Curcumin and major depression: a randomised, double-blind, placebo-controlled trial investigating the potential of peripheral biomarkers to predict treatment response and antidepressant mechanisms of change

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ABSTRACT

A recent randomised, double-blind, placebo controlled study conducted by our research group, provided partial support for the efficacy of supplementation with a patented curcumin extract (500mg, twice daily) for 8 weeks in reducing depressive symptoms in people with major depressive disorder. In the present paper, a secondary, exploratory analysis of salivary, urinary and blood biomarkers collected during this study was conducted to identify potential antidepressant mechanisms of action of curcumin. Pre and post-intervention samples were provided by 50 participants diagnosed with major depressive disorder, and the Inventory of Depressive Symptomatology self-rated version ($IDS\text{-}SR_{30}$) was used as the primary depression outcome measure. Compared to placebo, 8 weeks of curcumin supplementation was associated with elevations in urinary thromboxane B2 ($p < .05$), and substance P ($p < .001$); while placebo supplementation was associated with reductions in aldosterone ($p < .05$) and cortisol ($p < .05$). Higher baseline plasma endothelin-1 ($rs = -.587$; $p < .01$) and leptin ($rs = -.470$; $p < .05$) in curcumin-treated individuals was associated with greater reductions in $IDS\text{-}SR_{30}$ score after 8 weeks of treatment. Our findings demonstrate that curcumin supplementation influences several biomarkers that may be associated with its antidepressant mechanisms of action. Plasma concentrations of leptin and endothelin-1 seem to have particular relevance to treatment outcome. Further investigations using larger samples sizes are required to elucidate these findings, as the multiple statistical comparisons completed in this study increased the risk of type I errors.

INTRODUCTION

In medical and pharmaceutical practice biomarkers are regularly used to assist in the prediction, diagnosis and evaluation of treatments associated with disease (Biomarkers Definitions Working Group, 2001). However, in psychiatry, biomarkers are utilised primarily for research purposes and are seldom used in clinical practice. Greater understanding of biomarkers in psychiatry has the potential to enhance diagnostic accuracy, improve treatment-matching and evaluate treatment progress. Evaluation of biomarkers can also expand understanding into the mechanisms of change associated with specific treatments for depression (Lopresti et al., 2014b).

Several dysregulated biological pathways have been identified in major depressive disorder including disturbances in monoaminergic activity, immuno-inflammation, oxidative stress, hypothalamus-pituitary-adrenal (HPA) activity and neuroprogression (Leonard and Maes, 2012). Examination of biomarkers is particularly relevant as there are identified differences between depressed and healthy populations in markers of immuno-inflammation, such as C-reactive protein, interleukin-6 and tumor-necrosis factor-α (Howren et al., 2009); markers of oxidative stress such as malondialdehyde (MDA) (Galecki et al., 2009) and 8-Hydroxy-2-deoxyguanosine (8-OHdG) (Maes et al., 2009); and markers of HPA activity such as increased baseline or post-dexamethasone cortisol (Belvederi Murri et al., 2014).

Because of its effects on all of these pathways, interest in curcumin for the treatment of major depression has increased. In animal models of depression, curcumin has demonstrated antidepressant and anxiolytic effects (Lopresti et al., 2012). Three human-based trials on people with major depressive disorder have now been completed. In one study the addition of curcumin to antidepressant treatment provided no additional antidepressant benefit (Bergman et al., 2013), whereas in another study curcumin had similar antidepressant efficacy to fluoxetine (Sanmukhani et al., 2014). However, in this latter study there was no placebo-control or blinding of participants from treatment conditions. A recent randomised, double-blind, placebo controlled study conducted by our research team provided partial support for the efficacy of curcumin in reducing depressive symptoms in people with major depression, particularly in a subset of participants with atypical depression (Lopresti et al., 2014a). In the present paper, exploratory analysis of results from this study is provided with an emphasis on the effects of curcumin on blood, urinary and salivary biomarkers, and on the potential of biomarkers to predict treatment response. The primary goal of this exploratory-driven analysis was to identify potentially important biomarkers that will require validation in future, greater powered studies.

EXPERIMENTAL PROCEDURES

Study design

Details of this study have been previously published in Lopresti *et al.* (2014a). Briefly, this study was an 8-week, randomised, double-blind, placebo-controlled clinical trial (Figure 1). Investigators responsible for study administration, data collection, intervention allocation and data analysis were blinded to treatment conditions until the collection of all participant data. The trial protocol was approved by the Human Research Ethics Committee at Murdoch University, Western Australia and was registered with the Australian New Zealand Clinical Trials Registry (no. 12612001260819) and participants were recruited between February and November 2013, across the Perth, Western Australia metropolitan area. Participants were randomly and equally allocated into two groups (placebo and curcumin) using a randomisation calculator (http://www.randomization.com). Both curcumin and placebo capsules were packed in identical containers labelled by participant code numbers and allocation was assigned by the first author according to order of participant enrolment in the study.

As no previous clinical study has investigated the effect of curcumin on most of the measured biomarkers, data to complete an *a priori* power analysis could not be determined. However, to achieve a power of 0.8, sample size estimates were based on the assumption of a moderate effect size of 0.4 indicating a total sample size of approximately 50 was required for this study.

Participants

Full details of inclusion and exclusion criteria are outlined in Lopresti *et al.* (2014a). Briefly, male and female participants aged 18 to 65 years were eligible to participate if they met the DSM-IV criteria for current major depressive disorder and had an Inventory of Depressive Symptomatology self-rated version (IDS-SR_{30}) score ≥ 14. The diagnosis of major depression was made by the first author, an experienced clinical psychologist, using The Mini International Neuropsychiatric Interview 6.0 (MINI 6.0) (Sheehan et al., 1998). Participants with a psychotic disorder, bipolar disorder, comorbid obsessive-compulsive disorder, posttraumatic stress disorder, eating disorder, chronic fatigue syndrome, fibromyalgia, or any substance abuse or dependence disorder were excluded, as were participants assessed as high risk of suicide. Volunteers were also excluded if they suffered from medical illnesses including diabetes, autoimmune diseases, cardiovascular disease, hypertension, chronic fatigue syndrome, or asthma.

Interventions

Placebo (cellulose) and curcumin capsules were supplied by Arjuna Natural Extracts Ltd. Kochi, Kerala, India, and were identical in appearance. Curcumin was provided in a 500 mg capsule (BCM-95®) containing total curcuminoids 88% (curcumin, bisdemethoxycurcumin, demethoxycurcumin) and volatile oils 7% from rhizomes of *Curcuma longa* Linn. Participants were directed to take one capsule, twice daily with or without food for 8 weeks.

Outcomes

Depression questionnaire

Evaluation of depression and anxiety-related symptoms occurred through the administration of several self-report questionnaires as detailed in Lopresti *et al.* (2014a). The Inventory of Depressive Symptomatology self-rated version (IDS-SR_{30}) was used as the primary assessment of depressive symptoms. Baseline and delta change in total IDS-SR_{30} score was used to examine the relationship between evaluated biomarkers and depressive symptoms. The IDS-SR_{30} contains 30 items measuring depressive symptoms based on the DSM-IV criteria for major depressive episode (Rush et al., 1996). The IDS-SR_{30} has acceptable psychometric properties in depressed outpatients and correlates highly with common depression inventories such as the $HRSD_{17}$, BDI, and MADRS (Rush et al., 1996).

Laboratory assessments

Blood, urine and salivary specimens were collected from participants at baseline and endpoint (8 weeks). Participants were requested to collect blood and urine samples on the same day and salivary samples on the same day or within a day of their blood and urine collections. All collections occurred in the morning after an overnight fast. The biomarkers are described in Table 1.

Urinary collections: Participants were asked to moderately restrict fluid intake the day before testing. Participants collected overnight urine along with their first morning urine in a collection container. Samples were refrigerated until collection by researchers later that day.

Blood collections: Participants were instructed to visit a commercial local pathology centre to provide venous blood samples. They were requested to provide the blood sample after an overnight fast and before 10 am. 10mls of venous blood was drawn into lithium-heparin tubes

and was centrifuged at 3000 rpm for 10 min to obtain plasma. Plasma samples were then stored at −80°C for later analysis.

Salivary collections: Participants were instructed to dribble saliva in provided collection tubes within 10 minutes of awakening and 30 minutes later. Both salivary collections occurred before brushing the teeth and consumption of food or caffeinated beverages. Samples were refrigerated until collected by researchers later that day.

Urinary testing protocol: All urine samples were received on dry ice (-78°C) and stored at -80°C until testing. The samples were allowed to thaw to reach room temperature. Tbx-B2 was tested with an ELISA kit from R&D systems (cat. number KGE011), designed on the inhibition principle. LTB4 was tested with ELISA test kit from R&D systems (cat. number KGE006B). Midkine was tested with an ELISA kit from Cellmid (cat. number MKELISA), designed on the sandwich principle. Cortisol was tested with an ELISA kit from R&D systems (cat. number KGE008), designed on the inhibition principle. SUB-P was tested with an ELISA kit from R&D systems (cat. number HGE007), designed on the inhibition principle. HEVM was tested with a test from Ray Bio (cat. Number ELH-HVEM-001), designed on the sandwich principle. Aldosterone was tested with a test from LDN Germany (cat. Number MS E5200), designed on the inhibition principle. Preparation of the reagents and the testing was completed according to the procedure as described in the respective instructions for use available with all kits. The calculation of the concentrations were completed by linear interpolation uniformly for all tests. All urinary biomarkers were adjusted for creatinine concentrations.

Salivary testing protocol: Following transportation to the laboratory, saliva samples were stored at 2-8°C for up to 24 hours. Untreated saliva was used directly after centrifugation. Salivary cortisol was assayed on the Roche Cobas e® analyser (Roche Diagnostics, Basel, Switzerland) using a competitive polyclonal antibody immunoassay that employs a magnetic separation step followed by electrochemiluminescence quantification. 20 μL of sample was used and the assay had a measurement range of 0.5 to 1750 nmol/L (0.018-63.4 μg/dL). Results were determined via a 2-point calibration curve and a predefined master curve. The cortisol assay has a functional sensitivity of < 8.5 nmol/L (< 0.308 μg/dL) (defined as the lowest concentration reproducibly measured with precision coefficient of variation < 10%). The intra-assay coefficient of variation was < 3.6%.

Blood testing protocol: All plasma samples were received on dry ice (-78°C) and stored at -80°C until testing. The samples were thawed and allowed to reach room temperature, centrifuged 7000xg for 5 minutes to spin down and remove any clotted fibrin particles formed. Tbx-B2 was

tested with an ELISA kit from R&D systems (Cat. number KGE01) based on the inhibition principle. LTB4 was tested with an ELISA kit from R&D systems (cat. number KGE006B) based on the sandwich principle. Human leptin was tested with ELISA kit from Ray Bio (cat. number SKGE006B) based on the sandwich principle. Calprotectin was tested with an ELISA kit from Hycult Biotech (cat. number HK325-02) based on the sandwich principle. Midkine was tested with an ELISA kit from Cellmid (Cat. number MKELISA) based on the sandwich principle. ET-1 was tested with an ELISA kit from R&D systems (cat. number DET100) based on the sandwich principle. Cortisol was tested with an ELISA kit from R&D systems (cat. number KGE008), designed on the inhibition principle. EGF was tested with an ELISA kit from Ray Bio (cat. number ELH-EGF-00)1 based on the sandwich principle. Human TNF-R2 was tested with an ELISA kit from Ray Bio (cat. number ELH-STNFRII-001) based on the sandwich principle. SUB-P was tested with an ELISA kit from R&D systems (cat. number HGE007), based on the inhibition principle. Preparation of the reagents and the testing were completed according to the procedures as described in the respective instructions for use available with all kits. The calculation of the concentrations was completed by linear interpolation uniformly for all tests.

<< Insert table 1 near here>>

Statistical analysis

Effect of curcumin on evaluated biomarkers

Since several biomarkers checked by the inspection of Q-Q plots were not normally distributed and contained several outliers, logarithmic transformations were completed on non-normalised biomarker data. This significantly improved linearity. Separate MANOVAs were conducted for salivary, urinary and plasma biomarkers to examine overall biomarker changes between groups. When a significant MANOVA group x time interaction was identified, further repeated measures ANOVAs were completed to assess this interaction for individual biomarkers. An analysis of covariance (ANCOVA) was used if differences in baseline biomarkers across groups were identified. Independent samples t-tests were used to compare baseline biomarker levels across the treatment groups. Data from all participants were included in analyses (intention to treat, with multiple imputation for missing values).

Relationship between biomarkers, depression and treatment outcome

Due to the small sample sizes (approximately 20 in each condition for biomarker data), exploratory correlational analyses were conducted rather than multiple regression analysis. Correlations between delta change in IDS-SR_{30} (change from baseline to week 8) with baseline and delta change in plasma biomarkers were calculated. Fisher's r-to-z transformation was used to examine the significance of between-group differences in correlation coefficients. As data was not normalised and included outliers, Spearman's rho was used for correlational analyses. Where significant predictors were identified, further analyses were conducted to examine the influence of the biomarker on treatment outcome.

Significance values for statistical testing

All analyses are considered preliminary, requiring further confirmation in more highly powered studies. For all tests, the criterion of statistical significance was $P < 0.05$ (two-tailed). Since correlation coefficient values between 0.0 and 0.3 are considered weak, only correlations above 0.4 were considered clinically meaningful. All data were analysed using SPSS (version 21; IBM, Armonk, NY).

Study Population

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Baseline questionnaire and demographic information

Eighty people were screened for participation in the study and 50 people met inclusion/exclusion criteria. Twenty-five people were randomised into the placebo group and 25 into the treatment (curcumin) group. Forty-seven participants completed up to week 8. There were 3 drop-outs, all from the curcumin group. As detailed in Table 2, there were no significant differences between the two groups in IDS-SR_{30} scores or demographic variables, except for distribution of medical illnesses, with a greater number reporting medical illnesses in the placebo (n=14) than the curcumin (n=6) group (X^2 (1) = 5.33, $p \leq .05$).

<< Insert table 2 near here>>

Details of biomarker collections

Plasma biomarkers: 50 baseline plasma samples (25 from each condition) were collected for biomarker assessment. At baseline, EGF levels were higher in the curcumin than placebo group, but all other biomarkers were similar in the two treatment groups. Post-plasma collections were obtained from 43 participants (curcumin, n=20; placebo, n=23).

Urinary biomarkers: 50 baseline urinary samples (25 from each condition) were collected for biomarker assessment. At baseline, urinary biomarker levels were similar in the placebo and curcumin groups. Post-urinary collections were obtained from 46 participants (curcumin, n=22; placebo, n=24).

Salivary cortisol: 50 baseline salivary samples (25 from each condition) were collected for cortisol assessment. Cortisol could not be measured in two samples (one from each condition) due to insufficient saliva collection, leaving 24 pre-salivary cortisol measurements for each condition. Baseline cortisol levels were similar in the placebo and curcumin groups. Post-salivary collections were obtained from 45 participants (curcumin, n=22; placebo, n=23).

An examination of cortisol collection times revealed no differences in average time of awakening collection pre- and post-treatment (baseline mean awakening collection time = 7.17am; week 8 mean awakening collection time = 7.14am). Average time of collection of 30-minute salivary samples also remained similar over time (baseline 30-min collection = 36 min after awakening; week 8, 30-min collection = 36 min after awakening). At baseline, 7 participants collected 30 min samples greater than 40 minutes after awakening, and at week 8, this occurred for 4 participants. As exclusion of the samples did not influence statistical outcomes and cortisol measures were not significantly different from other collections, all data were used in the analyses below.

RESULTS

Outcome Measures

Biomarker changes across treatment condition

Urinary biomarkers: A repeated measures MANOVA conducted on all urinary biomarkers revealed a significant group x time interaction ($F_{8,41} = 2.62$, $p = .021$). Univariate tests exploring the effects of group by time on the different indiividual urinary biomarkers revealed significant group x time interactions for levels of Tbx-B2 ($F_{1,48} = 4.63$, $p = .036$), SUB-P ($F_{1,48} = 13.30$, $p = .001$),

cortisol ($F_{1,48}$ = 4.87, p = .032) and aldosterone ($F_{1,48}$ = 6.92, p = .011). In the curcumin group, treatment was associated with significant increases in SUB-P ($F_{1,24}$ = 11.67, p = .002) and Tbx-B2 ($F_{1,24}$ = 11.06, p = .003) while placebo treatment was associated with significant decreases in aldosterone ($F_{1,24}$ = 5.19, p = .032). Despite a significant group x time interaction for urinary cortisol, there were no significant within-group time effects. Results of ANOVA analyses and descriptive statistics are detailed in Table 3.

Plasma biomarkers: A repeated measures MANOVA conducted on all plasma biomarkers revealed a non-significant group x time interaction ($F_{10,39}$ = 1.28, p = .275). Results of univariate tests and descriptive statistics are detailed in Table 3.

<< Insert table 3 near here>>

Salivary cortisol: A repeated measures MANOVA conducted on all salivary cortisol biomarkers (awakening cortisol, 30 minute cortisol and cortisol awakening response) revealed a non-significant group x time interaction ($F_{3,44}$ = 0.35, p = .792). This indicates that there were no between-group differences in salivary cortisol changes over time.

Relationship between biomarkers, depression and treatment outcome

Correlations between IDS-SR_{30} total score and biomarker levels (baseline levels and change in levels) are detailed in Tables 4 and 5. A Fisher's r-to-z transformation revealed only two significant differences in Spearman's correlation coefficients between the curcumin and placebo groups. In the placebo group, lower baseline urinary cAMP was associated with a greater reduction in the IDS score after 8 weeks of treatment (r_s = -.480; p = .015; Fisher's z-score = 2.46; p = .014). In the curcumin group, higher baseline plasma ET-1 (r_s = -.587; p = .002; Fisher's z-score = 2.97; p = .003) was associated with greater reductions in the IDS score after 8 weeks of treatment.

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As baseline plasma ET-1 in the curcumin group and urinary cAMP in the placebo-group, were significantly associated with change in the IDS-SR_{30} score, further exploratory analyses were conducted. Participants with high baseline ET-1 (defined as an ET-1 level above the total sample mean of 1.47) (n=19) were selected for further analysis. Baseline demographics were similar in the

curcumin and placebo groups except for significant differences in age (mean difference = 9.20, 95% CI [0.41, 17.02], $t(17) = -2.27$, $p = .037$) and medical illness (X^2 (1) = 10.05, p- = .002). Hence, these variables were included as covariates in a repeated measures ANOVA. In participants with a high baseline ET-1, curcumin was more effective than placebo in reducing IDS-SR_{30} as evidenced by a significant group x time interaction ($F_{2,30} = 3.82$, p = .033; Cohen's d = 1.26) (Figure 2). No similar significant patterns were observed with baseline cAMP levels.

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DISCUSSION

Curcumin and its effect on biomarkers

Eight-weeks of supplementation with either curcumin or placebo was associated with changes in several measured urinary biomarkers; namely urinary levels of Tbx-B2, SUB-P, cortisol and aldosterone. In curcumin-treated individuals Tbx-B2 and SUB-P increased over time whereas in placebo-treated individuals aldosterone declined. Urinary cortisol levels trended downward following placebo-treatment and trended upward following curcumin treatment, resulting in significant between group differences. These changes in several urinary biomarkers contrast with findings of non-significance in all biomarkers measured in plasma and saliva.

SUB-P: SUB-P is a neuropeptide expressed predominantly in the basal forebrain, amygdala, hippocampus and diencephalon. It is associated with nociception, respiration, cardiovascular and thermo-regulation, gut motility, emetic response, and stress-related behaviour (Holmes et al., 2003). A role of SUB-P in depression is supported by evidence confirming that SUB-P and its preferred receptor neurokinin-1 (NK1) are activated by stressors which, in turn, stimulate HPA axis activity (Ebner and Singewald, 2006). They are also associated with the modulation of noradrenaline and serotonin (Blier et al., 2004). Although evidence is mixed, anxiolytic and antidepressant properties of NK1 antagonists have been identified both in animal and human studies (Holmes et al., 2003). Elevated concentrations of SUB-P in the cerebrospinal fluid (CSF) and serum of depressed patients have also been reported, and SUB-P levels decrease in some responders following antidepressant treatment (Alldredge, 2010).

Investigations into the effects of curcumin on SUB-P are limited. In a recent randomised double-blind placebo-controlled trial, curcuminoids as an adjunct to chemotherapy significantly

lowered serum SUB-P in patients with cancer (Panahi et al., 2014). Similar effects were observed following curcumin administration in patients treated for pruritic symptoms induced by sulphur mustard (Panahi et al., 2012). In the present study, curcumin administration was not associated with changes in plasma SUB-P, but was associated with increased urinary concentrations. This increase is contrary to the reduced levels reported in the aforementioned studies and from previous findings of increased plasma concentrations in major depression. However, urinary SUB-P has not been measured in any previous study. Potentially, urinary increases in SUB-P reflect increased excretion, followed by reduced plasma concentrations over the longer term. In addition, a urine collection comprising overnight and first morning voids may be more sensitive to changes in biomarkers than a single point, fasting plasma collection. Further investigation is necessary to help elucidate the relevance of this finding and how it relates to depression treatment.

Tbx-B2: Tbx-B2 is an inactive metabolite of thromboxane A2 (Tbx-A2). Tbx-A2, an unstable arachidonic acid metabolite, is produced by activated platelets that elicit diverse physiological and pathophysiological actions, including platelet aggregation and smooth muscle contraction (Sellers and Stallone, 2008). In the brain, Tbx-A2 also contributes to peripheral adrenal catecholamine secretion (Okada et al., 2003). Elevated levels of Tbx-B2 have been found in patients with major depression, with positive correlations between cortisol and Tbx-B2 (Piccirillo et al., 1994). Treatment with sertraline in individuals with cardiac illness and comorbid depression decreased Tbx-B2 significantly after 16 weeks (Serebruany et al., 2003).

Curcumin inhibits Tbx-B2 production in human platelets (Shah et al., 1999). In the present study, urinary levels increased after curcumin treatment but remained stable following placebo intervention. Urinary levels of Tbx-B2 have not been previously measured in people with major depression, so its relationship to the reported increased plasma concentrations in major depression is currently unknown. As hypothesised with SUB-P, urinary increases may be transient, reflecting increased excretion, or urinary levels may be a more sensitive measure than plasma concentrations.

Cortisol & Aldosterone: Abnormalities in the activity of the HPA axis are commonly observed in major depression. In relation to cortisol output, findings are regularly characterised by heightened cortisol secretion, and non-suppression of cortisol following the dexamethasone suppression test (Mokhtari et al., 2013). Alterations in aldosterone concentrations have also been reported in people with major depression (Holsboer et al., 1987), and in animal-models is

associated with depressive behaviour (Hlavacova et al., 2012). In the present study, compared to curcumin treatment, placebo supplementation was associated with decreases in urinary aldosterone. Urinary cortisol concentrations trended upward following placebo-treatment and trended downward with curcumin supplementation. Collectively this resulted in significant between-group differences. The relevance of these findings in relation to curcumin supplementation requires further investigation, as the within-group effect did not achieve statistical significance.

Baseline biomarkers and their relationship to change in depression following curcumin treatment

Compared to placebo treatment, in curcumin-treated individuals, higher baseline plasma levels of ET-1 or leptin were associated with greater improvements in depressive symptoms. Further support for the importance of ET-1 in curcumin treatment was obtained in additional exploratory analyses. When individuals with high baseline ET-1 levels were examined (i.e. greater than the total sample mean of 1.47 pg/ml), curcumin had significantly greater efficacy in lowering IDS-SR_{30} scores (indicated by a significant group x time interaction). A large Cohen's d effect size of 1.26 was observed in curcumin-treated individuals compared to placebo.

Given limitations associated with the small samples size in this study and the risk of type I error due to multiple correlational analyses, these findings should be considered preliminary and certainly require confirmation from studies with larger sample sizes. This is particularly relevant for findings associated with leptin. Although a higher baseline leptin level was associated with greater improvements in depressive symptoms following curcumin supplementation, between-group differences did not reach statistical significance, therefore impacting on the validity of this finding. However, if these relationships are replicated in future studies, it may facilitate greater treatment-matching. Potentially, depressed individuals with higher baseline ET-1 (and possibly leptin) have a greater likelihood of successful treatment with curcumin.

ET-1: ET-1, a peptide with potent vasoactive effects, is widely expressed in brain cells. Altered expression of ET-1 in reactive astrocytes has been observed in many pathological conditions including infarcts, Alzheimer's disease and inflammatory diseases of the brain (Schinelli, 2006). In the nervous system, ET-1 plays an important role in blood pressure regulation, blood-brain barrier permeability, respiratory control and renal sympathetic neuronal activity (Khimji and Rockey, 2010). There is also evidence that ET-1 and its associated receptors interact with dopamine release (van den Buuse and Webber, 2000). ET-1 also influences free

radical production (Piechota et al., 2010), HPA activity (Kiefer et al., 2000), and cytokine production (Giordano et al., 2011).

From research on the endothelin system in major depression, an impaired endothelial function in depressed individuals has been demonstrated (Wagner et al., 2012), and severity of depressive symptoms predicted ET-1 elevations in patients with coronary artery disease (Burg et al., 2011). In the CSF, ET-1 concentrations were also reduced by approximately 50% in patients with major depression (Hoffman et al., 1989). Bosentan, a mixed endothelin receptor antagonist, also demonstrated antidepressant activity in mice (Pinho-Ribeiro et al., 2014).

Investigations into the effects of curcumin on the endothelin system are limited although curcumin inhibited ET-1 induced mitogenic and proliferative signalling events in vascular smooth muscle cells (Kapakos et al., 2012), and in diabetes-induced rats curcumin increased ET-1 concentrations in microvascular endothelial cells (Farhangkhoee et al., 2006). In the present study curcumin did not alter ET-1 concentrations, suggesting that the enhanced antidepressant efficacy of curcumin in people with higher concentrations of ET-1 were not due to its ET-1-lowering effects. The effects of curcumin may therefore be related to influences on endothelin receptor sensitivity or on inflammatory, HPA axis or oxidative stress processes which have been shown to be influenced by ET-1 concentrations.

Recent conceptualizations of depression have highlighted a role for increased gut permeability (or leaky gut) and increased bacterial translocation with increased levels of lipopolysaccharides in the mesenteric lymph nodes or systemic circulation as contributing to the immune-inflammatory and autoimmune responses in depression (Maes et al., 2013). Interestingly, oral treatment with curcumin prevents bacterial translocation by maintaining tight junction function probably via suppression of nuclear factor kappa B (Weber-Mzell et al., 2006). Thus, it is hypothesized that part of the clinical efficacy of curcumin may be explained by its effects ameliorating leaky gut and bacterial translocation in depression (Maes et al., 2008). Moreover, in a rat model, ET-1 played a role in bacterial translocation (Xiao et al., 2009). This may be another interesting link between the findings of curcumin's enhanced antidepressant efficacy in patients with increased baseline ET-1.

Leptin: Leptin, a peptide hormone secreted from adipocytes, plays a significant role in suppressing food intake and stimulating energy expenditure. Leptin concentrations are elevated in obesity, although its appetite-suppressing effects in obese individuals are thought to be compromised (Zhou and Rui, 2013). Leptin resistance may occur at several levels due to impaired transport of leptin across the blood brain barrier, a reduction in the function of leptin receptors,

and damage to leptin signal transduction (Munzberg and Myers, 2005). Variation in leptin concentrations is linked with major depression, though there are inconsistences in the direction of change. In the majority of animal-based studies lower levels of leptin are associated with depressive behaviours. Human-based studies are inconsistent, with reports of increased and decreased leptin concentrations in depressed individuals (Lu, 2007, Zupancic and Mahajan, 2011). It is believed that a state of leptin resistance, and factors such as age, sex and BMI, are major reasons for this inconsistent data (Lu, 2007). A role of leptin in depression is also supported by studies showing antidepressant properties of leptin and its ability to influence monoamine activity, HPA regulation, neurotrophic actions, and immune responses (Lu, 2007).

Investigations into the effect of curcumin on leptin levels and signalling have confirmed its influential effects. In animal and *in vitro* models, curcumin lowered leptin concentrations induced by various dietary and inflammatory stressors (Lee et al., 2013). In a 6-month randomised, double-blind, placebo controlled trial on adults with type 2 diabetes, curcumin significantly decreased leptin levels (Chuengsamarn et al., 2014).

In the present study, the relationship between curcumin and leptin and its relevance to depression was further highlighted. At baseline, higher leptin levels were associated with greater antidepressant benefits from curcumin. While curcumin did not significantly lower leptin concentrations compared with placebo, benefits may have been derived from its effect on leptin receptor sensitivity or other metabolic factors associated with leptin. Alternatively, the lack of statistical power due to low sample sizes and lack of control for confounders (e.g., gender and BMI) may have hidden real changes in leptin induced by curcumin. Greater time intervals may also be required for curcumin to have an effect on leptin concentrations in plasma.

Relationship between change in biomarkers and change in depression following curcumin treatment

In placebo-treated individuals, increases in plasma concentrations of cortisol and urinary HVEM were associated with reductions in depressive symptoms. However, in curcumin-treated individuals, no biomarker changes were associated with treatment outcome. This indicates the following possibilities in relation to curcumin-treatment: (1) changes in the measured biomarkers have no relevance to change in depressive symptoms; (2) assessing change in a single biomarker (in isolation) is an ineffective way to examine biomarker relevance to depression outcome. Rather, a more effective approach may be to examine change through an evaluation of several biomarkers collectively. Unfortunately, the small sample size used in the study precluded an

adequately powered evaluation of this; (3) other biomarkers may have more relevance to depression and curcumin treatment, and; (4) the study simply lacked enough power, due to small sample sizes, to adequately examine the relevance of the findings.

LIMITATIONS & DIRECTIONS FOR FUTURE RESEARCH

The relatively small samples size and the large number of statistical analyses used in this study limits the reliability and statistical power associated with the findings. For evaluation of biomarker changes over time, 50 samples were collected and numbers were even lower when examining the antidepressant effects of curcumin on people with high ET-1. The likelihood of type 1 error is also increased due to multiple statistical testing. The results from this study therefore require replication with larger sample sizes using greater controls for type 1 errors.

Given problems associated with testing and biomarker stability, greater control around sample collection, storage, testing, and comparisons with non-depressed, matched samples will also be important. In the current study, participants were requested to collect fasting urine and saliva samples in their home and to visit a local pathology centre for blood collection before 10am. Although convenient for participants, compliance with this protocol could not be assessed and it is possible that there was significant variation in the time of collection, fasting state and storage conditions of samples.

Differences in findings across urine and plasma samples also require further clarification. In the present study, biomarker correlations across the plasma and urine samples were low and their response to curcumin supplementation was variable. In particular, greater understanding of urinary measurements are required to help decipher the most reliable and responsive sampling method for biomarker evaluation.

As several markers of oxidative stress and activated immune-inflammatory pathways are associated with depression, testing for additional markers such as malondialdehyde, 8-oxo-2'-deoxyguanosine, CRP, tryptophan catabolites along the kynurenine pathway, and selected cytokines would also be helpful (Lopresti et al., 2014b). Evaluation of biomarkers at more frequent intervals and comparisons with a healthy control group may also improve understanding about the mechanisms associated with the antidepressant actions of curcumin.

In this study the effect of curcumin on individual biomarkers was examined. However, given the multiple potential actions of curcumin, and the several mechanisms associated with major depression, it is unlikely that using a single biomarker in isolation will fully explain the antidepressant effects of curcumin. Improved sensitivity and specificity around diagnosis,

treatment matching, and evaluation of treatment progress is only likely when multiple markers are collectively examined. This requires further research using larger sample sizes and the use of pattern analysis to identify relevant biomarker algorithms.

While this 8-week study now represents the longest trial investigating the antidepressant effects of curcumin, greater treatment periods may be beneficial. Longer administration of curcumin may be necessary for curcumin to have effects on evaluated biomarkers. For example, in one study on people with osteoarthritis, reductions in IL-6, ESR and IL-1β continued for 8 months after treatment (Belcaro et al., 2010). Investigations to determine the optimal curcumin dosage and frequency of administration will also be useful.

Finally, greater control for potential covariates such as medication use, medical illnesses and BMI will increase the strength of future findings, as will the use of clinician-rated instruments and the improved monitoring of curcumin intake, possibly through blood analysis. Some form of measurement of the success of participant blinding would also be useful in future studies as no such measure was included in the current study design.

CONCLUSIONS

In this randomised, double-blind, placebo controlled study several important preliminary findings were identified:

1. Eight-weeks of supplementation with either curcumin or placebo was associated with changes in several measured biomarkers; namely urinary levels of Tbx-B2, SUB-P, cortisol and aldosterone. In curcumin-treated individuals Tbx-B2 and SUB-P increased over time whereas in placebo-treated individual's aldosterone declined. Urinary cortisol levels trended downward following placebo-treatment and trended upward following curcumin treatment, resulting in significant between group differences. Determining reasons for these differences are difficult as they were not related to treatment outcome. Investigations with larger samples and comparisons with non-depressed, matched populations will enhance understanding of these findings. The collective examination of biomarkers rather than in isolation may also increase clarification about the mechanisms associated with curcumin supplementation. In addition, an important finding that requires further investigation is that observed changes only occurred in biomarkers measured in urine. No biomarkers measured in plasma or saliva changed over time, indicating a greater sensitivity of urinary biomarkers.

2. In depressed individuals, higher baseline levels of plasma ET-1 or leptin were associated with an enhanced antidepressant benefit from curcumin supplementation. On the whole, people with high baseline ET-1 levels (above 1.47 pg/ml) experienced significant antidepressant benefits from curcumin. Interpreting these findings are difficult as curcumin supplementation was not associated with changes in ET-1 or leptin levels. Speculatively, curcumin may have enhanced endothelin or leptin receptor sensitivity or some other mechanism associated with biomarkers which, in turn, had antidepressant effects.

These findings need to be interpreted cautiously as multiple statistical comparisons were completed, thereby increasing the risk of type I errors. Further research with larger sample sizes is required.

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Table 1: Definition of measured urinary and plasma biomarkers.

	Plasma only
Endothelin-1 (ET-1)	Protein that constricts blood vessels and raises blood pressure. In the nervous system, ET-1 plays an important role in blood pressure regulation, blood-brain barrier permeability, respiratory control and renal sympathetic neuronal activity.
Calprotectin (Cal)	Released from activated leukocytes leading to increased concentrations during bacterial infections or inflammation in relevant organs.
Leptin	Adipokine that plays a key role in regulating energy intake and expenditure, including appetite and hunger, metabolism, and behaviour.
Tumor necrosis factor receptor 2 (TNF-R2)	Binding of tumour necrosis factor (TNF) to TNFR2 results in activation and recruitment of intracellular adaptor proteins that induce signal transduction, promoting cell proliferation and survival.
Epidermal growth factor (EGF)	Growth factor that stimulates cell growth, proliferation, and differentiation. Concentrations are high in the CNS, controlling proliferation and differentiation of nervous tissue during neurogenesis.
	Urine only
Cyclic adenosine monophosphate (cAMP)	Second messenger that affects transmembrane transport, cell growth and morphology, cellular adhesion, and cytoskeletal organisation.
Herpes virus entry mediator (HVEM)	Plays a critical role in the regulation of inflammation and also serves as one of the entry receptors of herpes simplex virus.
Aldosterone	Steroid hormone that plays a central role in the regulation of blood pressure. When dysregulated, aldosterone contributes to the development and progression of cardiovascular and renal disease by promoting sodium and water retention, and lowering plasma potassium concentrations.
	Urine and plasma
Cortisol (also collected in saliva)	Hormone associated with several important processes in the body including the stress response, inflammatory and immune processes, blood sugar regulation and fat, protein, and carbohydrate metabolism.
Substance P (SUB-P)	Neuropeptide expressed predominantly in the basal forebrain, amygdala, hippocampus and diencephalon associated with nociception, respiration, cardiovascular and thermo regulation, gut motility, emetic response, and stress-related behaviour.
Leukotriene B4 (LTB4)	Lipid inflammatory mediator, generated in leukocytes from membrane arachidonic acid.
Thromboxane B2 (Tbx-B2)	An inactive metabolite of thromboxane A2 (Tbx-A2), an unstable arachidonic acid metabolite produced by activated platelets that elicits diverse physiological and pathophysiological actions, including platelet aggregation and smooth muscle contraction. In the brain, Tbx-A2 also contributes to peripheral adrenal catecholamine secretion.
Midkine	Strongly induced during oncogenesis, inflammation and repair. Promotes cell survival and cell migration, and is deeply involved in cancer progression, the onset of inflammatory diseases and the preservation and repair of injured tissues.

Table 2: Demographic characteristics of curcumin and placebo participants.

	Placebo n=25	Curcumin n=25	p-value
Age (years) mean (S.D.)	48.44 (12.26)	43.00 (12.05)	0.12†
BMI (kg/m²) mean (S.D.)	27.01 (4.90)	25.98 (5.56)	0.50†
IDS-SR$_{30}$ Total Score	33.36 (12.05)	31.24 (9.10)	0.49†
Sex *n*			
Female	18	17	1.00#
Male	7	8	
Marital Status *n*			
Single	7	10	
Married	11	9	
De facto	4	4	.379#
Divorced	1	2	
Widowed	2	0	
Educational Status *n*			
Secondary	5	9	
Tertiary	16	14	.284#
Post-graduate	4	2	
General Health *n*			
Great	8	5	
Average	17	18	.257#
Poor	0	2	
Medical Illness *n*			
Yes	14	6	.021#
No	11	19	
Antidepressant Medication *n*			
Yes	9	11	.387#
No	16	14	
Exercise Frequency *n*			
Never/Rarely	6	4	
1-2 times week	8	9	.650#
3-5 times week	10	12	
6+ times week	1	0	
Injuries causing regular pain *n*			
Yes	11	9	.387#
No	14	16	

†Independent samples T-test; #Chi-square Test

Table 3: Change in biomarkers over time.

	Treatment	Baseline		Week 8		Treatment x time effect (p-value)
		Plasma Biomarkers				
LTB4[#]	Placebo (n=25)	51.34	(66.76)	48.27	(49.63)	.996
	Curcumin (n=25)	31.78	(21.07)	32.48	(23.05)	
ET-1	Placebo (n=25)	1.55	(0.52)	1.57	(0.43)	.802
	Curcumin (n=25)	1.40	(0.25)	1.44	(0.33)	
Tbx-B2[#]	Placebo (n=25)	4.50	(6.45)	3.77	(4.90)	.946
	Curcumin (n=25)	4.98	(7.50)	5.21	(6.51)	
Calprotectin	Placebo (n=25)	341.90	(180.30)	419.27	(216.76)	.652
	Curcumin (n=25)	331.56	(149.31)	382.11	(166.82)	
Leptin[#]	Placebo (n=25)	1211.56	(1044.14)	1088.76	(820.28)	.886
	Curcumin (n=25)	969.00	(1176.24)	1091.29	(1320.96)	
Midkine[#]	Placebo (n=25)	1139.76	(1235.93)	1094.25	(1066.96)	.811
	Curcumin (n=25)	941.38	(1006.88)	1007.56	(1029.05)	
TNF-R2	Placebo (n=25)	1907.40	(682.23)	1971.27	(753.69)	.184
	Curcumin (n=25)	1819.57	(416.28)	1735.53	(356.11)	
EGF	Placebo (n=25)	16.65	(11.71)	36.74**	(25.66)	.003
	Curcumin (n=25)	34.27	(22.49)	25.75	(15.22)	
SUB-P	Placebo (n=25)	410.80	(207.49)	361.91	(178.63)	.738
	Curcumin (n=25)	461.00	(439.74)	431.49	(282.88)	
Cortisol	Placebo (n=25)	28.88	(14.13)	27.15	(17.48)	.938
	Curcumin (n=25)	29.27	(8.94)	27.29	(11.78)	
		Urinary Biomarkers				
cAMP	Placebo (n=25)	624.91	(213.53)	589.16	(168.32)	.165
	Curcumin (n=25)	560.57	(202.57	622.96	(191.17)	
LTB4	Placebo (n=25)	70.54	(56.69)	47.20	(19.82)	.063
	Curcumin (n=25)	50.83	(36.10)	55.69	(30.40)	
HVEM	Placebo (n=25)	15.99	(7.00)	13.91	(7.23)	.084
	Curcumin (n=25)	14.88	(4.46)	16.71	(4.23)	
Tbx-B2[#]	Placebo (n=25)	0.79	(0.63)	0.80	(0.44)	.036
	Curcumin (n=25)	0.62	(0.66)	1.31**	(1.34)	
Midkine[#]	Placebo (n=25)	28.82	(17.20)	26.09	(7.93)	.121
	Curcumin (n=25)	33.22	(23.54)	37.06	(38.08)	
SUB-P	Placebo (n=25)	27.91	(33.19)	18.21	(21.73)	.001
	Curcumin (n=25)	26.28	(32.24)	78.28**	(61.24)	
Cortisol[#]	Placebo (n=25)	4.57	(3.05)	3.57	(1.85)	.032
	Curcumin (n=25)	4.52	(2.91)	5.36	(3.09)	
Aldosterone	Placebo (n=25)	376.90	(257.06)	273.96*	(183.23)	.011
	Curcumin (n=25)	339.68	(200.17)	390.08	(134.46)	

Significant within-group time effects * (p<.05); **(p<.01)
Data are shown as mean (S.D.)
Values expressed as plasma pg/mL and urine mg/creatinine
[#]Analyses completed on log-transformed scores

Table 4: Spearman's rank-order correlations between IDS-SR$_{30}$ total score and plasma biomarker levels (baseline levels and change in levels).

			LTB4	ET-1	Tbx-B2	Calprotectin	Leptin	Midkine	TNF-R2	EGF	Substance P	Cortisol
Change in IDS score and baseline biomarker level	Placebo	rs	.160	.022	-.078	.053	.030	.003	.094	-.146	.151	-.302
		p-value	.446	.916	.712	.801	.888	.987	.655	.488	.473	.142
		n	25	25	25	25	25	25	25	25	25	25
	Curcumin	rs	.166	-.587##	.026	-.126	-.470#	-.077	-.188	-.099	.055	.134
		p-value	.426	.002	.902	.550	.018	.716	.369	.638	.793	.522
		n	25	25	25	25	25	25	25	25	25	25
	Fisher r-to-z transformation	Z-score	-0.02	2.97**	-0.35	0.6	1.79	0.27	0.94	-0.16	0.32	-1.48
		p-value	.984	.003	.726	.549	.074	.787	.347	.873	.749	.139
Change in IDS score and change in biomarkers level	Placebo	rs	-.332	-.357	-.364	-.367	.158	-.299	-.280	-.017	-.085	-.472#
		p-value	.105	.080	.074	.072	.451	.146	.175	.935	.686	.017
		n	25	25	25	25	25	25	25	25	25	25
	Curcumin	rs	-.171	-.158	-.262	-.139	.341	-.060	.188	.261	-.157	-.052
		p-value	.412	.450	.206	.508	.095	.777	.369	.207	.453	.804
		n	25	25	25	25	25	25	25	25	25	25
	Fisher r-to-z transformation	Z-score	-0.57	-0.71	-0.38	-0.81	-0.65	-0.82	-1.59	-1.46	0.24	-1.53
		p-value	.569	.478	.704	.418	.516	.412	.112	.144	.810	.126

Significant between group differences (Fisher r-to-z transformation) *p<.05; **p<.01
Significant within-group time effects (Fisher r-to-z transformation) #p<.05; ##p<.01

Table 5: Spearman's rank-order correlations between IDS-SR$_{30}$ total score and urinary biomarker levels (baseline levels and change in levels).

			cAMP	LTB4	HVEM	Tbx-B2	Midkine	Substance P	Cortisol	Aldosterone
Change in IDS score and baseline biomarker level	Placebo	rs	.480#	.215	.349	.144	-.041	.318	.208	.085
		p-value	.015	.302	.087	.493	.845	.122	.320	.685
		n	25	25	25	25	25	25	25	25
	Curcumin	rs	-.215	-.190	-.111	-.256	.170	-.024	.219	-.013
		p-value	.303	.363	.559	.216	.418	.908	.292	.949
		n	25	25	25	25	25	25	25	25
	Fisher r-to-z transformation	Z-score	2.46*	1.36	1.58	1.35	-0.71	1.17	-0.04	0.33
		p-value	.014	.174	.114	.177	.478	.242	.968	.741
Change in IDS score and change in biomarkers level	Placebo	rs	-.255	-.375	-.407#	.009	-.030	-.151	-.179	-.044
		p-value	.219	.065	.044	.964	.885	.473	.393	.836
		n	25	25	25	25	25	25	25	25
	Curcumin	rs	-.181	-.053	.126	.037	-.304	-.116	-.084	-.274
		p-value	.387	.803	.550	.861	.139	.582	.688	.185
		n	25	25	25	25	25	25	25	25
	Fisher r-to-z transformation	Z-score	-0.26	0.65	-1.85	-0.09	0.94	-0.12	-0.32	0.79
		p-value	.795	.516	.064	.928	.347	.905	.749	.430

Significant between group differences (Fisher r-to-z transformation) *p<.05
Significant within-group time effects (Fisher r-to-z transformation) #p<.05

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Contributors

Adrian Lopresti, Peter Drummond and Garth Maker designed the study and Adrian Lopresti wrote the protocol. Adrian Lopresti managed the literature searches and analyses. Adrian Lopresti and Peter Drummond undertook the statistical analysis, and Adrian Lopresti wrote the first draft of the manuscript. All authors provided comment and assisted in the revision of following manuscripts. All authors contributed to and have approved the final manuscript.

Conflict of Interest

The authors report no biomedical financial interests or potential conflicts of interest.

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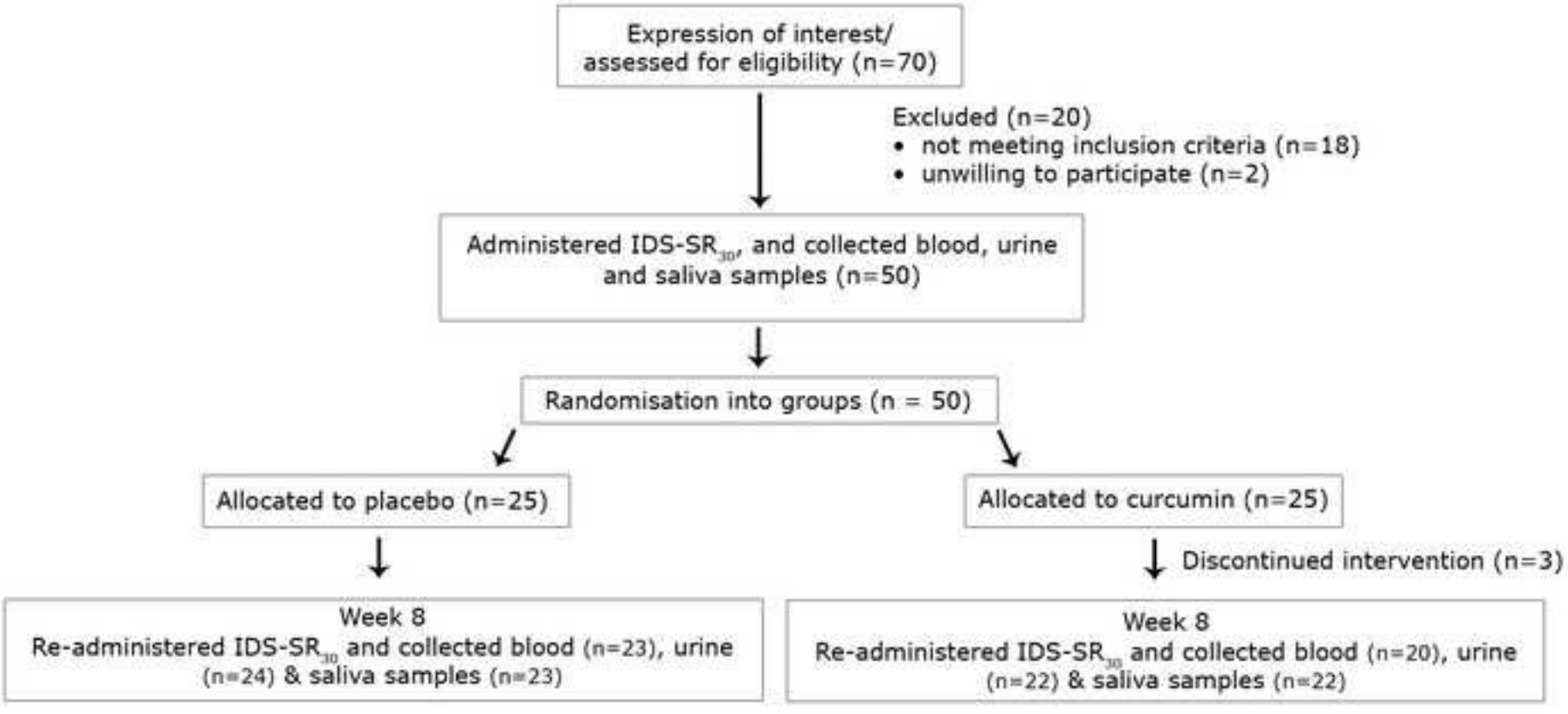
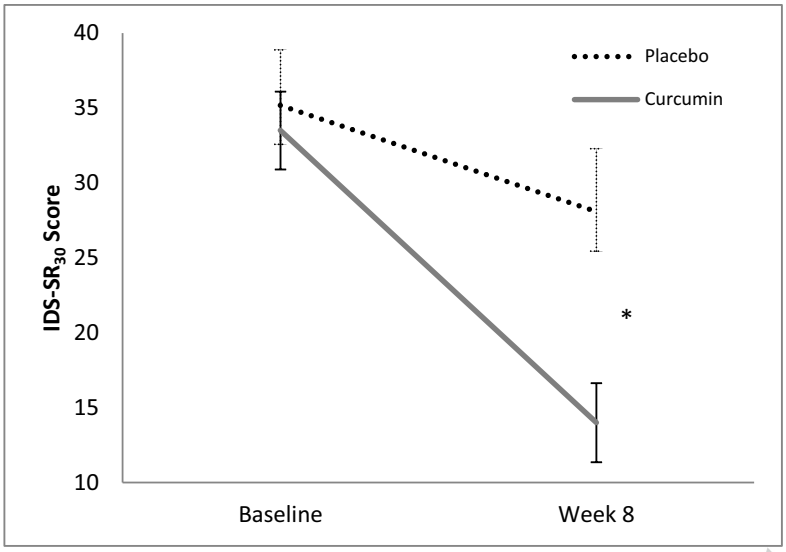
Figure 1. Systematic illustration of study design

Figure 2. Change in IDS-SR_{30} total score over time *(± std. error)* across curcumin and placebo groups in participants with a high ET-1 level at baseline (> 1.47). * indicates significant group x time interaction ($p < 0.05$).

Figure 1





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