan 96-well array plates containing six predesigned inventoried TaqMan assays (*DNMT1*: *Hs00154749_m1*, *DNMT3A*: *Hs01027166_m1*, *DNMT3B*: *Hs00171876_m1*, *DNMT3L*: *Hs01081364_m1*, *EZH2*: *Hs01016789_m1*, *IL-6*: *Hs00985639_m1*) and an endogenous control gene (*ACTB: Hs99999903_m1*) were used. Quantitative real-time PCR assays consisted of cDNA (4 ng) and the 2XTaqMan Universal PCR master mix, and they were performed in triplicate under standard real-time cycling conditions on a 7500HT real-time PCR. Gene expression analysis was carried out on SDS 7500 softwareV2.0 (Applied Biosystems, Carlsbad, California, USA). The levels of target gene expression in participant samples were calculated using the ΔC_T method (normalized to the endogenous control gene, *ACTB*), and target fold change, relative to a calibrator (average C_T value of the nonanxious group), was used to generate relative quantification (RQ) values.

Global DNA methylation quantification in participant peripheral blood mononuclear cells

Global DNA methylation quantification was performed as per the manufacturer's instructions using the Methylflash Methylated DNA Quantification Kit (Epigentek, Farmingdale, New York, USA). Briefly, methylated DNA was detected using capture and detection antibodies to 5-mC and then quantified colorimetrically by reading the absorbance at 450 nm using a Multiskan-EX Microplate Photometer (Thermofisher Scientific Inc., Waltham, Massachusetts, USA). The absolute amount of methylated DNA (proportional to the optical density intensity) was measured and was quantified using a standard curve plotting the optical density values versus five serial dilutions of control methylated DNA (0.5–10 ng).

Statistical analyses

A sample size of 52 has 80% power to detect significant changes in global DNA methylation and gene expression, which have a large effect size ($d = 0.8$, $\alpha = 0.05$). This study may be underpowered to detect significant small–medium changes in global DNA methylation and gene expression between the two groups. Power calculations were performed using G*Power version 3.1 (Faul *et al.*, 2007). A nonparametric Mann–Whitney *U*-test was performed to calculate the differences in mean ΔC_T values, global DNA methylation levels (5-mC%) and age in anxious versus nonanxious participants. Pearson's χ^2-test was used to compare frequencies of men, women and smokers among anxious individuals and controls. Spearman's rank-correlation was used to assess relationships between continuous variables. Multiple regression analysis was used to control for potential confounders and to explore the relationship between variables associated with HADS-A scores in anxious participants and *IL-6* mRNA levels. Logistic regression analysis was carried out to evaluate whether global DNA methylation levels predicted anxiety status, correcting for potential confounders.

False discovery rate-adjusted *P*-values ($P_{adjusted}$) were calculated as described previously (Hochberg and Benjamini, 1990) and are listed together with the unadjusted *P*-values. Statistical analyses were carried out using SPSS (PASW statistics 18, Chicago, Illinois, USA). For all tests, significance was ascribed at *P* less than 0.05.

Results

Clinical and psychological features of the study population

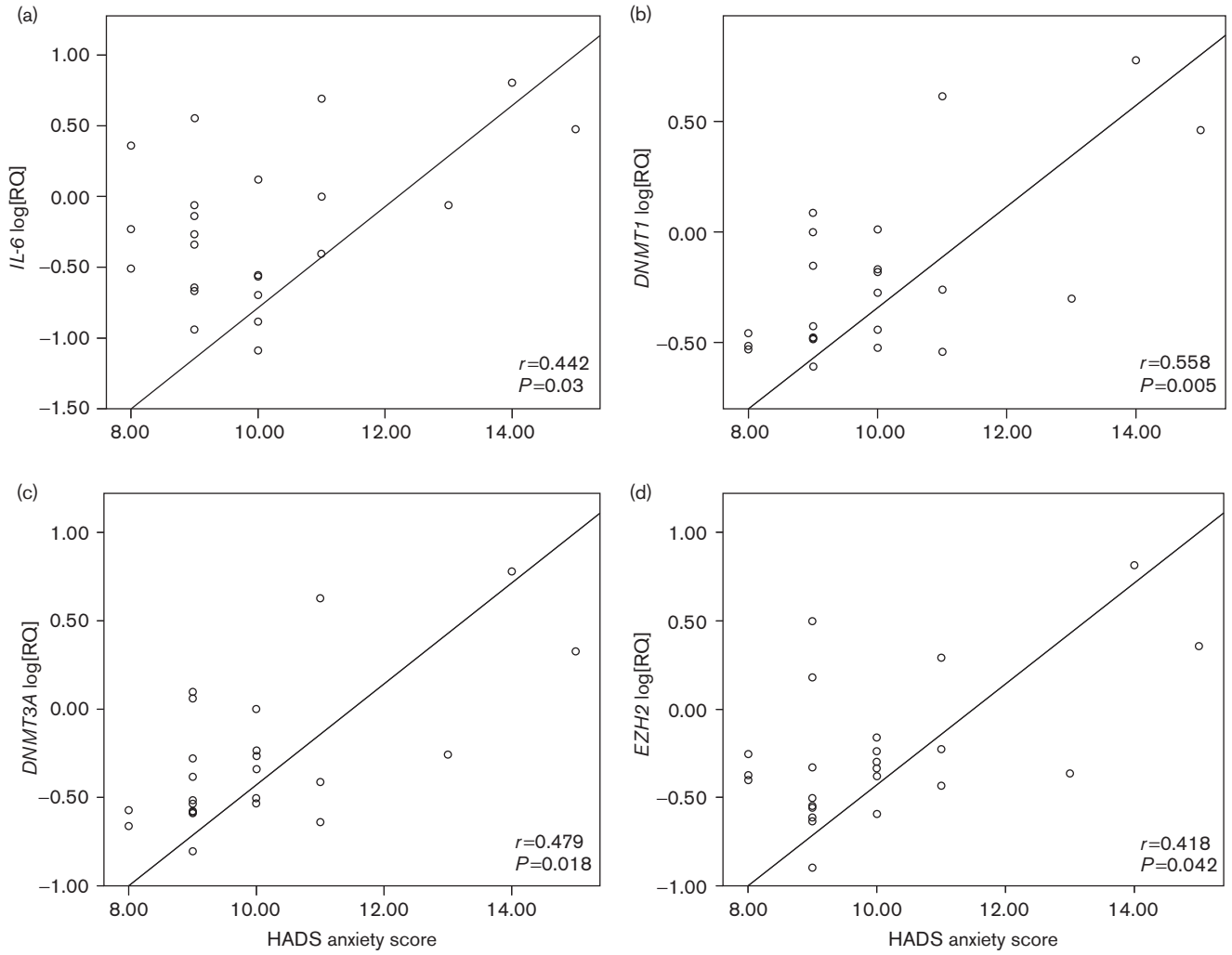
The mean age of the anxious ($n = 25$) and nonanxious ($n = 22$) groups used in the current study did not differ significantly (mean = 33.04 years vs. mean = 32.73 years, respectively; $Z = -0.771$, $P = 0.441$). The anxious cohort included 17 women and eight men. The nonanxious cohort included 14 women and eight men (Pearson's $\chi^2 = 0.99$, $d.f. = 1$, $P = 0.753$). The mean HADS-A score of the anxious and nonanxious cohorts was 9.92 (HADS-A score range: 8–15) and 5 (HADS-A score range: 2–7, $P < 0.0001$), respectively. The frequency of smokers in the anxious group was significantly higher (6/25) than in the nonanxious group (0/22; Pearson's $\chi^2 = 5.38$, $d.f. = 1$, $P = 0.01$). The groups did not differ significantly with respect to HADS-D scores ($Z = -1.301$, $P = 0.193$).

DNMTs and *EZH2* mRNA levels in anxious versus nonanxious individuals

There was no statistically significant difference in the mean levels of *DNMTs*, *EZH2* and *IL-6* gene expression (mean ΔC_T values) between anxious individuals and controls (Mann–Whitney *U*-test; $P = 0.93$, 0.4, 0.39, 0.32, 0.76, respectively). *DNMT3L* was undetectable in all samples analysed.

Interestingly, *IL-6* mRNA levels (logRQ values) were positively correlated with the HADS-A score in anxious participants ($r = 0.44$, $P = 0.03$, $P_{adjusted} = 0.08$; Fig. 1a). This trend was not observed in the nonanxious controls ($P = 0.1$). Similarly, *DNMT1/3A* and *EZH2* mRNA levels (logRQ values) were correlated significantly with the HADS-A score among anxious participants only ($r = 0.48$, $P = 0.018$,

Fig. 1



Relationship between the gene panel and HADS-A scores. (a–d) Scatter plot illustrating differences in *IL-6*, *DNMT1*, *DNMT3A* and *EZH2* gene expression levels (log[RQ] values) and HADS-A scores in anxious individuals. The correlation was assessed using Spearman's rank-correlation analysis. DNMT, DNA methyltransferase; EZH2, Enhancer of Zeste Homolog 2; HADS-A, Hospital Anxiety and Depression Scale-Anxiety; IL-6, interleukin-6; RQ, relative quantification.

$P_{\text{adjusted}} = 0.07$; $r = 0.56$, $P = 0.005$, $P_{\text{adjusted}} = 0.025$; $r = 0.42$, $P = 0.04$, $P_{\text{adjusted}} = 0.08$, respectively; Fig. 1b–d).

Multiple regression analysis was used to examine a prediction model of HADS-A scores in anxious participants only. The model, which included logRQ values for *DNMT1*, *DNMT3A*, *DNMT3B* and *EZH2* as independent variables and controlled for potential confounders (age, sex, HADS-D scores, smoking and total cell count), was a significant predictor of HADS-A scores in the anxious cohort ($F = 8.464$, *d.f.* $= 9$, $P = 0.001$). This model explained 76.8% (adjusted $R^2 = 0.768$) of the variance in the HADS-A scores in the anxious group. However, mRNA expression of *DNMT1*, *DNMT3A/3B* and *EZH2* in PBMCs was highly correlated in our cohort (Supplementary Figure 1, Supplemental digital content 1, *http://links.lww.com/PG/A116*). Thus, the contribution of each gene to the model could not be accurately accessed. The logRQ values of each epigenetic regulatory gene and *IL-6* were entered into the model separately (controlling for potential confounders), and their contribution to the model was examined (Supplementary Table 1, Supplemental digital content 2, *http://links.lww.com/PG/A117*). No genes were statistically significant independent predictors of HADS-A scores among anxious individuals (Supplementary Table 1, Supplemental digital content 2, *http://links.lww.com/PG/A117*).

Correlation of mRNA expression levels of *DNMT1/3A/3B* and *EZH2* with *IL-6* mRNA

Spearman's rank-correlation analysis demonstrated a significant positive correlation between the gene expressions of *DNMT1/3A/3B/EZH2* and *IL-6* (*DNMT1*: $r = 0.56$, $P \leq 0.001$, $P_{\text{adjusted}} \leq 0.002$; *DNMT3A*: $r = 0.55$,

$P \leq 0.001$, $P_{adjusted} \leq 0.002$; *DNMT3B*: $r = 0.33$, $P = 0.03$, $P_{adjusted} = 0.03$, *EZH2:* $r = 0.55$, $P \leq 0.001$, $P_{adjusted} \leq 0.002$; Fig. 2). Multiple regression analysis revealed that *DNMT1*, *DNMT3A* and *DNMT3B* were significantly associated with *IL-6* gene expression ($P < 0.001$, < 0.001, $= 0.002$, respectively), controlling for age, sex and total cell count (Supplementary Table 2, Supplemental digital content 3, *http://links.lww.com/PG/A118*). In the anxious participants, an increase in the logRQ value of *DNMT1, DNMT3A* and *DNMT3B* by 1 SD (0.58, 0.35 and 0.47, respectively) coincided with an increase in the *IL-6* logRQ value by 0.44, 0.39 and 0.27, respectively. In this study, we found no significant correlation between *DNMT1*, *DNMT3A/3B*, *EZH2* and *IL-6* mRNA expression in PBMCs and protein IL-6 levels in the serum (data not shown).

Global methylation (5-mC%) quantification in anxious versus nonanxious controls

Global methylation (5-mC%) analyses showed that anxious participants had significantly higher levels of global DNA methylation compared with controls (median 5-mC% ± SD: patients, 0.74 ± 0.25; controls, 0.42 ± 0.21; $Z = -3.197$, $P = 0.001$, Mann–Whitney test; Fig. 3). Controlling for potential confounders (age, sex, total cell number, HADS-D scores and smoking), global 5-mC% was found to be significantly associated with anxiety (odds ratio = 48.5; 95% confidence interval, 1–2537.1; $P = 0.05$). In contrast, a negative correlation between 5-mC% and expression of *DMNT1*, *DNMT3A*, *DNMT3B* and *EZH2* was observed ($r = -0.31$, $P = 0.055$, $P_{adjusted} = 0.275$; $r = -0.50$, $P = 0.001$, $P_{adjusted} = 0.005$; $r = -0.43$, $P = 0.007$, $P_{adjusted} = 0.035$; $r = -0.52$, $P = 0.001$, $P_{adjusted} = 0.005$, respectively). Thus, increased global 5-mC levels are associated with reduced expression of the epigenetic regulatory genes. No significant correlation between 5-mC levels and PBMC mRNA and protein serum IL-6 levels was observed in our cohort ($r = -0.18$, $P = 0.26$; $r = -0.125$, $P = 0.46$, respectively).

Discussion

We have, for the first time, identified differential global DNA methylation levels in PBMCs between anxious and nonanxious individuals. We also found that *IL-6* gene expression in PBMCs correlates strongly with *DNMTs/EZH2* expression in anxious individuals and that the expression of these genes increases with increasing HADS-A scores in the anxious cohort.

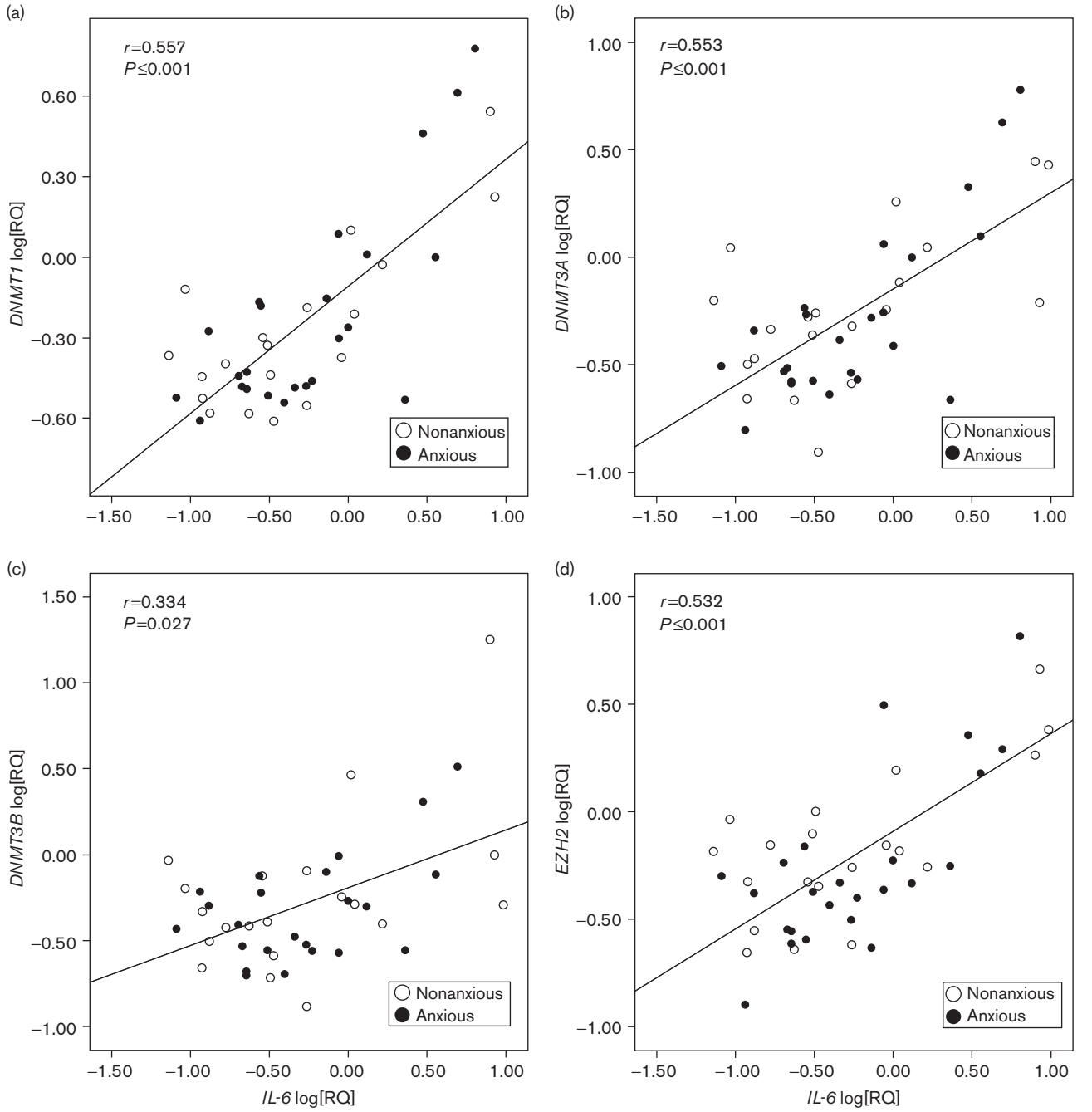
In this study, we examined mRNA expression of *DNMT1*, *DNMT3A*, *DNMT3B*, *DNMT3L*, *EZH2* and *IL-6* genes in cohorts of anxious ($n = 25$) and nonanxious ($n = 22$) individuals and found no statistical difference in the mean gene expression (ΔC_T) levels between the two groups. *DNMT3L* was undetectable in all samples analysed. Previously, *DNMT3L* was found not to be expressed in peripheral blood leucocytes in a small cohort of individuals ($n = 5$; Minami *et al.*, 2010), and our results confirm this finding. Intriguingly, in the anxious cohort, *DNMT1*, *DMNT3A*, *EZH2* and *IL-6* expression increased with increasing anxiety severity scores on the HADS-A. After adjustment for multiple testing only *DNMT3A* remained significantly correlated with HADS-A scores in the anxious cohort. This trend was not observed in the nonanxious group, which had low scores on the HADS-A. A model including the expression levels of all epigenetic regulatory genes and controlling for potential confounders was a significant predictor of HADS-A scores in anxious participants, suggesting a synergistic effect of the epigenetic regulatory genes on HADS-A scores in anxious individuals. As the expression of our genes was highly correlated in our cohort (a finding supported by previous studies that have also shown highly correlated expression of these enzymes; Robertson *et al.*, 1999; Balada *et al.*, 2008), the contribution of each gene to the prediction model could not be accurately accessed. These results warrant future investigation in a larger cohort of anxious individuals.

Interestingly, we found a significant correlation between the expression of *DNMTs* and *EZH2* and *IL-6* mRNA levels. These findings support previous studies that have demonstrated a link between *DNMT1/EZH2* and IL-6 in human disease (Croonquist and Van Ness, 2005; Foran *et al.*, 2010), and highlight a potential relationship between inflammatory cytokines and important epigenetic regulatory enzymes in anxiety. This relationship supports a biological embedding model of stress (Miller *et al.*, 2011), whereby inflammatory cytokines interact (directly or indirectly) with epigenetic enzymes, potentially resulting in the alteration of an individual's methylome in a way that contributes to and perhaps exacerbates both the anxiety and the associated inflammation phenotypes.

In this study, we found no association between *IL-6* mRNA and protein IL-6 levels in the serum. These findings are similar to those of a previous study of obese individuals, in which *IL-6* mRNA levels in PBMCs did not reflect serum IL-6 protein levels (O'Rourke *et al.*, 2006). This could be a result of post-transcriptional regulation of *IL-6* expression and/or the presence of IL-6 in the serum from sources other than PBMCs (e.g. liver and adipose tissue; O'Rourke *et al.*, 2006).

Examination of global methylation (5-mC%) levels indicated that anxious individuals had significantly higher levels of 5-mC compared with nonanxious controls, suggesting that anxious individuals have global hypermethylation of their genome. Moreover, our findings suggest that aberrant DNA methylation patterns in anxious individuals can be identified by examining methylation profiles in peripheral blood. A recent intraindividual cross-tissue study suggested that some interindividual epigenetic variation may be correlated between the blood and the brain, demonstrating that blood may act as a proxy in studies of certain neurobiological phenotypes (Davies *et al.*, 2012). Moreover, studies analysing global methylation in a number of cancers have proposed that examination of the 5-mC content in blood could

Fig. 2

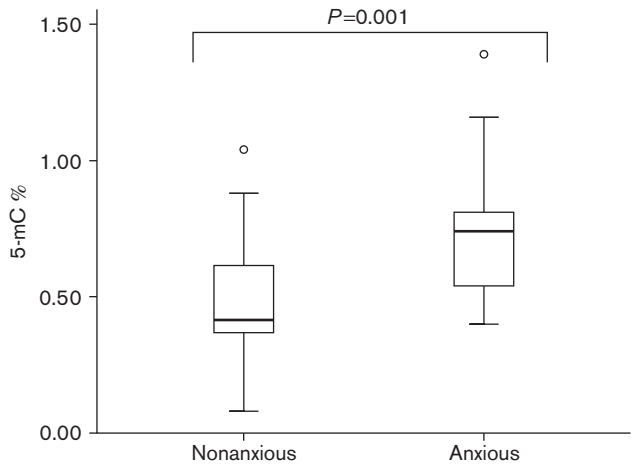


Relationship between epigenetic regulatory genes and IL-6 gene expression. Scatter plot illustrating correlation between *DNMT1*, *DNMT3A*, *DNMT3B* and *EZH2* gene expression levels and *IL-6* gene expression levels. The correlation was assessed using Spearman's rank-correlation analysis. DNMT, DNA methyltransferase; EZH2, Enhancer of Zeste Homolog 2; HADS-A, Hospital Anxiety and Depression Scale-Anxiety; IL-6, interleukin-6; RQ, relative quantification.

act as a reliable biomarker of certain cancers (Woo and Kim, 2012); thus, global hypermethylation (as assessed by 5-mC %) may represent a novel biomarker of anxiety. Blood 5-mC % levels observed in this study (< 1.5%) are consistent with those in previous studies by our group and others examining 5-mC content using an antibody to 5-mC in blood and various other tissues (Kinney and Pradhan, 2011; Nestor *et al.*, 2012; Woo and Kim, 2012; Murphy *et al.*, 2013).

DNMT and *EZH2* gene expression levels were inversely correlated with global methylation levels. Although paradoxical, these findings are in line with those of

Fig. 3



Box plot comparison of global methylation levels between anxious participants and nonanxious controls. Anxious participants had significantly higher levels of global DNA methylation [5-methylcytosine% (5-mC%)] compared with nonanxious controls ($P = 0.001$; Mann–Whitney test). Circles represent outliers.

previous studies. An inverse correlation between *DNMT1* and *DNMT3A/3B* gene expression and global methylation levels was found in patients with lupus (Balada *et al.*, 2008; Liu *et al.*, 2011). In mice, DNA methylation appears to act as a feedback regulation mechanism of *DNMT1* gene expression (Slack *et al.*, 1999). If a similar process occurs in humans, it could provide an explanation for the inverse correlation found in this study. Expression of *DNMT1/3B* is, however, associated with increased promoter hypermethylation of specific genes in both cancer and suicidal depression (Poulter *et al.*, 2008; Foran *et al.*, 2010). Therefore, future studies examining the association between *DNMT* expression and promoter hypermethylation of important neurobiological and immunological genes that may play an important role in anxiety could yield important findings.

A limitation of the current study is that the cross-sectional design does not allow us to determine the direction of the relationship observed between anxiety, IL-6 and epigenetic regulatory gene expression, and DNA methylation changes. However, Foran *et al.* (2010) have shown that treatment of colon cancer cells with IL-6 resulted in an increase in *DNMT1* expression and promoter hypermethylation of specific genes. Hence, it is likely that in severely anxious individuals increased *IL-6* gene expression (perhaps through IL-6-dependent expression of miRNAs) increases expression of the *DNMTs/EZH2* genes, and this may lead to aberrant regulation of locus-specific DNA methylation profiles.

In conclusion, this study, although preliminary, provides novel insights into the relationship between anxiety, epigenetics and the inflammatory cytokine IL-6, and provides a basis for future epigenetic studies examining gene-specific methylation changes. Moreover, individuals with severe anxiety, elevated IL-6 levels and aberrant *DNMT/EZH2* expression may be candidates for treatment with anti-IL-6 drugs or cognitive behavioural therapy (also shown to reduce proinflammatory signalling, Antoni *et al.*, 2012). Decreasing IL-6 levels in these individuals may modify epigenetic mechanisms, potentially reducing their risk for developing inflammation-related diseases in the future.

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Conflicts of interest

There are no conflicts of interest.

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