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**Altered Peripheral Toll-like Receptor Responses in Irritable
Bowel Syndrome**

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Editor-in-Chief,
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February 16th 2011

Dear Prof Talley,

Please find attached our revised manuscript entitled “ Altered Peripheral Toll-like Receptor Responses in Irritable Bowel Syndrome – A potential biomarker? We thank the referee for their constructive comments and suggestions all of which we have taken on board as outlined below. Please find below a point by point response to each of the reviewer’s comments. We hope you will consider this revised manuscript acceptable for your journal.

We look forward to hearing from you in due course,

With best regards,

Declan McKernan, PhD.

For Peer Review

EDITOR'S COMMENTS TO AUTHOR:

Please address the reviewer comments below which we feel are very important.

REVIEWERS' COMMENTS TO AUTHOR:

Reviewer:

Comments for Transmission to the Authors

On re-analysis the authors found that LPS stimulation (TLR4) induced cytokine released significantly more IL1beta, IL6 and TNFalpha in female IBS compared to male IBS patients. This sentence has been included in the results section. However, no discussion of this finding is made. Was this also observed in their mucosal studies made previously and if so how relevant may be to point out that IBS is much more frequent in females.

This finding has now been included in the discussion. This finding was not observed in the mucosal studies as the number of patients was lower and the study was not powered to detect sex differences in either the control or IBS cohorts.

Where cortisol levels significantly different between males and females with IBS. Would distribution between males and females explain differences previously reported by other authors

No sex differences were found for plasma cortisol levels or cytokine levels measured in this study. This has been mentioned in the results section.

Finally I do not understand their Conclusion in their abstract: -" Taken together, these data demonstrate the presence of low-grade inflammation in the periphery of IBS patients" This is difficult to conclude and no referenceto this has been made in the discussion. Probably these TLRs agonsit mediate cytokine release does not contribute but is the result of an immune dysfunction in IBS to the intestinal flora.

The abstract conclusion has now been rephrased and now reads – “Taken together, these data demonstrate elevated cytokine levels and TLR activity in the periphery of IBS patients indicating some immune dysregulation in IBS patients.”

Altered Peripheral Toll-like Receptor Responses in Irritable Bowel Syndrome

Running Title: Alterations in TLR activity in IBS.

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Abstract:

Background & Aims: Irritable bowel syndrome (IBS) is a stress-related disorder with disturbed brain-gut communication, gastrointestinal homeostasis and, based on recent evidence, low grade inflammation and an altered microbiota. The immune system is a critical regulator of the brain-gut axis. Toll-like receptors (TLRs) are pattern recognition molecules regulating innate immunity. The aim of this study was to characterize TLR activity in IBS.

Methods: Thirty IBS patients and 30 healthy controls (HC) were recruited. Venous blood was collected, and cultured with a panel of TLR agonists for 24 hours. Cell supernatants were analysed using a multiplex ELISA approach to measure IL1 β , IL6, IL8 and TNF α . Plasma was analysed for levels of inflammatory cytokines and cortisol.

Results: TLR agonist-induced cytokine (IL1 β , IL6, IL8 and TNF α) release was markedly enhanced in stimulated whole blood from IBS (n=30) patients compared with healthy controls (n=30). An exaggerated response to the TLR8 agonist for all cytokines investigated was seen in IBS patients. In addition, enhanced TLR2-induced TNF α release, TLR3-induced IL-8 release, TLR4-induced IL1 β and TNF α release, TLR5-induced IL1 β and TNF α release and TLR7-induced IL-8 release were also observed in IBS patients. No differences in TLR1, TLR6 or TLR9 activity were detected. In addition, plasma levels of cortisol, IL-6 and IL-8 were significantly increased in IBS patients.

Conclusion: Taken together, these data demonstrate elevated cytokine levels and TLR activity in the periphery of IBS patients indicating some immune dysregulation in IBS patients.

Key Words: Irritable Bowel Syndrome; Toll-like receptors; stress; cytokine; inflammation.

Introduction:

Irritable Bowel Syndrome (IBS) is a highly prevalent functional disorder of the gastrointestinal tract characterized by the presence of abdominal pain, an alteration in bowel habit and the absence of reproducible biomarkers¹⁻³. Altered visceral perception and gut dysmotility are important contributors to symptom expression^{4, 5}. IBS is a disorder of the brain-gut axis with stress being a frequently invoked precipitant of symptoms among sufferers^{6, 7}. IBS subjects have been shown to demonstrate exaggerated motor and sensory response to a variety of stressors⁸⁻¹⁰. Patients also display an over activation of the hypothalamic-pituitary adrenal (HPA) axis¹¹⁻¹³. Immune dysfunction in IBS patients in both mucosal and systemic compartments has been reported^{11, 14-16}. We have shown that plasma levels of the inflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) are elevated in IBS patients^{11, 16}. Others have reported that levels of tumour necrosis factor α (TNF α), interleukin-1 β (IL-1 β) and IL-6 were elevated in diarrhoea predominant IBS patients (IBS-D)¹⁴.

Toll-like receptors (TLRs) are activated by various bacterial and viral cell components¹⁷. Receptor binding results in transcription of inflammatory cytokines¹⁸⁻²⁰. TLRs are found in high numbers on mucosal surfaces (e.g. the colonic epithelium) and on monocytic cells²¹. Recently, we have also shown that the mRNA expression of certain TLRs is increased in the colonic mucosa of rat models of visceral hypersensitivity²² and have demonstrated increased expression of TLR-4 in mucosal biopsies from IBS patients²³. However, the relevance of these mucosal findings to the immune activation observed in the systemic circulation is unclear. In these studies we sought to characterize the activity of various TLRs on peripheral blood cells to explore the possibility that, as in the colonic mucosa, IBS patients may be primed for an inflammatory response via certain TLR ligands.

Materials & Methods:**Subject Population:**

The study protocol (APC020 2009), was approved by the University College Cork Clinical Research Ethics committee of the Cork University Hospital. IBS patients were recruited from gastroenterology and rheumatology clinics at Cork University Hospital, while healthy controls were recruited from the laboratory, institute or hospital staff. Each healthy control and patient donated 20 ml of venous blood between 11:00 and 13:00 hours to avoid diurnal variations. Thirty IBS patients aged between 18-65 years of age who satisfied Rome II criteria for the diagnosis of IBS were studied. No patient was categorised as post-infectious IBS (PI-IBS). Individuals with inflammatory diseases, celiac disease, lactose intolerance, immunodeficiency, individuals who had undergone any abdominal surgery, with the exception of hernia repair and appendectomy, and those with a psychiatric history were excluded. No attempt was made to select IBS patients on the basis of predominant bowel habit. Each potentially eligible patient was evaluated by a full review of clinical history including current health status, vitals, BMI, family history and current medication. Bowel habit was defined as constipation-dependent (IBS-C), diarrhoea-predominant (IBS-D) or alternating (IBS-A) according to Rome II subclassifications. In addition, a questionnaire was given to participants that assessed IBS severity. The IBS severity scoring system (IBS-SS)²⁴ assesses abdominal pain, abdominal distension (bloating), bowel habit (including frequency and stool consistency) and the effect of IBS on quality of life. A cumulative score (maximum is 500) was calculated and symptom severity was assigned as mild (75-175), moderate (175-300) or severe (>300). Current mood was assessed using the patient health questionnaire (PHQ-9)²⁵ and interview with a psychiatrist.

Plasma Isolation & Whole Blood Culture:

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3 Collected whole blood (15 ml) was added to an equal volume of Histopaque 1077 (Sigma, St.
4 Louis, MO, USA) in a sterile 50 ml tube and centrifuged at 400xg for 30 mins at room
5 temperature. Plasma on the upper layer was transferred to a separate tube and stored at -80°C for
6 future use. Collected whole blood (2 ml) was diluted 1:10 in Dulbecco's Modified Eagle's
7 Medium (DMEM) (Gibco, Dublin, Ireland). Blood was aliquoted into 24-well plates and cultured
8 in 37°C incubator with 5% CO₂. Each blood sample was cultured in DMEM cell culture medium
9 supplemented with 10% Fetal Calf Serum (Sigma, Dublin, Ireland) with or without the following
10 Toll-like receptor ligands from Human TLR agonist kit (InvivoGen, San Diego, CA, USA) for 24
11 hours: TLR1/2 – Palmitoyl-3-cysteine-serine-lysine 4 (Pam3Cys); TLR2 – Heat-killed *Listeria*
12 *Monocytogenes* (HKLM); TLR3 – Polyriboinosinic polyribocytidylic acid (Poly I:C); TLR4 –
13 lipopolysaccharide (LPS); TLR5 – *Salmonella typhimurium* Flagellin; TLR6/2: FSL-1; TLR7 -
14 Imiquimod; TLR8 – ssRNA40. Agonists were reconstituted in endotoxin-free water (supplied)
15 with a final concentration of 1 µg/ml except for HKLM (10⁸ cells) and Poly I:C 10 µg/ml. Each
16 treatment was carried out in duplicate. After the indicated culture period, supernatant from both
17 untreated and stimulated cells was aspirated and stored at -80°C.

41 **Enzyme-linked Immunosorbent Assay (ELISA):**

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43 Measurements of IL-1β, IL-6, IL-8 and TNFα in both plasma and following TLR agonist
44 stimulation of whole blood was carried out using an electrochemiluminescence multiplex system
45 with the Mesoscale Discovery (MSD) 4-plex Human Proinflammatory Kit II. Measurements of
46 IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IFN-γ in plasma were carried out in duplicate using the
47 MSD 7-plex Human Th1/Th2 kit. ELISA plates were analysed using the Sector 2400 imager from
48 Mesoscale Discovery (Gaithersburg, MD, USA). This is an ultra-sensitive method which has a
49 detection limit for IL-1β of 0.3pg/ml, IL-6 of 0.3pg/ml, IL-8 of 1.0pg/ml, IL-10 of 1.9pg/ml, IL-
50 12p70 of 1.1pg/ml, IL-13 of 2.8pg/ml, TNFα of 0.3pg/ml, and IFNγ of 0.8pg/ml. Plasma cortisol
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3 was measured using the Cortisol Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI,
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5 USA) with a detection limit of 26.99 pg/ml.
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10 **Statistical Analysis:**

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12 The sample size was determined by a power calculation based on of our previous data and aimed
13 at detecting differences between IBS patients and healthy controls at the 0.05 level. ELISA data
14 was expressed as mean +/- SEM. All statistical analysis was carried out using GraphPad Prism for
15 Windows (Version 4). Plasma cortisol cytokine levels were compared using an unpaired two-
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two-way ANOVA with a Bonferroni post-hoc test.

Results:**Demographic Data:**

Demographic data for IBS patients and control subjects are presented on Table 1. There were no significant differences between the groups in terms of age, gender distribution or body mass index (BMI). According to Rome II subclassification in terms of predominant bowel habit, this cohort had 11 alternating (IBS-A), 10 constipation-predominant (IBS-C) and 9 diarrhoea-predominant (IBS-D) patients. IBS symptoms were assessed using the IBS-SS, it was found that the average cumulative score for the IBS patients was 258.8 (moderate) compared to 4.83 (out of 500) for the control subjects; as expected, the difference was highly significant ($p < 0.0001$) (Table 1). Additionally, 2 of the control group had a family history of IBS compared to 13 of the IBS group (Table 1).

Plasma cytokine and cortisol levels:

Plasma from both healthy controls and IBS patients was analyzed for eleven cytokines and cortisol. Student's t-tests (with correction for multiple comparisons) confirmed that there were significantly elevated levels of IL-6 ($p = 0.008$), IL-8 ($p = 0.028$) and cortisol ($p = 0.048$) in the plasma of IBS patients compared to healthy controls (Figure 1). Levels of other cytokines tested (IL-1 β , TNF α , IL-2, IL-4, IL5, IL-10, IL-12, IL-13 and IFN γ) were not found to be significantly different between groups (Table 2). **No sex differences were found for plasma cortisol or cytokines measured.**

TLR-agonist cytokine release:

Supernatants from whole blood treatments with TLR agonists of both healthy controls and IBS patients were analysed for levels of four different pro-inflammatory cytokines IL-1 β , IL-6, IL-8

1
2
3 and TNF α . Two-way ANOVA demonstrated a significant effect of both disease state ($F_{1, 1684} =$
4 38.10, $p < 0.001$) and TLR agonist treatment ($F_{8, 1684} = 115.7$, $p < 0.001$) with a significant
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6 interaction ($F_{8, 1684} = 5.98$, $p < 0.001$) between the two factors for IL-1 β levels. A significant
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8 effect of both disease state ($F_{1, 1717} = 10.41$, $p = 0.0013$) and treatment ($F_{8, 1717} = 119.7$, $p < 0.001$)
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10 with a significant interaction ($F_{8, 1717} = 2.142$, $p = 0.0293$) between the two factors was found for
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12 IL-6 levels. Two-way ANOVA demonstrated a significant effect of both disease state ($F_{1, 1725} =$
13 66.93, $p < 0.001$) and treatment ($F_{8, 1725} = 31.50$, $p < 0.001$) with a significant interaction ($F_{8, 1725}$
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15 = 8.321, $p < 0.001$) between the two factors for IL-8 levels. A significant effect of both disease
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17 state ($F_{1, 1709} = 51.71$, $p < 0.001$) and treatment ($F_{8, 1709} = 124.3$, $p < 0.001$) with a significant
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19 interaction ($F_{8, 1709} = 6.515$, $p < 0.001$) between the two factors for TNF α levels also. When age
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21 was entered as a co-variate the differences between the groups are still significant.
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30 Post-hoc analysis of individual treatments revealed significant differences between
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32 IBS patients and healthy controls. In the untreated blood, levels of IL8 were found to be
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34 significantly higher in IBS patients compared to healthy controls (HC) (1287 +/- 119.7 vs. 2135
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36 +/- 107.4pg/ml; $p < 0.001$) (Figure 2a). No significant differences were found in cytokine release
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38 following treatment with the TLR1/2 agonist Pam3Csk (Figure 2b). TNF α release was found to
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40 be significantly elevated in IBS compared to HC blood (1462 +/- 127.9 vs. 934.6 +/- 94.93 pg/ml;
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42 $p < 0.001$) following treatment with the TLR2 agonist HKLM (Figure 2c). IL-8 release was found
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44 to be significantly elevated in IBS compared to HC blood (2582 +/- 122.7 vs. 2015 +/- 168.3
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46 pg/ml; $p < 0.05$) following treatment with the TLR3 agonist Poly I:C (Figure 2d). IL-1 β was
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48 found to be significantly elevated in IBS compared to HC (1715 +/- 100.8 vs. 1157 +/- 95.4
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50 pg/ml; $p < 0.001$) following treatment with the TLR4 agonist LPS as well as TNF α levels (2427
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52 +/- 175.5 vs. 1723 +/- 118.5 pg/ml; $p < 0.001$) (Figure 2e).
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58 IL-1 β release was found to be significantly elevated in IBS compared to HC (947.8
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60 +/- 91.27 vs. 570.8 +/- 65.95 pg/ml; $p < 0.001$) as well as TNF α (1354 +/- 128.8 vs. 921.7 +/-

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3 85.61 pg/ml; $p < 0.01$) following treatment with the TLR5 agonist Flagellin (Figure 3a). No
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5 significant differences were found following treatment with the TLR6/1 agonist FSL1 (Figure
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7 3b). IL-8 release was found to be significantly elevated in IBS compared to HC blood (3408 +/-
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9 112.2 vs. 2128 +/-147.9 pg/ml; $p < 0.001$) following treatment with the TLR7 agonist Imiquimod
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11 (Figure 3c). IL-1 β release was found to be significantly elevated following treatment with the
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13 TLR8 agonist ssRNA40 (1404 +/- 118.5 vs. 913.8 +/- 88.15 pg/ml; $p < 0.001$), IL6 release was
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15 found to be significantly elevated (2037 +/- 144.3 vs. 1326 +/- 137.6 pg/ml; $p < 0.01$); in addition
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17 IL-8 release was found to be significantly elevated (3438 +/- 126.9 vs. 2477 +/- 146.8 pg/ml; $p <$
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19 0.001); finally, TNF α release was found to be significantly elevated in IBS compared to HC blood
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21 following treatment with the ssRNA40 (1944 +/- 161.2 vs. 1076 +/- 110.3 pg/ml; $p < 0.001$)
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23 (Figure 3d). No significant changes in cytokine levels were detected following treatment with the
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25 TLR9 agonist ODN2006 (Figure 3e). A separate analysis was carried out of male and female
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27 participants. There was no significant difference between these groups with the exception of LPS
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29 (TLR4) induced cytokine release, where there was significantly more IL1 β , IL6 and TNF α
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31 released in female IBS compared to male IBS patients (Data not shown).
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Discussion:

The exact pathophysiology of IBS is still unknown. It has been suggested to have an immune component²⁶, with low level immune activation reported^{11, 14, 15, 27-30}. These studies have shown increases in proinflammatory cytokines,^{11, 14, 15, 30} as well as alterations in certain immune cell populations in IBS patients^{15, 27-29}. Given the key role of toll-like receptors in the activation of cytokine release, we hypothesized that TLR activity may be altered in IBS patients. IBS patients were shown to have elevated plasma cortisol levels, in agreement with previous reports from this laboratory¹¹. Cortisol is a product of the hypothalamic–pituitary-adrenal (HPA) axis and an important hormonal indicator of stress. The HPA axis has been demonstrated to be dysregulated in IBS patients by a number of groups including our own¹¹⁻¹³. Stress is known to disrupt the function of the immune system, including cytokine levels³¹. Of cytokines measured, only IL-6 and IL-8 were significantly elevated in IBS patients, in agreement with previous studies^{11, 30}.

The effects of elevated cytokine levels on the pathophysiology of IBS remain to be defined. Cytokines play a role in central sensitization. IL-1 β , IL-6 and TNF α have been shown to affect transmission in the spinal cord resulting in hyperalgesia³². This finding is relevant to IBS, in which both elevations in cytokines and hypersensitivity to pain have been demonstrated³³. Furthermore, cytokines are known to have an effect on the HPA-axis and stress response³⁴. IL-1 β , IL-6 and TNF α have all been reported to stimulate secretion of corticotrophin-releasing hormone (CRH) in rat as well as in humans^{35, 36}. Cytokines can cross the blood brain barrier and stimulate cells at the barrier to release more cytokines within the brain³⁶. However, it is not clear whether the elevations in cytokine levels observed in IBS patients are responsible for the alteration in HPA-axis activity or *vice versa*.

Whole blood stimulations revealed a distinct pattern of peripheral TLR activity in IBS patients. Accordingly, we demonstrated an association between increased TLR4, 5 and 8 activities and IL1 β production; these receptors recognise bacterial lipopolysaccharide (LPS),

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3 bacterial flagellin and viral nucleic acid. Increased TLR8 activity was associated with IL-6
4 release. IL-8 release was greater following activation with agonists for TLR3, 7 and 8. All of
5 these receptors recognise viral nucleic acids. Finally, increased TNF α release was related to
6 increased TLR2, 4, 5 and 8 activities. Enhanced TLR8-mediated cytokine release was the
7 common factor in relation to each of these cytokines. At present, viral nucleic acids are the only
8 known ligand for TLR8 ¹⁷, although a yet undiscovered endogenous or exogenous ligand(s) may
9 also activate this receptor. In addition, there were some differences between male and female
10 patients for LPS stimulation, indicating changes in TLR4 activity may be linked not only to the
11 condition but also sex, and further studies are needed to investigate this matter.
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25 IBS patients have been shown to have a different microbiota to that of healthy
26 controls ³⁷. The gut microbiota are known to have an influence on a number of physiological
27 parameters including the immune system and may prime immune cells that enter the peripheral
28 circulation ³⁸. This may impact on the TLR expression profile of immune cells and result in
29 altered TLR activity. Indeed, our group has recently reported increased expression of TLR4 in
30 mucosal biopsies from IBS patients ²³. Secondly, IBS patients appear to be particularly
31 susceptible to stress ¹¹ and this seems to have effect on gut motility ^{9, 10}. It is apparent from both
32 animal models and clinical studies that chronic stress may affect the immune response ³¹.
33 Repeated social defeat stress in mice enhanced the bactericidal activity of splenic macrophages,
34 and led to an increase in the expression of TLR2 and TLR4 on these cells ³⁹. Social defeat can
35 also activate splenic dendritic cells and enhance TLR activity on these cells as assessed by
36 cytokine output in response to ligand stimulation ⁴⁰. Furthermore, chronic restraint stress resulted
37 in immune suppression which was mediated by TLR4 ^{41, 42}. Elevations in the expression of TLR
38 mRNA was observed in the colonic mucosa of two different rat models of stress-induced visceral
39 hypersensitivity when compared to normosensitive rats ²². These rats develop visceral
40 hypersensitivity, changes in immune response and differences in the gut microbial populations ⁴³.
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3 Thirdly, cytokines and glucocorticoids may synergise to alter TLR expression ^{44, 45}. Plasma
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5 cytokines and cortisol have been shown to be elevated in this cohort of patients and this
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7 potentially may explain why there are increases in TLR activity in these patients.
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10 A limitation of the current study is that the TLR receptor activity in the periphery
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12 may not resemble that of the gut mucosa. This study has provided novel insights into relationships
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14 between TLR activation, plasma cytokine levels and clinical phenotype among a well
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16 characterised group of IBS patients. We have described for the very first time, distinctive patterns
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18 of TLR activity in the periphery. Based on observed relationships between certain phenotypic
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20 features and demonstrated associations between stress and TLR expression and activity in animal
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22 models, it is plausible to suggest that there maybe a link between stress, TLR activation and
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24 cytokine profiles in IBS patients.
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Figure Legends:**Table 1:**

Shown is a comparison of demographic data collected on the study's cohort of healthy controls and IBS patients. Data was collected from medical and family history questionnaires while additional information was taken from specific questionnaires regarding IBS severity (IBS-SS). Data is expressed as mean +/- SEM. Student's t-test was used to determine significant differences (p-values shown).

Table 2:

Shown is a table of cytokine and cortisol levels in the plasma of healthy controls and IBS patients. Data is expressed as mean +/- SEM. Student's t-test was used to determine significant differences.

Figure 1:

Shown is a graph of plasma (a) cytokine and (b) cortisol levels in healthy controls and IBS patients. Data shown is expressed as mean +/- SEM. Statistical differences between treatments and untreated were determined using Student's t-test. * $p < 0.05$, *** $p < 0.001$ vs Ctrl.

Figure 2:

Shown is a graph of IL-1 β , IL-6, IL-8 and TNF α release in whole blood supernatants taken from healthy controls and IBS patients measured using ELISA following (a) no treatment, or treatment with the (b) TLR1/2 agonist Pam3Csk, (c) TLR2 agonist HKLM, (d) TLR3 agonist Poly I:C or the (e) TLR4 agonist LPS for 24 hrs. Data shown is expressed as mean +/- SEM. Statistical differences between treatments and disease state was determined using two-way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.05$ and *** $p < 0.001$ vs Ctrl.

Figure 3:

Shown is a graph of IL-1 β , IL-6, IL-8 and TNF α release in whole blood supernatants taken from healthy controls and IBS patients measured using ELISA following treatment with the (a) TLR5 agonist Flagellin, (b) TLR6/1 agonist FSL1, (c) TLR7 agonist Imiquimod (d) TLR8 agonist ssRNA40 and the (e) TLR9 agonist ODN2006 for 24 hrs. Data shown is expressed as mean \pm SEM. Statistical differences between treatments and disease state was determined using two-way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.05$ and *** $p < 0.001$ vs Ctrl.

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Table 1:

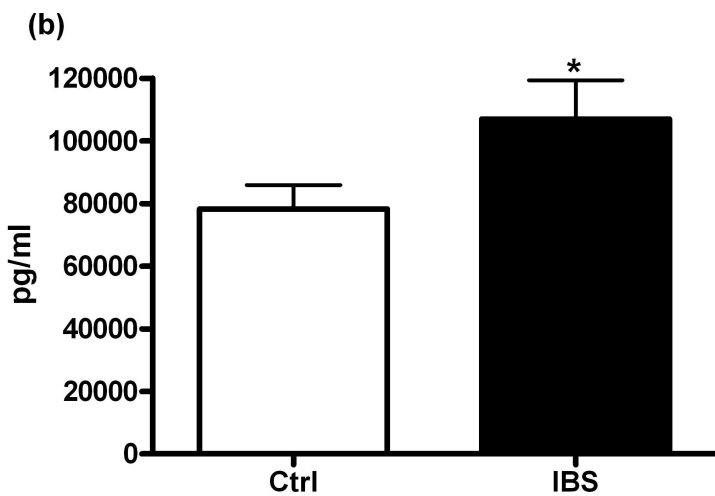
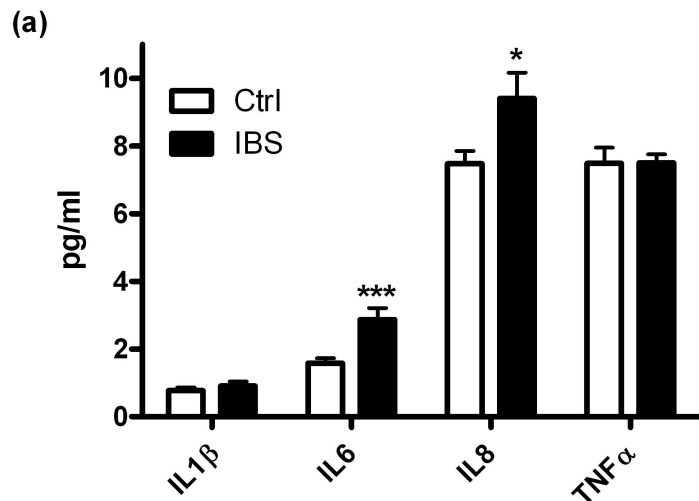
	Healthy Controls	IBS	p value
Age	36.24 +/- 1.835	40.9 +/- 2.034	0.09
Sex	10 M, 20 F	5 M, 25 F	
Height	1.71 +/- 0.017	1.67 +/- 0.017	0.0876
Weight	68.4 +/- 2.558	69.74 +/- 2.673	0.7192
BMI	23.19 +/- 0.6084	24.89 +/- 0.7459	0.0827
Family History IBS	2	13	
IBS Subtype	N/A	11A, 10C, 9D	
IBS-SS	4.83 +/- 4.83	258.8 +/- 13.58	p < 0.0001

Table 2:

	Control	IBS	p-value
Cortisol	78220 ± 7590	107000 ± 12340*	0.0488
<i>Cytokine</i>			
IL-1β	0.7751 ± 0.09349	0.9111 ± 0.1270	0.3922
IL-6	1.581 ± 0.1462	2.875 ± 0.3350***	0.0008
IL-8	7.474 ± 0.3745	9.397 ± 0.7667 *	0.0282
TNFα	7.487 ± 0.4606	7.500 ± 0.2472	0.9808
IL-2	0.7668 ± 0.09624	0.9327 ± 0.2535	0.5430
IL-12	8.227 ± 2.990	13.84 ± 4.923	0.3342
IFNγ	4.381 ± 0.7624	5.484 ± 0.9830	0.3789
IL-4	2.114 ± 0.6566	1.120 ± 0.2286	0.1585
IL-5	3.708 ± 0.7518	5.121 ± 1.387	0.3745
IL-10	16.62 ± 2.327	20.50 ± 3.762	0.3840
IL-13	14.32 ± 4.894	9.615 ± 3.226	0.4257

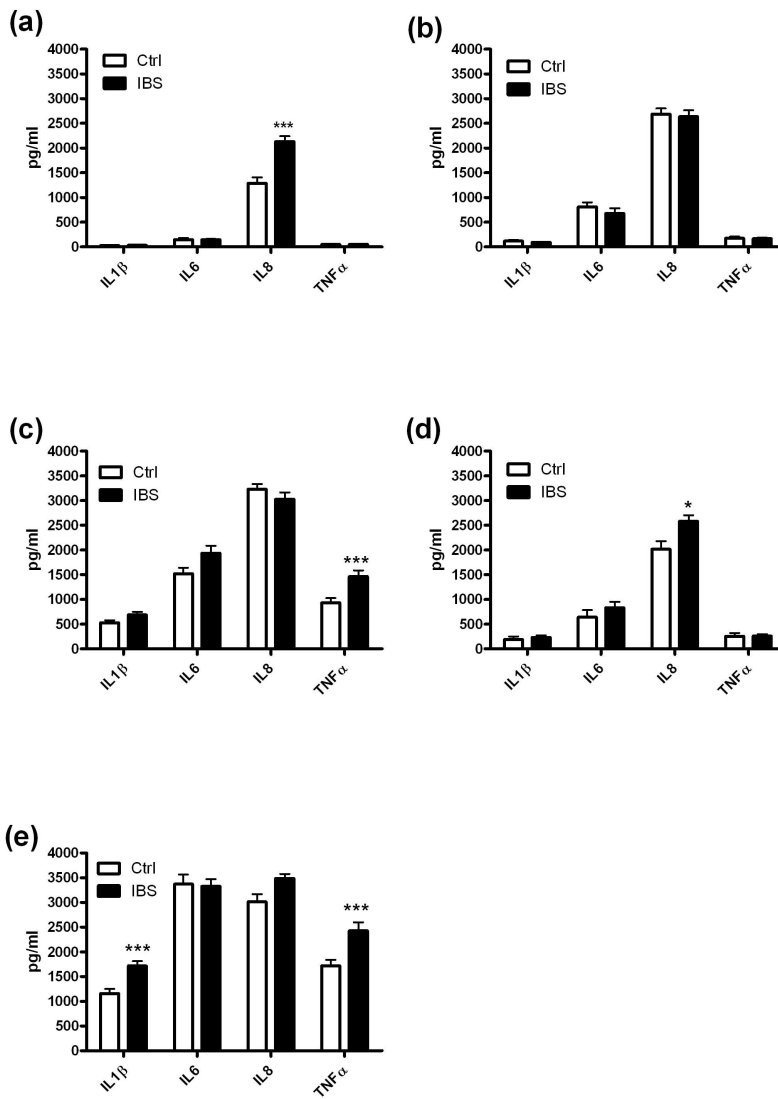
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Figure 1



205x287mm (300 x 300 DPI)

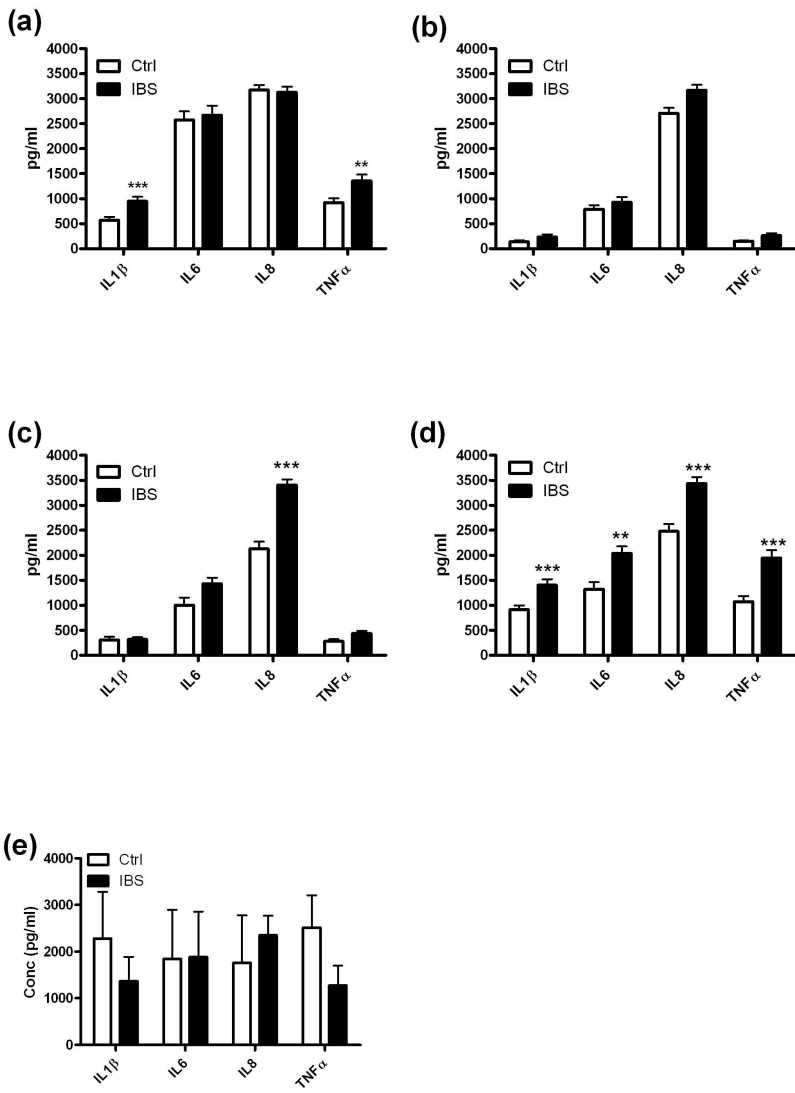
Figure 2



181x263mm (300 x 300 DPI)

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Figure 3



181x260mm (300 x 300 DPI)