



(51) International Patent Classification:

A23L 1/29 (2006.01) A61P 3/02 (2006.01)
A23L 1/305 (2006.01) A61K 31/593 (2006.01)
A61P 1/00 (2006.01)

(21) International Application Number:

PCT/AU2014/001117

(22) International Filing Date:

12 December 2014 (12.12.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

2013904917 12 December 2013 (12.12.2013) AU

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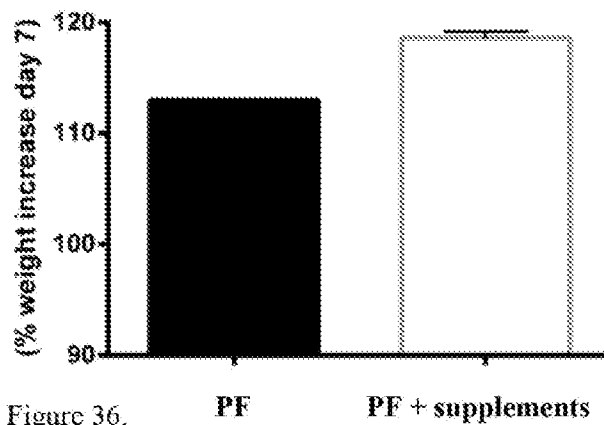
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: PHARMACONUTRIENT COMPOSITION



(57) Abstract: The invention relates to a pharmaconutrient composition for treating intestinal inflammation in a subject, the pharmaconutrient composition comprising at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid. The invention also relates to a nutritional formulation for treating intestinal inflammation in a subject, the nutritional formulation comprising the pharmaconutrient composition. The invention further relates to a method of treating intestinal inflammation in a subject, the method comprising administering to the subject at least two pharmaconutrients having similar mechanisms of pharmacological activity.

PHARMACONUTRIENT COMPOSITION

FIELD

The present invention relates to a pharmaconutrient composition and uses thereof for treating intestinal inflammation, such as Inflammatory Bowel Disease, such as Crohn's disease.

BACKGROUND

Inflammatory Bowel Disease (IBD), which includes Crohn's disease (CD), ulcerative colitis (UC) and inflammatory bowel disease unclassified (IBDU), is a chronic illness affecting the gastrointestinal tract. CD and UC, although having some similar features, have distinctive immunological, endoscopic and histological characteristics. As indicated by the name, intestinal inflammation is a major component of IBD.

The current best-accepted hypothesis for development of IBD is that an initiator, either gastrointestinal microorganisms or their by-products, in association with a disruption of the gastrointestinal epithelium, stimulates and subsequently drives a dysregulated immune response, e.g. the nuclear factor- κ B (NF- κ B) pathway, in genetically susceptible subjects. Thus, the host genetic makeup, the gastrointestinal immune system and the intestinal flora are likely to be important in the pathogenesis of IBD.

IBD is currently incurable and causes significant morbidity, which can be amplified when the disease occurs during childhood or adolescence. This is particularly significant, because, although IBD may present at any age, the peak period of presentation for IBD is the second and third decades of life, especially adolescence. CD, for example, is characterised by a variable chronic relapsing course, in which periods of disease control are interrupted by periods of increased disease activity.

Because IBD is incurable, the available therapeutic options aim to control the disease and prevent adverse outcomes. Current pharmacological therapies comprise agents used to induce remission and those used to maintain remission. These include antibiotics, corticosteroids, aminosalicylates and immunosuppressive agents. Although all these current therapies modulate inflammatory responses, none cure IBD, few lead to histological healing and most have associated side effects, which can have significant impact in children with IBD.

An alternative approach to inducing remission of IBD in adults and children, perhaps desirably in children, is the use of exclusive enteral nutrition (EEN). EEN involves the provision of a liquid diet using elemental formulation or polymeric formulation (PF), given exclusively over a prolonged period of up to 12 weeks, without eating food. Meta-analysis of paediatric studies shows that EEN has equivalent efficacy to steroids in the induction of remission. However, three meta-analyses have shown that steroids are superior to EEN as primary treatment in adult IBD subjects. In addition to anti-inflammatory benefits, EEN leads to superior mucosal healing and nutritional improvement, has fewer side effects, and avoids medication-related side effects. However, EEN in adults may suffer from poor compliance, in part due to poor palatability compounded by prolonged treatment. Furthermore, disease relapse often occurs when the therapy is ceased. For example, it has

been reported that 12 month relapse rates for both EEN and steroids when used as induction therapy is greater than 60%. Therefore, there is a need for improved treatment of intestinal inflammation. Such improvements may relate to better compliance, more effective induction of remission compared to steroids (the current “gold” standard), particularly in adults, reduced steroid dependency, and maintenance of remission. Although palatability *per se* may be difficult to improve, improved efficacy may offset the effect of poor palatability on compliance.

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

SUMMARY

The invention relates to a pharmaconutrient composition for treating intestinal inflammation in a subject.

A first aspect provides a pharmaconutrient composition for treating intestinal inflammation in a subject, the pharmaconutrient composition comprising at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.

A second aspect provides a nutritional formulation for treating intestinal inflammation in a subject, the nutritional formulation comprising the pharmaconutrient composition of the first aspect.

A third aspect provides a method for producing a nutritional formulation that treats intestinal inflammation in a subject, the method comprising supplementing a nutritional formulation with the pharmaconutrient composition of the first aspect, or supplementing a nutritional formulation with at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid, or supplementing a nutritional formulation comprising one of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid with at least one other of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.

A fourth aspect provides a nutritional formulation produced by the method of the third aspect.

A fifth aspect provides a method of treating intestinal inflammation in a subject, the method comprising administering to the subject at least two pharmaconutrients having similar mechanisms of pharmacological activity. The at least two pharmaconutrients may be selected from arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.

Also disclosed as an alternative to the fifth aspect is use of at least two pharmaconutrients having similar mechanisms of pharmacological activity in the manufacture of a medicament for treating intestinal inflammation in a subject. The at least two pharmaconutrients may be selected from arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.

Also disclosed as an alternative to the fifth aspect is at least two pharmaconutrients having similar mechanisms of pharmacological activity for use in a method of treating intestinal inflammation in a subject. The at least two pharmaconutrients may be selected from arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.

A sixth aspect provides a method of treating intestinal inflammation in a subject, the method comprising administering to the subject a pharmaconutrient composition or a nutritional formulation comprising at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.

Also disclosed as an alternative to the sixth aspect is use of at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid in the manufacture of a pharmaconutrient composition or a nutritional formulation for treating intestinal inflammation in a subject.

Also disclosed as an alternative to the sixth aspect is a pharmaconutrient composition or a nutritional formulation comprising at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid for use in a method of treating intestinal inflammation in a subject.

A seventh aspect provides a kit comprising the pharmaconutrient composition of the first aspect or the nutritional formulation of the second or fourth aspect.

Advantages of the invention disclosed herein include improved efficacy of nutritional therapy both *per se* and relative to steroidal treatment with respect to both induction of remission and maintenance of remission, and reduced rates of relapse, which together improve subject compliance.

While not wishing to be bound by theory, the inventors believe that molecules that may act as pharmaconutrients have at least two concentration windows. When concentrations are low within a first concentration window, the molecules act as nutrients for the cell (nutrient concentrations). When concentrations are increased, however, the molecules then have additional activity within the cell (pharmacological concentrations) and then behave as pharmaconutrients.

The invention relates to two or more pharmaconutrients that have similar mechanisms of pharmacological activity that in combination 1) increase the pharmacological effect and/or 2) reduce the pharmacological concentration window required for the pharmacological effect.

In one embodiment, the combination is a pharmaconutrient composition that may be administered in any suitable form, e.g. solid, powder, tablet, capsule, liquid, suspension, solution. In one embodiment, the pharmaconutrient composition is a nutritional formulation. In a preferred embodiment, the pharmaconutrient composition comprises at least two pharmaconutrients selected from arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid. In a more preferred embodiment, the pharmaconutrient composition comprises glutamine, arginine, and curcumin. However, the invention is not limited to these pharmaconutrients, or to the NFκB pathway, or to intestinal inflammation as other pharmaconutrients may be employed in the pharmaconutrient composition and other forms of tissue inflammation may benefit from administration of the pharmaconutrient composition.

Although not wishing to be bound by theory, the inventors consider that in one embodiment of the invention, glutamine, arginine and curcumin are actively taken up into epithelial cells. Uptake is not rate limiting as concentrations within the cell are similar to concentrations adjacent to the cell, even with high concentration of glutamine, arginine and curcumin. Once within the cell, glutamine and arginine may be metabolised, however metabolism is not necessary for their anti-inflammatory

activity. Metabolites of glutamine, arginine or curcumin may be anti-inflammatory. Within the cell, glutamine, arginine and curcumin may interact with components of the Nuclear Factor kappa light chain enhancer of activated B cells (NF κ B) pathway. In particular, glutamine, arginine and curcumin may prevent activated I κ B Kinase (I κ K) from phosphorylating, and therefore activating, Inhibitory protein of NF- κ B (I κ B). This essentially blocks the NF κ B pathway and hence suppresses inflammation. Glutamine, arginine and curcumin may compete with or prevent adenosine triphosphate (ATP) from binding to the ATP binding site of the I κ K complex. Because this represents competitive binding, concentration of glutamine, arginine and curcumin may be important. Furthermore, because glutamine, arginine and curcumin appear have similar mechanisms or behave similarly towards I κ K, this anti-inflammatory effect may be amplified when glutamine, arginine and curcumin in combination are present within the cell. Also, glutamine, arginine and curcumin may also interact with other components of the NF κ B pathway.

The invention allows physiological efficacy to be maintained (or enhanced) by decreasing individual nutrient concentrations, but increasing the number of physiologically active nutrients that have similar mechanisms of pharmacological activity. Therefore, physiological efficacy is maintained or enhanced, but toxicity or side effects are decreased.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments, incorporating all aspects of the invention, will now be described by way of example only with reference to the accompanying drawings.

Figure 1 is a schematic illustrating the etiology of malnutrition in subjects with CD. (A): Drug therapy and inflammatory response. (B): gastrointestinal inflammation, pain and decreased appetite. (C): Leaky gut, surgical resection decreased absorptive area, increased mucosal turn over and bleeding loss.

Figures 2 to 11 relate to Example 1.

Figure 2 provides scatter plots A and B illustrating the effect of four constituents of a whole protein 1 Cal/mL nutritionally complete PF (glutamine 12.7 mM, arginine 1.8 mM, vitamin D3 3.8 nM and α -linolenic acid (ALA, an-3 fatty acid) 0.72 mM) on IL-8 production by HT29 cells subsequently exposed to 50 ng/mL TNF- α for 6 hours. Glutamine, arginine and vitamin D3, but not ALA, significantly reduced IL-8 production from TNF- α stimulated HT29 cells. The IL-8 levels were further attenuated when these nutrients were added in combination (A* P<0.05, ** P<0.01, *** P<0.001, and ns not significant; B * P<0.01, ** P<0.001, *** P<0.0001, and ns not significant). IL-8 levels were compared in treated groups to its level in the positive control group using one-way ANOVA test followed by Fischer least significance post hoc test, P <0.05 was considered significant. Negative: Negative control group. Positive: Positive control group (only TNF- α). Glu: Glutamine treated group. Arg: Arginine treated group. VitD: Vitamin D3 treated group. ALA: α -linolenic acid treated group. Combination: combination treated group (glutamine of 12.7 mM, arginine of 1.8 mM, vitamin D3 of 3.8 nM and ALA of 0.72 mM). Values represent mean $\pm$ standard error of mean (SEM)

of 4 replicates for each group.

Figure 3 is a scatter plot illustrating the effect of a combination of four constituents of a whole protein 1 Cal/mL nutritionally complete (standard) PF (glutamine 12.7 mM, arginine 1.8 mM, vitamin D3 3.8 nM, and ALA 0.72 mM) versus standard PF on IL-8 production by HT29 cells exposed to 50 ng/mL TNF- α for 6 hours. Both the combination and standard PF reduced IL-8 level compared to positive control group ($P < 0.0001$ for both groups). Further, in both groups IL-8 levels remained higher than the negative control group ($P = 0.0136$ for PF and $P < 0.0001$ for the combination treatment vs negative control group). However, PF was superior to the combination in reducing the IL-8 response ($P = 0.0057$). Multiple comparisons were carried out between all groups for comparing IL-8 levels using one-way ANOVA test followed by Fischer least significance post hoc test, $P < 0.05$ was considered significant. Negative: Negative control group. Positive: Positive control group (only TNF- α). Combination: combination treated group (glutamine 12.7 mM, arginine 1.8 mM, vitamin D3 3.8 nM, and ALA 0.72 mM). Standard PF: Polymeric formulation treated group. Values represent mean $\pm$ SEM of 4 replicates for each group.

Figure 4 is a line graph illustrating viability of HT29 cells treated with four constituents of PF (glutamine 12.7 mM, arginine 1.8 mM, vitamin D3 3.8 nM, and ALA 0.72 mM) separately or in combination, or PF as determined by Trypan Blue exclusion. In all groups, cell viability was maintained above 90% and remained comparable to the untreated cells group ($P > 0.05$ for treated groups vs control group using one-way ANOVA test followed by Fischer least significance post hoc test). Control: untreated cells group. Glu: Glutamine 12.7 mM treated group. Arg: Arginine 1.8 mM treated group. VitD: Vitamin D3 3.8 nM treated group. ALA: Alpha-linolenic acid 0.72 mM treated group. Combination: combination treated group (glutamine 12.7 mM, arginine 1.8 mM, vitamin D3 3.8 nM and ALA 0.72 mM). Standard PF: Polymeric formulation treated group. Values represent the mean $\pm$ SEM of 4 replicates for each group.

Figure 5 is a dose response scatter plot illustrating the effect of glutamine on IL-8 production by HT29 cells exposed to TNF- α (50 ng/mL) and cultured simultaneously with increasing glutamine concentrations (0.5, 1, 2.5, 5, 10, 15, 50, 120 and 240 mM) for 6 hours. Cells treated with 15mM glutamine and above showed a significant reduction in IL-8 level as compared with the positive control group. (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and ns $P > 0.05$ using one-way ANOVA test followed by Fischer least significance post hoc test). (-): Negative control group. (+): Positive control group (only TNF). (0.5 to 240): mM glutamine treated groups. Values represent mean $\pm$ SEM of 4 replicates for each group.

Figure 6 is a line graph illustrating viability of HT29 cells treated with increasing glutamine concentrations (0.5, 1, 2.5, 5, 10, 15, 50, 120 and 240 mM) for 24 hours as determined by Trypan Blue exclusion. Glutamine treated groups showed no significant drop in the cell viability compared to control group ($P > 0.05$; groups were compared to control group using one-way ANOVA test followed by Fischer least significance test). (0): 0 mM glutamine concentration (control group). (0.5 to 240):

mM glutamine treated groups. Values represent mean $\pm$ SEM of 4 replicates for each group.

Figure 7 provides dose response scatter plots A and B illustrating the effect of arginine on IL-8 production by HT29 cells exposed to TNF- α (50 ng/ml) and cultured simultaneously with increasing arginine concentrations (0.5, 2, 2.5, 5, 10, 20, 40, and 50 mM) for 6 hours. In the presence of arginine, IL-8 was reduced in a dose dependent fashion (A * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, ns $P > 0.05$, B * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$, ns $P > 0.05$, arginine treatment groups were compared to positive control group). Statistical analysis of data was conducted using one-way ANOVA test followed by Fischer least significance post hoc test. (-): Negative control group. (+): Positive control group (only TNF). (0.5 to 50): mM arginine treated groups. Values represent mean $\pm$ SEM of 4 replicates for each group.

Figure 8 provides scatter plots A and B illustrating viability of HT29 cells treated with increasing arginine concentrations (0.5, 2, 2.5, 5, 10, 20, 40 and 50 mM) for 24 hours as determined by Trypan Blue exclusion. Arginine showed no toxic effect even at high concentrations ($P > 0.05$; groups were compared to the control group using one-way ANOVA test followed by Fischer least significance test). (0): 0 mM arginine (control group). (0.5, 2, 2.5, 5, 10, 20, 40 and 50): mM arginine treated groups. Values represent mean $\pm$ SEM of 4 replicates for each group.

Figure 9 is a dose response scatter plot illustrating the effect of vitamin D3 on IL-8 production by HT29 cells exposed to TNF- α (50 ng/mL) and cultured simultaneously with increasing vitamin D3 concentrations (1, 10, 30 and 100 nM) for 6 hours. Vitamin D3 treated cells showed a dose dependent reduction in IL-8 production in response to TNF- α stimulation (** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ using one-way ANOVA test followed by Fischer least significance post hoc test). (-): Negative control group. (+): Positive control group (only TNF). (1, 10, 30 and 100): nM vitamin D3 treated groups. Values represent mean $\pm$ SEM of 4 replicates for each group.

Figure 10 is a line graph illustrating viability of HT29 cells treated with increasing vitamin D3 concentrations (1, 10, 30 and 100 nM) for 24 hours as determined by Trypan Blue exclusion. Vitamin D3 treatment showed no drop in the cell viability compared to control group ($P > 0.05$; groups were compared to control group using one-way ANOVA test followed by Fischer least significance test). (0): 0 nM of vitamin D3 (control group). (1, 10, 30 and 100): nM vitamin D3 treated groups. Values represent mean $\pm$ SEM of 4 replicates for each group.

Figure 11 is a dose response scatter plot illustrating the effect of alpha-linolenic acid (ALA) on IL-8 production by HT29 cells exposed to TNF- α (50 ng/mL) for 6 hours. HT29 cells were pre-incubated with increasing concentrations of ALA (0.3, 0.7, 1.4, 3.5 and 7 mM) for 48 hour before exposure to TNF- α . ALA had a negligible anti-inflammatory effect at all tested concentrations ($P > 0.05$; groups were compared to the positive control group using one-way ANOVA test followed by Fischer least significance test). (-): Negative control group. (+): Positive control group (only TNF). (0.3, 0.7, 1.4, 3.5 and 7): mM ALA treated groups. Values represent mean $\pm$ SEM of 4 replicates.

Figures 12 to 19 relate to Example 2.

Figure 12 is a scatter plot illustrating viability of HT29 cells treated with increasing concentrations of glutamine {0, 1, 10, 50, 100 or 240 mM} (A) or arginine {0, 1, 5, 10, 20 or 50 mM} (B) for 24 hours as determined by Trypan Blue exclusion. Values represent mean $\pm$ SEM of 4 replicates for each group. Cell viability in all treated groups was equivalent to the control ($P > 0.05$ in all groups vs control group (0mM concentration treated group, only media) using one-way ANOVA test followed by Fischer least significance test).

Figure 13 is a scatter plot illustrating viability of Caco2 cells treated with increasing concentrations of glutamine {0, 1, 10, 50, 100 or 240 mM} (A) or arginine {0, 1, 5, 10, 20 or 50 mM} (B) for 24 hours as determined by Trypan Blue exclusion. Values represent mean $\pm$ SEM of 4 replicates for each group. Cell viability in all treated groups was equivalent to the control ($P > 0.05$ in all groups vs control group (0mM concentration treated group, only media) using one-way ANOVA test followed by Fischer least significance test).

Figure 14 is a scatter plot illustrating viability of HT29 cells treated with increasing concentrations of glutamine {0, 1, 10, 50, 100 or 240 mM} (A) or arginine {0, 1, 5, 10, 20 or 50 mM} (B) for 24 hours as determined by MTT assay. Values represent mean $\pm$ SEM of 4 replicates of each treated group.

Figure 15 provides scatter plots A and B illustrating the effect of glutamine (240 mM) or arginine (50 mM) individually or in combination on IL-8 production, and scatter plot C illustrating the effect of glutamine (240 mM) and arginine (50 mM) in combination on IL-8 mRNA production, by HT29 cells concurrently incubated with TNF- α (50 ng/mL) for 6 hours. Values represent mean $\pm$ SEM of 5 replicates for each group and the experiment repeated twice. (Neg): negative control (neither treatment nor TNF- α). (Pos): positive control (only TNF- α). (Glu): glutamine 240 mM. (Arg): arginine at 50 mM. (Glu/Arg): a combination of 240 mM glutamine and 50 mM arginine. Each of glutamine and arginine significantly attenuated IL-8 level in response to TNF- α (A, $P < 0.0001$ versus positive control group). Combined glutamine and arginine resulted in further reduction in IL-8 level (A, $P = 0.0051$ and B, * $P < 0.05$ for glutamine treated group and A, $P = 0.025$ and B, * $P < 0.05$ for arginine treated group vs combined glutamine and arginine treated group). Indeed, complete abrogation of IL-8 was evident in the combination treatment group (A, $P = 0.9536$ versus negative control group). C, Glutamine plus arginine inhibited IL-8 mRNA expression (* $P < 0.05$). Analysis of data was conducted using one-way ANOVA followed by Fischer least significance test.

Figure 16 is a scatter plot illustrating the effect of glutamine (240 mM) or arginine (50 mM) individually or in combination on IL-8 production from Caco2 cells concurrently incubated with a mixture of 50 ng/mL TNF- α , 50 ng/mL INF- γ , 25 ng/mL IL-1 β and 1 μ g/mL LPS for 24 hours. Values represent mean $\pm$ SEM of 5 replicates for each group and the experiment repeated twice. (Neg): negative control (neither treatment nor TNF- α). (Pos): positive control (only TNF- α). (Glu): glutamine at 240 mM. (Arg): arginine at 50 mM. (Glu/Arg): a combination of 240 mM

glutamine and 50 mM arginine. Each of glutamine and arginine significantly attenuated IL-8 level in response to given inflammatory stimuli. Combined glutamine and arginine resulted in further reduction in IL-8 (* $P < 0.01$, ** $P < 0.001$ and *** $P < 0.0001$ versus positive control group). Analysis of data was conducted using one-way ANOVA followed by Fischer least significance test.

Figure 17 provides line graphs quantifying by densitometry the corresponding Western blot images presented under each graph of I κ B and phosphorylated I κ B (ph-I κ B) responses in HT29 cells exposed to 100 ng/mL TNF- α (A), or 100 ng/mL TNF- α and 240 mM glutamine (B), or 100 ng/mL TNF- α and 50 mM arginine (C) for 5, 15 or 30 minutes. In TNF- α exposed cells, I κ B level peaked at 5 minutes that corresponded with an absence of ph-I κ B. At 15 minutes, the majority of I κ B was phosphorylated, consistent with the return of I κ B to baseline levels at 15 minutes. Glutamine prevented increases in I κ B expression and production of ph-I κ B over 30 minutes. Arginine prolonged I κ B expression and delayed production of ph-I κ B over 30 minutes.

Figure 18 provides Western blot images of total I κ B and phosphorylated I κ B (Ph I κ B) responses in HT29 cells exposed to 100 ng/mL TNF- α (A) or 100 ng/mL TNF- α and 240 mM glutamine (B) for 5, 15, 30 or 60 minutes. After adding TNF- α , I κ B was partially degraded at 5 minutes, corresponding to the appearance of phosphorylated I κ B at 5 minutes. An I κ B level was increased at 15 and 30 minutes but was equivalent to baseline at 60 minutes that was consistent with disappearance of phosphorylated I κ B. Glutamine prevented the early TNF- α induced I κ B degradation at 5 minutes. Phosphorylated I κ B was not detected in the presence of glutamine along with continuous rise of I κ B over the 60 minutes of TNF- α exposure.

Figure 19 provides line graphs quantifying by densitometry the corresponding Western blot images presented under each graph of total I κ B in HT29 cells exposed to 100 ng/mL TNF- α (A) or 100 ng/mL TNF- α and 50 mM arginine (B) for 5, 15, 30 or 60 minutes. An initial drop was evident at 5 minutes that normalized over 60 minutes. In the presence of arginine, the early drop in the I κ B seen with TNF- α was less evident; instead cells showed an accumulation at 15 minutes followed by accelerated I κ B degradation from 15 minutes with complete loss of I κ B at 60 minutes.

Figures 20 to 28 relate to Example 3.

Figure 20 provides scatter plots illustrating viability of HT29 (A) and INT407 (B) cells treated for 24 hours with curcumin at 10, 25, 50, 75 or 100 μ M dissolved in DMSO (final concentration of 0.1% v/v) as determined by Trypan Blue exclusion. Values represent mean $\pm$ SEM of 4 replicates for each group. Curcumin at 10, 25 or 50 μ M in both cell lines maintained cell viability above 90% comparable to the control group ($P > 0.05$). In contrast, cells of both lines treated with 75 or 100 μ M curcumin showed a significant drop in the cell viability ($P < 0.0001$ of 75 and 100 μ M as compared with 0 μ M curcumin group (DMSO only) using one way ANOVA test followed by Fischer least significance).

Figure 21 is a scatter plot illustrating viability of HT29 cells treated for 24 hours with curcumin at 0, 20, 40, 50, 60, 75 or 100 μ M dissolved in DMSO (final concentration of 0.1% v/v)

determined by MTT assay. Values represent mean $\pm$ SEM of 4 replicates of each treated group. Curcumin at 60 μ M and above significantly reduced the cellular viability compared to the control group (0 μ M curcumin) { $P=0.001$, 0.0002 and 0.0002 for 60, 75 and 100 μ M treated groups, respectively as compared with 0 μ M curcumin group (DMSO only) using one way ANOVA test followed by Fischer least significance}.

Figure 22 provides scatter plots illustrating the effect of pre-treatment with curcumin (10 μ M, 25 μ M or 50 μ M) on IL-8 production in HT29 (A) and INT407 (B) cells exposed to TNF- α (50 ng/mL) for 6 hours (HT29 cells) or 24 hours (INT407 cells). Values represent mean of 4 replicates of each group. Curcumin at 50 μ M (x with solid line) showed abrogation of IL-8 level from HT29 cells (A) in response to TNF- α ($P<0.001$ for 24 hours pre-incubation, $P=0.001$ for 6 hours pre-incubation, $P<0.0001$ for 1 hour pre-incubation and $P=0.0001$ for 0 hour pre-incubation, respectively as compared to positive control group). Similar effects were seen in INT407 cells (B) in which IL-8 were levels totally attenuated in the presence of curcumin (50 μ M) ($P<0.0001$ for all treatment groups compared to positive control group using one way ANOVA test followed by Fischer least significance). (–) no treatment control, (+) TNF- α exposure, no curcumin, (*) TNF- α exposure, no curcumin, 0.1% v/v DMSO control, $\circ$ with dotted line is 10 μ M curcumin, $\circ$ with dashed line is 25 μ M curcumin, and x with solid line is 50 μ M curcumin.

Figure 23 is a photograph of Western blots illustrating the I κ B response of HT29 cells pre-treated for 1 hour with curcumin before exposure to TNF- α (100 ng/mL) or exposed simultaneously to curcumin and TNF- α (100 ng/mL) then incubated for 5, 15 or 30 minutes. Curcumin treatment with or without pre-incubation blocked I κ B phosphorylation and degradation in TNF- α stimulated HT29 cells.

Figure 24 is a photograph of Western blots illustrating the I κ B response of INT407 cells pre-treated for 1 hour with curcumin before exposure to TNF- α (100 ng/mL) or exposed simultaneously to curcumin and TNF- α (100 ng/mL) then incubated for 5, 15 or 30 minutes.. Similar to HT29 cells (Figure 23), curcumin treatment with or without pre-incubation blocked I κ B phosphorylation and degradation in TNF- α stimulated INT407 cells.

Figure 25 provides scatter plots illustrating the effect of a whole protein 1 Cal/mL nutritionally complete (standard) PF versus PF supplemented with increasing concentrations of glutamine and arginine (glutamine/ arginine: 50/2, 50/10, 50/20, 50/25, 50/30 and 240/50 mM/ mM) on IL-8 production by HT29 cells exposed simultaneously to TNF- α (50 ng/mL) and incubated for 6 hours. The PF and glutamine/arginine concentrations were 1/5 of the concentrations tested previously to simulate intestinal dilution. Thus, for example, 50/20 corresponds with 250/100 (the calculated highest tolerable concentration) and 240/50 corresponds with 1200/250. Values represent mean $\pm$ SEM of 4 replicates of each group. The two plots represent the same data plotted against the positive control (A) or the negative control (B). Standard PF treatment significantly reduced IL-8 level in response to TNF- α ($P<0.0001$ versus positive control group). Increasing glutamine and arginine

supplementation in PF resulted in further reduction of IL-8 up to the highest tested concentrations 240 mM glutamine and 50 mM arginine (ns $P>0.05$, * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ versus standard PF). However, IL-8 level in the group treated with highest tolerable concentrations (i.e. 50/20 glutamine, 50 mM and arginine 20 mM) remained greater than the negative control (P=0.0026 versus negative control). Analysis was conducted using one-way ANOVA followed by Fischer least significance test. (-): negative control (neither treatment nor TNF- α). (+): positive control (only TNF- α). (Stand PF): standard PF. (50/2, 50/10, 50/20, 50/25, 50/30 and 240/50) glutamine/ arginine mM/ mM.

Figure 26 is a column graph illustrating the effect of curcumin 50 μ M, standard PF, PF supplemented with glutamine 50 mM and arginine 20 mM, or PF supplemented with glutamine 50 mM, arginine 20 mM and curcumin 50 μ M on IL-8 production by HT29 cells exposed simultaneously to TNF- α (50 ng/mL) and incubated for 6 hours. Standard PF comprised glutamine 12.7 mM and arginine 1.8 mM. Values represent mean + SEM of 4 replicates of each group. The two graphs represent the same data plotted against the positive control (A) or the negative control (B). All treatments significantly reduced IL-8 level in response to TNF- α ($P<0.0001$ for all treated groups as compared to the positive control group). However, only PF supplemented with glutamine, arginine and curcumin completely blocked IL-8 level (ns $P>0.05$, * $P<0.01$ and ** $P<0.001$, respectively versus negative control group). Analysis of data was conducted using one-way ANOVA followed by Fischer least significance test. (-): negative control (neither treatment nor TNF- α). (+): positive control (only TNF- α). (Standard PF): standard PF (12.7/1.8 glutamine/ arginine mM/ mM). (Curcumin): curcumin (50 μ M) was dissolved first in PF and then was added to media to give 0.1% v/v PF final concentration in media. (Enriched-PF): PF supplemented with glutamine 50 mM and arginine 20 mM. (Novel formula): PF supplemented with glutamine 50 mM, arginine 20 mM and curcumin 50 μ M.

Figure 27 is a scatter plot illustrating viability of HT29 cells incubated with (A) PF supplemented with increasing concentrations of glutamine and arginine (glutamine/arginine 50/2, 50/10, 50/20, 50/25, 50/30 or 240/50 mM/ mM) or (B) curcumin 50 μ M, standard PF, PF supplemented with glutamine 50 mM and arginine 20 mM, or PF supplemented with glutamine 50 mM, arginine 20 mM and curcumin 50 μ M for 24 hours as determined by Trypan Blue exclusion. Values represent mean $\pm$ SEM of four replicates for each group. Treatment groups showed no significant difference in the viability as compared to control group ($P>0.05$ treated groups vs untreated group using one-way ANOVA followed by Fischer least significance test). (-): negative control (no treatment). (Standard PF): standard PF (12.7/1.8 glutamine/ arginine mM/ mM). (50/2, 50/10, 50/20, 50/25, 50/30 and 240/50) PF supplemented with glutamine/ arginine mM/ mM). (Curcumin): curcumin (50 μ M) was dissolved first in PF and then was added to media to give 0.1% v/v PF final concentration in media. (Enriched-PF): PF supplemented with glutamine and arginine (50/20 glutamine/arginine mM/ mM). (Novel formula): PF supplemented with glutamine 50 mM,

arginine 20 mM and curcumin 50 μ M.

Figure 28 is a scatter plot illustrating viability of HT29 cells incubated with curcumin 50 μ M, standard PF, PF supplemented with glutamine 50 mM and arginine 20 mM, or PF supplemented with glutamine 50 mM, arginine 20 mM and curcumin 50 μ M for 24 hours as determined by MTT assay. Values represent mean $\pm$ SEM of 4 replicates of each treated group. Standard PF had lower activity than control group (* $P < 0.05$). Statistical analysis of data was carried out using one way ANOVA test followed by Fischer least significance). (–): negative control (no treatment). (Standard PF): standard PF (12.7/1.8 glutamine/ arginine mM/ mM). (Curcumin): curcumin (50 μ M) was dissolved first in PF and then was added to media to give 0.1% v/v PF final concentration in media. (Enriched-PF): PF supplemented with glutamine and arginine (50/20 glutamine/ arginine mM/ mM). (Novel formula): PF supplemented with glutamine 50 mM, arginine 20 mM and curcumin 50 μ M.

Figures 29 and 30 relate to Example 4.

Figure 29 provides dose response scatter plots for HT29 cells exposed to TNF- α (50 ng/mL) and varying concentrations of glutamine (A), arginine (B), curcumin (C), or standard PF (PF) or PF supplemented with glutamine (250 mM), arginine (50 mM), and curcumin (50 μ M) (PF + G/A/C) (D) for 6 hours. There was no significant difference in IL-8 levels between the Negative control and TNF stimulated PF + G/A/C treated cells ($p > 0.05$). Arrow – glutamine/ arginine/ concentration in standard PF. Circle – target glutamine/ arginine/ curcumin concentration for supplemented PF in this experiment; elsewhere target concentrations include 240 mM or 250 mM glutamine, 100 mM arginine, 50 μ M curcumin.

Figures 30 and 31 relate to Example 5.

Figure 30 provides photographs of Western blots analysing NF- κ B signalling proteins. Confluent HT29 cells were differentially exposed to TNF- α (50ng/ml) glutamine (240mM) or arginine (50mM) and incubated for 5, 15, or 30 minutes before cytosolic and nuclear cell lysates were collected. Membranes were probed with: anti-I κ B or anti-ph I κ B antibodies (A); anti-I κ B or anti-ph I κ B antibodies (B); anti-P65 antibodies (C); or anti-P38 antibodies or anti-ph P38 antibodies (D). β -actin was included as a loading control. Protein bands were visualised by chemiluminescence.

Figure 31 provides photographs of cells grown on glass slides for 3 days then analysed by immunohistochemistry for expression and nuclear migration of P65 subunit of NF- κ B. HT29 cells were unstimulated (A) or stimulated with TNF- α (50ng/ml) for 1 hour (B), or pre-incubated with either 240mM glutamine (C) or 50mM arginine (D) for 1 hour then stimulated with TNF- α (50ng/ml) for another 1 hour. Slides were then incubated with rabbit polyclonal anti-human P65 antibody and detected using 488 Alexa (green) secondary goat anti-rabbit antibodies. Nuclei were counter stained with DAPI fluorescence (blue). The slides were visualized by Axioplan 2 immunofluorescent microscope (40x magnification) illustrating epithelial monolayer histology (1), P65 expression (2) and P65 expression with nuclei counterstaining (3).

Figure 32 relates to Example 6.

Figure 32 provides scatter plots showing I κ B kinase (I κ BK) enzyme activity in response to A glutamine or B arginine. Increasing concentrations of glutamine (10, 50, 100 or 240mM) or arginine (10, 20 or 50mM) were added to the reaction buffer. No enzyme control (no I κ BK) and synthetic inhibitor control at 10mM concentration (K252a) were included. Data represent mean $\pm$ SEM of 5 replicates.

* P<0.001, ** P<0.0001, ns not significant vs. I κ BK control.

Figure 33 relates to Example 7.

Figure 33 provides a scatter plot A and a column graph B showing that glutamine, arginine and curcumin in combination can completely suppress I κ BK activity. No enzyme control (no I κ BK) and synthetic inhibitor control at 10mM concentration (K252a) were included. Final concentrations were: glutamine 12mM (Glu 1) or 50mM (Glu 2); arginine 2mM (Arg 1) or 20mM (Arg 2); curcumin 50 μ M (Cur). Raw data are presented in A and data normalised to positive control are presented in B. The combination of glutamine, arginine and curcumin (Glu 2/Arg 2+Cur) abolished I κ BK activity (P=0.66 vs. K252). Analysis of data was conducted using one-way ANOVA test followed by Fischer's least significance post hoc test (* P<0.01, ** P<0.0001 vs I κ BK control).

Figures 34 and 35 relate to Example 8.

Figure 35 provides scatter and column plots of LDH activity in colonic biopsies in tissue culture. Intestinal biopsies (10 per group) collected from normal subjects without inflamed bowel (negative control) and from patients with active CD were incubated with media alone (positive control), standard PF or the nutritional formulation comprising glutamine 50 mM, arginine 20mM, curcumin 50 μ M (labelled "Novel formula"). Lactate dehydrogenase (LDH) activity (A) was measured in the culture media and converted to % enzyme release (B). LDH release, as an indicator of tissue viability, was not significantly different between groups. Data represent mean $\pm$ SEM. Analysis by one way ANOVA with least Fischer sufficient test; P#>0.05 vs. negative control.

Figure 35 provides scatter plots showing TNF (A), IL-8 (B) and IL-6 (C) release from colonic biopsies in tissue culture. Colonic biopsies were collected from normal subjects without inflamed bowel (negative control) and from patients with active CD and were incubated with media alone (positive control), standard PF or the nutritional formulation comprising glutamine 50 mM, arginine 20mM, curcumin 50 μ M (labelled "Novel formula"). Concentrations of TNF- α (A), IL-8 (B) and IL-6 (C) were measured in the supernatants using ELISA. Levels of the measured mediators were significantly higher in the positive control compared to the negative control. The nutritional formulation decreased the concentration of cytokine/chemokines to negative control levels. Standard PF showed only numerical reduction for the three cytokine/chemokines. Data represent mean $\pm$ SEM and were analysed by Kruskal-Wallis test; (*) P<0.05 vs. negative control; (&) P>0.05; (#) P<0.05 vs. positive control.

Figure 36 relates to Example 9.

Figure 36 is a column graph illustrating weight gain in a mouse colitis model treated with PF

supplemented with glutamine (250 mM), arginine (50 mM), and curcumin (50 μ M). Eight week old BALB/c mice were injected with 2.5 mg TNBS in 45% ethanol per rectal and then fed with standard PF or supplemented PF for 7 days. Presented as % weight increase at day 7 compared to day 0.

DETAILED DESCRIPTION

5 The inventors have utilised *in vitro*, *ex vivo* and animal models of IBD to define a pharmaconutrient composition comprising at least two pharmaconutrients having similar mechanisms of pharmacological activity that is useful for treating intestinal inflammation. The at least two pharmaconutrients may be selected from arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid. In one embodiment, the inventors have defined a nutritional formulation
10 supplemented with the at least two of the pharmaconutrients arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid, in one preferred embodiment glutamine, arginine and curcumin. The inventors have shown that this supplemented nutritional formulation can completely abrogate the inflammatory response to TNF- α in intestinal epithelial cells in cell culture.

15 Currently, EEN is as effective as steroids in induction of remission in childhood CD. Importantly, nutritional therapy has many additional benefits compared to steroids. Nutritional therapy provides nutritional support, which is often essential in childhood CD, has fewer side-effects and leads to high rates of mucosal healing. However, 12 month relapse rates for both EEN and steroids when used as induction therapy are greater than 60%.

20 The present invention significantly enhances the efficacy of EEN by providing a nutritional formulation supplemented with pharmaconutrients that actively suppresses inflammation without compromising the benefits of EEN. The significance of this is that it: 1) produces a therapy that is more effective than steroids, the current accepted “gold standard” induction therapy for IBD; 2) assists in reducing steroid dependency; and 3) allows for better
25 disease control and improved subject outcomes.

Nutritional therapy

The inventors have demonstrated induction of remission with EEN as sole therapy in 80% of a group of children with newly diagnosed CD. In these children the inventors noted falling disease activity (mean Pediatric Crohn’s Disease Activity Index (PCDAI) decreased from 37.1 ± 10.8 to $6.7 \pm$
30 5.1) and decreasing inflammatory markers (including C-reactive protein) after eight weeks of therapy.

Although the clinical benefits of EEN for CD have been well defined, the mechanism(s) of these effects remains unclear. Proposed putative mechanisms include relative bowel rest, alteration of the intestinal microflora and direct anti-inflammatory effects. Other potential mechanisms include reversal of malnutrition by EEN with secondary reversal of gut inflammation. The initial use of
35 elemental feeds (containing modified “pre-digested” proteins) for EEN lead to the suggestion that gut rest could be important. Subsequently, polymeric feeds (containing whole, unmodified proteins) were

shown to be equally beneficial. Consequently, gut rest alone would appear unlikely as a predominant beneficial mechanism, as PF would be expected to require more digestive activity than elemental formulae. The two most likely mechanisms attributed to EEN in treating CD therefore appear to be direct anti-inflammatory effects and alteration of the intestinal microflora leading to modification of host bacterial interactions.

The inventors have shown that PF reduces colonic epithelial cell chemokine responses to pro-inflammatory cytokines using an *in vitro* two-compartment model where colonic epithelial cells were grown to confluence on a support membrane that was separated by apical and basal wells. To replicate *in vitro* epithelial inflammation, TNF- α (100 ng/ml) was added to the basal well with PF added to the apical well. In the absence of PF 10,230 $\pm$ 1113 pg/ml of IL-8 was released by epithelial cells, however when PF was added to the apical well IL-8 release was reduced 5-fold to 2205 $\pm$ 554 pg/ml ($P < 0.0001$). Further investigations indicated that PF may interact with the NF- κ B pathway by limiting I κ B degradation.

Elsewhere, direct anti-inflammatory effects of enteral feeds have come from mucosal biopsies of adults with CD, UC and control subjects incubated with elemental formulae for up to 24 hours. Tissues from subjects with CD showed an increased ratio of interleukin-1 receptor antagonist (IL-1RA) to interleukin-1 β compared to the ratio in control samples ($P < 0.05$). Changes in this cytokine ratio were not observed in biopsy samples from subjects with UC or non-inflammatory controls. These results correspond to *in vitro* human data showing changes in mucosal anti-inflammatory and pro-inflammatory proteins as a consequence of EEN.

Accordingly, the pharmaconutrient composition or nutritional formulation may be used to induce remission of IBD in a subject.

Glutamine

Glutamine is the most readily available of the “non-essential” amino acids. Glutamine is profoundly depleted in cases of critical illness such as in the intensive care setting, where glutamine supplementation can greatly improve subject outcomes. Glutamine modulates intra-cellular activity through stimulating the mitogen-activated protein kinase (MAPK) pathway and the synthesis of heat shock proteins (HSP), which protect cells under stress and is suggested to reduce pro-inflammatory cytokines by suppressing the NF- κ B pathway. Indeed, glutamine leads to I κ B accumulation following TNF- α stimulation (Figure 18). In the absence of supplement, TNF- α stimulation of HT29 cells causes an initial degradation, then replenishment of I κ B as a result of NF- κ B activation (Figure 18A). However in the presence of glutamine I κ B accumulates, indicating that NF- κ B activation is suppressed (Figure 18B).

In addition, glutamine has activity specific to the intestine as it is a primary metabolic fuel for the small intestine, prevents apoptosis of intestinal epithelial cells and contributes to maintaining TJ integrity. Thus, sufficient glutamine availability is an important factor in protection from bacterial endotoxin challenge. In the setting of IBD, decreased serum glutamine levels have been reported in

children with CD. Using animal models of CD, glutamine supplementation has been shown to reduce inflammation. Nevertheless, glutamine concentrations in enteral formulation are generally low, because the art teaches that glutamine is a non-essential amino acid and need not be supplemented. Therefore, glutamine supplementation of enteral formulation provides surprising benefits for subjects with intestinal inflammation.

Arginine

Arginine is a dibasic amino acid that affects immune defence and wound healing and is reported to have therapeutic effects when enriched in diets. The Nestle formulation IMPACT, used for feeding intensive care and burns unit subjects, is arginine enriched. A meta-analysis of arginine-supplemented diets found that there were no adverse effects observed in the arginine supplemented subjects, but were associated with significant reductions in post-operative infectious complications, significant decrease in length of stay and in GI surgical subjects, and anastomotic leaks were 46% less prevalent.

The art teaches that glutamine is a non-essential amino acid and need not be supplemented. However, like glutamine, arginine may become a conditionally essential amino acid, meaning that under normal circumstances arginine is not essential, but becomes so during injury or stress, such as in critical illness. Nevertheless, when tested in numerous animal model of IBD, arginine showed inconsistent findings in terms of enhancing mucosal healing and improving survival rates of mice, even increasing the severity of intestinal inflammation. Therefore, the art teaches away from glutamine supplementation of enteral formula, and arginine supplementation provides surprising benefits for subjects with intestinal inflammation.

Over the time course tested herein, arginine did not interact with the NF- κ B pathway as shown by the complete degradation of I κ B at 60 minutes following TNF- α stimulation (Figure 19B). In the absence of supplements, TNF initially induces degradation of I κ B, but this is counter balanced by upregulation and replenishment of I κ B. Although not wishing to be bound by theory, the inventors propose that arginine exerts an effect following I κ B degradation and NF- κ B release, and limits NF- κ B from promoting gene expression and production of I κ B as well as inflammatory proteins.

Curcumin

Curcumin is the yellow pigment in turmeric and has been reported to have anti-inflammatory, anti-oxidant, anti-carcinogenic, and anti-microbial actions. In the setting of IBD, curcumin ameliorates gross and histological alterations of the colon, prevents bloody diarrhoea, improves tissue oedema and reduces serum TNF levels in DSS and TNBS mouse models of IBD. The mechanism of action of curcumin has been proposed as blocking protein kinase B/ MAPK and the NF- κ B pathway, therefore preventing nuclear translocation of the p65 NF- κ B subunit. These findings suggest that curcumin is a potential therapy for IBD treatment and indeed early stage clinical trials look promising. Importantly, curcumin confers this benefit without known side effects.

Previously, the inventors tested PF supplemented with curcumin in a similar model of

intestinal inflammation and observed no effect on concurrent inflammation. However, when the intestinal epithelial cells were pre-treated with PF supplemented with curcumin, the inflammatory response was greatly reduced. Thus, the inventors concluded that curcumin may be suitable to maintain remission of intestinal inflammation, but not induce remission of intestinal inflammation.

Herein, the inventors have demonstrated that curcumin attenuated pro-inflammatory cytokine (IL-8) production by the cultured intestinal cell lines in response to inflammatory stimuli (TNF- α), irrespective of the timing of supplementation. Subsequently, the addition of curcumin to a glutamine and arginine enriched PF completely inhibited pro-inflammatory cytokine production in response to inflammatory stimuli, without having a negative impact on cell viability below 60 μ M.

Given that the timing of curcumin supplementation had no observable effect on the anti-inflammatory effect, the inventors have shown that curcumin can induce and maintain remission of intestinal inflammation.

Pharmaconutrient Composition

The inventors have demonstrated that glutamine, arginine and vitamin D3 are the active ingredients of PF that attenuate intestinal inflammation. Further, the inventors have demonstrated that the anti-inflammatory activities of glutamine, arginine and vitamin D3 are dose dependent; increasing concentrations resulted in more considerable reduction in pro-inflammatory cytokine (IL-8) production from cultured epithelial monolayers without having any detrimental effect on cell viability. In addition, PF enriched with a combination of glutamine and arginine was superior to standard PF in ameliorating TNF- α induced inflammatory response in intestinal epithelial cells, again with no negative impact upon cell viability. These data indicate the usefulness of these nutritional agents at higher concentrations than currently used in the conventional nutritional formulae.

However, the high protein content could lead to safety concerns, thereby limiting implementation in clinical practice. Thus, for developing a safe nutritional formula, glutamine and arginine concentrations were reduced, and the nutritional formulation further supplemented with curcumin, thereby achieving superior efficacy to standard PF.

The person skilled in the art understands that PF is intended for nutritional support, for example in patients requiring intensive care. The person skilled in the art would not be motivated to supplement PF for treating intestinal inflammation, let alone to supplement PF with the agents or combination of agents disclosed herein.

Given the high concentrations of glutamine and arginine required to reach maximal inhibition of intestinal inflammation (e.g. IL-8 production), in light of the constraint of the maximum protein (including amino acid) intake, the person skilled in the art would not be motivated to *reduce* the concentrations of glutamine and arginine. Further, because it has been shown that curcumin does not induce remission of intestinal inflammation, the person skilled in the art would not have been motivated to combine curcumin with glutamine and arginine, let alone to reduce the concentrations of glutamine and arginine and then combine glutamine and arginine with curcumin. Therefore, the

present disclosure is unexpected and advantageous over the prior art.

As disclosed herein, a pharmaconutrient composition or nutritional formulation for treating intestinal inflammation in a subject comprises at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid. Thus, the pharmaconutrient composition or nutritional formulation may
5 comprise: arginine and glutamine; arginine and curcumin; arginine and vitamin D3; arginine and an n-3 fatty acid; glutamine and curcumin; glutamine and vitamin D3; glutamine and an n-3 fatty acid; curcumin and vitamin D3; curcumin and an n-3 fatty acid; or vitamin D3 and an n-3 fatty acid.

As used herein, a “pharmaconutrient” refers to a nutrient that has an effect on an inflammatory, immunological, metabolic, and other pathophysiological processes of a subject. In turn,
10 “nutrient” refers to a substance that a subject needs to live and grow, or a substance used in a subject’s metabolism, which must be taken in from the environment. Examples of nutrients include carbohydrates, fats, proteins (or amino acids), and vitamins. Dietary minerals, water, and oxygen may also be considered nutrients. A nutrient is “essential” if the subject cannot synthesize the nutrient or produces the nutrient in insufficient quantity to maintain health. Thus, as used herein, a

15 “pharmaconutrient” refers to a nutrient used as a pharmacological substance.

The at least two pharmaconutrients may be administered simultaneously or sequentially. The at least two pharmaconutrients may comprise a pharmaconutrient composition or a nutritional formulation. The at least two pharmaconutrients may administered by more than one route, e.g. any combination or oral, rectal, enteral and parenteral, or in more than one form, e.g. solid, liquid,
20 emulsion, suspension, solution, tablet, capsule, gel-cap, powder etc. For oral administration, the at least two pharmaconutrients may be administered as a food.

A pharmaconutrient differs from a “nutraceutical”, because a “nutraceutical” refers to a substance isolated or purified from a foodstuff, and is provided independent of food. Although a “nutraceutical” has a physiological benefit for or provides protection against chronic disease to a
25 subject, the health benefit of the “nutraceutical” is in addition to the basic nutrients found in food, and again is differentiated from a pharmaconutrient.

A pharmaconutrient composition or a nutritional formulation differs from a “functional food”, because a functional food refers to a food that is consumed as part of the normal diet of a subject, even though the “functional food” comprises a substance that offers the potential of enhanced
30 health or reduced risk of disease to the subject. In contrast, a pharmaconutrient composition or a nutritional formulation is not consumed as part of the normal diet of the subject. The pharmaconutrient composition or nutritional formulation serves the specific role of inducing and/or maintaining remission of intestinal inflammation and is not considered part of the subject’s normal diet. Nevertheless, the pharmaconutrient composition or nutritional formulation may be consumed in
35 addition or supplementary to the subject’s normal diet in order to induce and/or maintain remission of intestinal inflammation.

As used herein, “pharmaconutrient composition” refers to a composition that may be added

to a nutritional formulation to supplement or enrich the nutritional formulation with the selected pharmaconutrients. The term “pharmaconutrient composition” is not intended to exclude the term “nutritional formulation”; instead the term “pharmaconutrient composition” includes the term “nutritional formulation”. Therefore, a “pharmaconutrient composition” may also be a nutritional formulation supplemented or enriched for the selected pharmaconutrients, for example, in unit dosage.

The pharmaconutrient composition or nutritional formulation may comprise three of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid. Thus, the pharmaconutrient composition or nutritional formulation may comprise: arginine, glutamine and curcumin; arginine, glutamine and vitamin D3; arginine, glutamine and an n-3 fatty acid; arginine, curcumin and vitamin D3; arginine, curcumin and an n-3 fatty acid; arginine, vitamin D3 and an n-3 fatty acid; glutamine, curcumin and vitamin D3; glutamine, curcumin and an n-3 fatty acid; or curcumin, vitamin D3 and an n-3 fatty acid.

The pharmaconutrient composition or nutritional formulation may comprise four of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid. Thus, the pharmaconutrient composition or nutritional formulation may comprise: arginine, glutamine, curcumin and vitamin D3; arginine, glutamine, curcumin and an n-3 fatty acid; arginine, glutamine, vitamin D3 and an n-3 fatty acid; arginine, curcumin, vitamin D3 and an n-3 fatty acid; or glutamine, curcumin, vitamin D3 and an n-3 fatty acid.

The pharmaconutrient composition or nutritional formulation may comprise arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.

The pharmaconutrient composition or nutritional formulation may comprise curcumin and at least one of arginine, glutamine, vitamin D3, and an n-3 fatty acid. The pharmaconutrient composition or nutritional formulation may comprise curcumin and at least two of arginine, glutamine, vitamin D3, and an n-3 fatty acid. The pharmaconutrient composition or nutritional formulation may comprise curcumin and at least three of arginine, glutamine, vitamin D3, and an n-3 fatty acid. The pharmaconutrient composition or nutritional formulation may comprise curcumin and arginine, glutamine, vitamin D3, and an n-3 fatty acid.

In one embodiment, the pharmaconutrient composition or nutritional formulation comprises arginine, glutamine, and curcumin.

Preferably, arginine is L-arginine. Preferably, glutamine is L-glutamine. However, D-arginine and/or D-glutamine are also contemplated.

Arginine and/or glutamine, or other amino acid with pharmaconutrient activity, may be administered as free amino acid, peptide, or polypeptide.

In one embodiment, the pharmaconutrient composition or nutritional formulation comprises vitamin D3 (cholecalciferol). However, vitamin D3 may be substituted in part or in full for any Vitamin D, for example vitamin D2 (ergocalciferol), 25-hydroxyvitamin D3 (calcidiol, calcifediol,

25-hydroxycholecalciferol), 25-hydroxyvitamin D2 (25-hydroxyergocalciferol), or calcitriol (1,25-dihydroxycholecalciferol, 1,25-dihydroxyvitamin D3).

In one embodiment, the pharmaconutrient composition or nutritional formulation comprises an n-3 fatty acid. The n-3 fatty acid may comprise hexadecatrienoic acid (HTA), α -linolenic acid (ALA), stearidonic acid (SDA), eicosatrienoic acid (ETE), eicosatetraenoic acid (ETA), eicosapentaenoic acid (EPA), heneicosapentaenoic acid (HPA), docosapentaenoic acid (DPA), clupanodonic acid, docosahexaenoic acid (DHA), tetracosapentaenoic acid, or tetracosahexaenoic acid. Preferably, the n-3 fatty acid comprises α -linolenic acid (ALA), docosahexaenoic acid (DHA), or eicosapentaenoic acid (EPA). ALA may be derived from plant oils, for example canola, flaxseed or linseed. DHA and EPA may be derived from marine oils, for example fish or krill. As used herein, “n-3 fatty acid” refers to a fatty acid also known to the person skilled in the art as an “omega-3 fatty acid” or an “n-3 fatty acid” and defined as a polyunsaturated fatty acid with a C=C double bond at the third carbon atom from the terminal carbon of the carbon chain.

The pharmaconutrient composition or nutritional formulation may comprise about 1 mM to about 600 mM arginine. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise about 50 mM to about 550 mM, about 100 mM to about 500 mM, about 150 mM to about 450 mM, about 200 mM to about 400 mM, about 250 mM to about 350 mM, or about 300 mM arginine. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, about 100, about 101, about 102, about 103, about 104, about 105, about 106, about 107, about 108, about 109, about 110, about 111, about 112, about 113, about 114, about 115, about 116, about 117, about 118, about 119, or about 120 mM arginine. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise about 1, about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 260, about 270, about 280, about 290, about 300, about 310, about 320, about 330, about 340, about 350, about 360, about 370, about 380, about 390, about 400, about 410, about 420, about 430, about 440, about 450, about 460, about 470, about 480, about 490, about 500, about 510, about 520, about 530,

about 540, about 550, about 560, about 570, about 580, about 590, or about 600 mM arginine. In one embodiment, the pharmaconutrient composition or nutritional formulation comprises greater than 90, greater than 91, greater than 92, greater than 93, greater than 94, greater than 95, greater than 96, greater than 97, greater than 98, greater than 99, or greater than 100 mM arginine. Alternatively, the pharmaconutrient composition may comprise arginine in an amount sufficient to produce any one of the foregoing concentrations of arginine, for example when the pharmaconutrient composition is used to supplement a nutritional formulation.

The pharmaconutrient composition or nutritional formulation may comprise about 1 mM to about 1200 mM glutamine. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise about 50 mM to about 1150 mM, about 100 mM to about 1100 mM, about 150 mM to about 1050 mM, about 200 mM to about 1000 mM, about 250 mM to about 950 mM, about 300 mM to about 900 mM, about 350 mM to about 850 mM, about 400 mM to about 800 mM, about 450 mM to about 750 mM, about 500 mM to about 700 mM, about 550 mM to about 650 mM, or about 600 mM glutamine. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise about 201, about 202, about 203, about 204, about 205, about 206, about 207, about 208, about 209, about 210, about 211, about 212, about 213, about 214, about 215, about 216, about 217, about 218, about 219, about 220, about 221, about 222, about 223, about 224, about 225, about 226, about 227, about 228, about 229, about 230, about 231, about 232, about 233, about 234, about 235, about 236, about 237, about 238, about 239, about 240, about 241, about 242, about 243, about 244, about 245, about 246, about 247, about 248, about 249, about 250, about 251, about 252, about 253, about 254, about 255, about 256, about 257, about 258, about 259, about 260, about 261, about 262, about 263, about 264, about 265, about 266, about 267, about 268, about 269, about 270, about 271, about 272, about 273, about 274, about 275, about 276, about 277, about 278, about 279, or about 280 mM glutamine. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise about 1, about 20, about 40, about 60, about 80, about 100, about 120, about 140, about 160, about 180, about 200, about 220, about 240, about 260, about 280, about 300, about 320, about 340, about 360, about 380, about 400, about 420, about 440, about 460, about 480, about 500, about 520, about 540, about 560, about 580, about 600, about 620, about 640, about 660, about 680, about 700, about 720, about 740, about 760, about 780, about 800, about 820, about 840, about 860, about 880, about 900, about 920, about 940, about 960, about 980, about 1000, about 1020, about 1040, about 1060, about 1080, about 1100, about 1120, about 1140, about 1160, about 1180, or about 1200 mM glutamine. In one embodiment, the pharmaconutrient composition or nutritional formulation comprises greater than 90, greater than 91, greater than 92, greater than 93, greater than 94, greater than 95, greater than 96, greater than 97, greater than 98, greater than 99, greater than 100, greater than 101, greater than 102, greater than 103, greater than 104, greater than 105, greater than 106, greater than 107, greater than 108, greater than 109, or greater than 110 mM glutamine.

Alternatively, the pharmaconutrient composition may comprise glutamine in an amount sufficient to

produce any one of the foregoing concentrations of glutamine, for example when the pharmaconutrient composition is used to supplement a nutritional formulation.

The pharmaconutrient composition or nutritional formulation may comprise about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, or about 100 nM vitamin D3 or calcitriol. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise greater than 40, greater than 41, greater than 42, greater than 43, greater than 44, greater than 45, greater than 46, greater than 47, greater than 48, greater than 49, greater than 50, greater than 51, greater than 52, greater than 53, greater than 54, greater than 55, greater than 56, greater than 57, greater than 58, greater than 59, or greater than 60 nM vitamin D3 or calcitriol. As used herein, 1 µg vitamin D3 is equivalent to 40 IU of vitamin D3. Alternatively, the pharmaconutrient composition may comprise vitamin D3 or calcitriol in an amount sufficient to produce any one of the foregoing concentrations of vitamin D3 or calcitriol, for example when the pharmaconutrient composition is used to supplement a nutritional formulation.

The pharmaconutrient composition or nutritional formulation may comprise about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, or about 20 mM of an n-3 fatty acid. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise greater than 1, greater than 2, greater than 3, greater than 4, greater than 5, greater than 6, greater than 7, greater than 8, greater than 9, greater than 10, greater than 11, greater than 12, greater than 13, greater than 14, greater than 15, greater than 16, greater than 17, greater than 18, greater than 19, or greater than 20 mM of an n-3 fatty acid. Alternatively, the pharmaconutrient composition may comprise an n-3 fatty acid in an amount sufficient to produce any one of the foregoing concentrations of an n-3 fatty acid, for example when the pharmaconutrient composition is used to supplement a nutritional formulation.

The pharmaconutrient composition or nutritional formulation may comprise about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33,

about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, or about 100 μ M curcumin. Alternatively, the pharmaconutrient composition or nutritional formulation may comprise greater than greater than 1, greater than 2, greater than 3, greater than 4, greater than 5, greater than 6, greater than 7, greater than 8, greater than 9, greater than 10, greater than 11, greater than 12, greater than 13, greater than 14, greater than 15, greater than 16, greater than 17, greater than 18, greater than 19, greater than 20, greater than 21, greater than 22, greater than 23, greater than 24, greater than 25, greater than 26, greater than 27, greater than 28, greater than 29, greater than 30, greater than 31, greater than 32, greater than 33, greater than 34, greater than 35, greater than 36, greater than 37, greater than 38, greater than 39, greater than 40, greater than 41, greater than 42, greater than 43, greater than 44, greater than 45, greater than 46, greater than 47, greater than 48, greater than 49, greater than 50, greater than 51, greater than 52, greater than 53, greater than 54, greater than 55, greater than 56, greater than 57, greater than 58, greater than 59, greater than 60, greater than 61, greater than 62, greater than 63, greater than 64, greater than 65, greater than 66, greater than 67, greater than 68, greater than 69, greater than 70, greater than 71, greater than 72, greater than 73, greater than 74, greater than 75, greater than 76, greater than 77, greater than 78, greater than 79, greater than 80, greater than 81, greater than 82, greater than 83, greater than 84, greater than 85, greater than 86, greater than 87, greater than 88, greater than 89, greater than 90, greater than 91, greater than 92, greater than 93, greater than 94, greater than 95, greater than 96, greater than 97, greater than 98, greater than 99, or greater than 100 μ M curcumin. Preferably, the pharmaconutrient composition or nutritional formulation comprises about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, or about 60 μ M curcumin. Alternatively, the pharmaconutrient composition may comprise curcumin in an amount sufficient to produce any one of the foregoing concentrations of curcumin, for example when the pharmaconutrient composition is used to supplement a nutritional formulation.

In one embodiment, the pharmaconutrient composition or nutritional formulation comprises about 1 mM to about 1200 mM glutamine, about 1 μ M to about 100 μ M curcumin, about 1 nM to about 100 nM vitamin D3, or about 0.1 mM to about 10 mM of an n-3 fatty acid. Alternatively, the pharmaconutrient composition comprises arginine, glutamine, curcumin, vitamin D3, or an n-3 fatty acid sufficient to produce about 1 mM to about 1200 mM glutamine, about 1 μ M to about 100 μ M curcumin, about 1 nM to about 100 nM vitamin D3, or about 0.1 mM to about 10 mM of an n-3 fatty

acid, for example when the pharmaconutrient composition is used to supplement a nutritional formulation.

In one embodiment, the pharmaconutrient composition or nutritional formulation comprises about 50 mM arginine, about 240 mM or about 250 mM glutamine, about 50 μ M or about 54 μ M curcumin, about 100 nM vitamin D3, or about 7 mM of an n-3 fatty acid. Alternatively, the pharmaconutrient composition comprises arginine, glutamine, curcumin, vitamin D3, or an n-3 fatty acid sufficient to produce about 50 mM arginine, about 240 mM or about 250 mM glutamine, about 50 μ M or about 54 μ M curcumin, about 100 nM vitamin D3, or about 7 mM of an n-3 fatty acid, for example when the pharmaconutrient composition is used to supplement a nutritional formulation.

In one embodiment, the pharmaconutrient composition or nutritional formulation comprises greater than 94 mM arginine, greater than 103 mM glutamine, greater than 52 nM vitamin D3, or greater than 8.6 mM of an n-3 fatty acid. Alternatively, the pharmaconutrient composition comprises arginine, glutamine, vitamin D3, or an n-3 fatty acid sufficient to produce greater than 94 mM arginine, greater than 103 mM glutamine, greater than 52 nM vitamin D3, or greater than 8.6 mM of an n-3 fatty acid, for example when the pharmaconutrient composition is used to supplement a nutritional formulation.

It is advantageous to formulate the pharmaconutrient composition or nutritional formulation in unit dosage for ease of administration and uniformity of dosage. Unit dosage also facilitates supplementation of a nutritional formula with the pharmaconutrient composition. Unit dosage as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated, each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect. Alternatively, the pharmaconutrient composition or nutritional formulation may be presented in multi-dosage form.

A unit dosage may be contained in a sachet, a sealed ampoule, or a sealed vial. Each may be stored in a dried condition requiring only the addition of the liquid, such as the nutritional formulation to be supplemented, immediately prior to use.

The pharmaconutrient composition or nutritional formulation may also be included in a container, pack, or dispenser together with instructions for administration.

In one embodiment the unit dosage comprises about 2.2 g arginine, about 9.1 g glutamine, and about 5 mg curcumin. When added to 250 mL of nutritional formula, these quantities provide about 50 mM arginine, about 250 mM glutamine, and about 54 μ M curcumin. In one embodiment, the unit dosage is a sachet.

As used herein, a “nutritional formulation” refers to a liquid diet that replaces food in a normal diet, and comprises for example protein or amino acids, fats, sugars, vitamins, and minerals. A nutritional formulation may be “enteral”, i.e. an “enteral nutritional formulation” delivered “enterally” through a tube into the stomach or small intestine, or may be “parenteral”, i.e. a “parenteral nutritional formulation” delivered “parenterally” intravenously bypassing the intestine.

In one embodiment, the nutritional formulation for treating intestinal inflammation in a subject comprises a pharmaconutrient composition as disclosed herein. In one embodiment, a nutritional formulation may be supplemented with a pharmaconutrient composition as disclosed herein. It follows that disclosed herein is a method for producing a nutritional formulation that treats intestinal inflammation in a subject, the method comprising supplementing a nutritional formulation with the pharmaconutrient composition of the disclosure. Alternatively, the method for producing a nutritional formulation that treats intestinal inflammation in a subject may comprise supplementing a nutritional formulation with at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid. Also disclosed is a nutritional formulation produced by this method.

As used herein, an “elemental nutritional formulation” is a nutritional formulation that lacks whole or partial proteins, and optionally may lack complex carbohydrates. In contrast, a “polymeric nutritional formulation” is a nutritional formulation that comprises whole or partial proteins, and optionally may comprise complex carbohydrates.

As used herein, the nutritional formulation may be provided exclusively to the subject with intestinal inflammation, without food of any other kind. This is referred to as “exclusive enteral nutrition (EEN)”; thus the nutritional formulation may be an EEN formulation.

As used herein, “protein” refers to proteins, polypeptides, peptides, and/ or amino acids. Therefore, when the concentration of a protein is referred to, the concentration includes proteins, polypeptides, peptides, and/ or amino acids.

In one embodiment, the nutritional formulation comprises about 30 g/L to about 90 g/L, about 40 g/L to about 80 g/L, about 50 g/L to about 70 g/L, or about 60 g/L protein. The nutritional formulation may comprise about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, or about 90 g/L protein. Alternatively, the nutritional formulation may comprise greater than 30, greater than 31, greater than 32, greater than 33, greater than 34, greater than 35, greater than 36, greater than 37, greater than 38, greater than 39, greater than 40, greater than 41, greater than 42, greater than 43, greater than 44, greater than 45, greater than 46, greater than 47, greater than 48, greater than 49, greater than 50, greater than 51, greater than 52, greater than 53, greater than 54, greater than 55, greater than 56, greater than 57, greater than 58, greater than 59, greater than 60, greater than 61, greater than 62, greater than 63, greater than 64, greater than 65, greater than 66, greater than 67, greater than 68, greater than 69, greater than 70, greater than 71, greater than 72, greater than 73, greater than 74, greater than 75, greater than 76, greater than 77, greater than 78, greater than 79, greater than 80, greater than 81, greater than 82, greater than 83,

greater than 84, greater than 85, greater than 86, greater than 87, greater than 88, greater than 89, or greater than 90 g/L protein.

Examples of commercially available polymeric nutritional formulations include OSMOLITE, IMPACT and IMPACT GLUTAMINE, details of which are provided below.

OSMOLITE

Facts

Nutrient density	(Cal/mL)	1.0
	(kJ/mL)	4.2
Protein	(% Cal)	15.94
Carbohydrate	(% Cal)	54.03
Fat	(% Cal)	30.03
kcal/N ratio		160:1
Non-protein kcal/N ratio		134:1
Kosher		Yes
Gluten-free		Yes
Lactose		0.01g/100mL
Renal Solute Load (mOsm/L)		342
Osmolality (mOsm/kg H ₂ O)		288
N-6:N-3 Ratio		4.1:1
RDI/AI volume		1550mL [#]

[#]Recommended Dietary Intake (RDI) and Adequate Intake (AI) volumes based on 'Nutrient Reference Values for Australia and New Zealand 2005' Male 31-50y, specified volume meets 100% RDI/AI for micronutrients excluding electrolytes with the exception of Folate (89% RDI), Magnesium (74% RDI).

Ingredients

Water, maltodextrin, sodium and calcium caseinates, high oleic sunflower oil, canola oil, MINERALS (potassium citrate, calcium phosphate tribasic, magnesium chloride, potassium chloride, sodium citrate, potassium phosphate dibasic, magnesium sulphate, ferrous sulphate, zinc sulphate, manganese sulphate, cupric sulphate, sodium molybdate, chromium chloride, sodium selenate, potassium iodide), soy protein isolate, MCT oil, soy lecithin, VITAMINS (choline chloride, ascorbic acid, dl-alpha tocopheryl acetate, niacinamide, calcium pantothenate, pyridoxine hydrochloride, thiamin hydrochloride, riboflavin, vitamin A palmitate, folic acid, biotin, phylloquinone, vitamin D3, cyanocobalamin), carrageenan. May contain sodium chloride.

Nutrient Information

Nutrient	Unit	Avg Qty Per 100 mL	Avg Qty Per 1000 mL
Energy	kJ	422	4223
	kcal	100	1004
Protein	g	4.00	40
Carbohydrate	g	13.56	136
Sugars	g	0.63	6.3
Fat, total	g	3.40	34
Saturated fat	g	0.84	8.4
Monounsaturated fat	g	1.93	19.3
Polyunsaturated fat	g	0.48	4.8
Dietary fibre, total	g	0	0
Water	g	84.9	849
VITAMINS			
Vitamin A	µg RE	108	1080
Vitamin D3	µg	0.73	7.3
Vitamin E	mg α-TE	2.1	21
Vitamin K1	µg	5.2	52
Vitamin C	mg	10	100
Folic acid	µg	23	230
Thiamin (Vitamin B1)	mg	0.16	1.6
Riboflavin (Vitamin B2)	mg	0.18	1.8
Vitamin B6	mg	0.22	2.2
Vitamin B12	µg	0.34	3.4
Niacin	mg NE	1.7	17
Choline	mg	56	560
Biotin	µg	4.6	46
Pantothenic acid	mg	0.78	7.8
MINERALS			
Sodium	mg	88	880
	mmol	3.83	38.3
Potassium	mg	148	1480
	mmol	3.79	37.9
Chloride	mg	136	1360
	mmol	3.84	38.4
Calcium	mg	68	680

Phosphorus	mg	68	680
Magnesium	mg	20	200
Iodine	µg	11	110
Manganese	mg	0.38	3.8
Copper	µg	170	1700
Zinc	mg	1.3	13
Iron	mg	1.4	14
Selenium	µg	6.0	60
Chromium	µg	6.5	65
Molybdenum	µg	12	120

IMPACT

Facts

5	Nutrient density	(kcal/mL) 1.0
		(kJ/mL) 4.2
	Protein	(% kcal) 22
	Carbohydrate	(% kcal) 53
	Fat	(% kcal) 25
10	Non-protein kcal/N ratio	71:1
	Osmolality (mOsm/kg H ₂ O)	375
	N-6:N-3 Ratio	1.4:1
	RDI volume	1550mL [#]
	Protein Source	sodium and calcium caseinates (milk), L-arginine
15	Water	85%
	EPA/DHA	1.7 g/L
	Supplemental L-Arginine	12.5 g/L
	Dietary Nucleotides	1.2 g/L
	[#] 100% RDI for 24 key micronutrients	

Ingredients

20	Water, Maltodextrin, Sodium Caseinate (from Milk), Palm Kernel Oil, L-Arginine, Calcium Caseinate, Refined Fish Oil (Anchovy, Sardine), and less than 1% of Citric Acid, High Linoleic Safflower Oil, Magnesium Chloride, Potassium Citrate, Calcium Phosphate, Sodium Citrate, Yeast Extract, Cellulose Gel, Hydroxylated Soy Lecithin, Potassium Chloride, Potassium Phosphate, High Oleic Sunflower Oil, Sodium Ascorbate, Choline Chloride, Carrageenan, Cellulose Gum, Alpha-	
25	Tocopheryl Acetate, Zinc Sulfate, Ferrous Sulfate, Niacinamide, Vitamin A Palmitate, Copper Gluconate, Calcium Pantothenate, Vitamin D3, Manganese Sulfate, Thiamine Hydrochloride, Beta Carotene, Pyridoxine Hydrochloride, Riboflavin, Folic Acid, Chromium Chloride, Sodium	

Molybdate, Biotin, Sodium Selenite, Potassium Iodide, Phytonadione, Vitamin B₁₂.

Nutrition Information		250 mL (1 Carton)	1000 mL
Calories	kcal	250	1000
Total Fat	g	7	28
Sodium	mg (mEq)	240 (10.4)	960 (41.7)
Potassium	mg (mEq)	400 (10.2)	1600 (41)
Total Carbohydrate	g	33	132
Protein*	g	14	56
Vitamin A**	IU	1675	6700
Vitamin C	mg	20	80
Calcium	mg	200	800
Iron	mg	3	12
Vitamin D	IU	133	532
Vitamin E	IU	15	60
Vitamin K	mcg	16.7	66.8
Thiamin	mg	0.5	2
Riboflavin	mg	0.43	1.7
Niacin	mg	5	20
Vitamin B ₆	mg	0.37	1.5
Folic Acid	mcg	100	400
Vitamin B ₁₂	mcg	2	8
Biotin	mcg	50	200
Pantothenic Acid	mg	1.7	6.8
Phosphorus	mg	200	800
Iodine	mcg	25	100
Magnesium	mg	67.5	270
Zinc	mg	3.75	15
Selenium	mcg	25	100
Copper	mg	0.42	1.7
Manganese	mg	0.5	2
Chromium	mcg	25	100
Molybdenum	mcg	50	200
Chloride	mg (mEq)	325 (9.2)	1300 (36.6)
Choline	mg	67.5	270
Water	mL	213	853

*Includes protein from caseinates and L-arginine

**Includes 50% vitamin A activity from beta carotene

mcg = µg

IMPACT GLUTAMINE

5	Nutrient density	(kcal/mL)	1.3
		(kJ/mL)	4.2
10	Protein	(% kcal)	24
	Carbohydrate	(% kcal)	46
	Fat	(% kcal)	30
	Non-protein kcal/N ratio		62:1
15	Osmolality (mOsm/kg H ₂ O)		630
	N-6:N-3 Ratio		1.4:1
	RDI volume		1000mL [#]
	Protein Source		wheat protein hydrolysate, free amino acids, sodium caseinate (milk)
	Water		81%
	EPA/DHA		2.7 g/L
	Supplemental L-Arginine		16.4 g/L

L-glutamine (inherent)	15 g/L
Dietary Nucleotides	1.5 g/L
Fiber Content (Source)	10 g/L (NUTRISOURCE FIBER, soy fiber)

* 100% RDI for 24 key micronutrients

5

Ingredients

Water, Maltodextrin, Wheat Protein Hydrolysate, contains less than 2% of Sodium Caseinate (from Milk), Palm Kernel Oil, Refined Fish Oil (Anchovy, Sardine), L-Arginine, Partially Hydrolyzed Guar Gum (soluble fibre), L-Lysine, Soy Fiber, Citric Acid, High Linoleic Safflower Oil, Potassium Citrate, Calcium Phosphate, Mono- and Diglycerides, Yeast Extract, Sodium Hexametaphosphate, Choline Bitartrate, Cellulose Gel and Gum, L-Leucine, L-Threonine, High Oleic Sunflower Oil, Sodium Ascorbate, Potassium Chloride, Magnesium Oxide, L-Histidine, L-Tryptophan, Salt, Carrageenan, L-Methionine, Alpha-Tocopheryl Acetate, L-Valine, L-Carnitine, Taurine, Zinc Sulfate, Ferrous Sulfate, Vitamin A Palmitate, Niacinamide, Copper Gluconate, Calcium Pantothenate, Vitamin D3, Manganese Sulfate, Thiamine Hydrochloride, Pyridoxine Hydrochloride, Riboflavin, Beta Carotene, Folic Acid, Biotin, Chromium Chloride, Potassium Iodide, Sodium Molybdate, Phytonadione, Sodium Selenite, Vitamin B₁₂.

Nutrition Information		250 mL (1 Carton)	1000 mL
Calories	kcal	325	1300
Total Fat	g	10.8	43.2
Sodium	mg (mEq)	300 (13)	1200 (52)
Potassium	mg (mEq)	450 (11.5)	1800 (46.1)
Total Carbohydrate	g	37	148
Dietary Fiber	g	2.5	10
Protein*	g	19.5	78
Vitamin A**	IU	2200	8800
Vitamin C	mg	65	260
Calcium	mg	300	1200
Iron	mg	4.5	18
Vitamin D	IU	200	800
Vitamin E	IU	19.5	78
Vitamin K	mcg	20	80
Thiamin	mg	0.4	1.6
Riboflavin	mg	0.43	1.7
Niacin	mg	5.0	20
Vitamin B ₆	mg	0.5	2.0
Folic Acid	mcg	100	400
Vitamin B ₁₂	mcg	1.5	6.0
Biotin	mcg	75	300
Pantothenic Acid	mg	2.5	10
Phosphorus	mg	300	1200
Iodine	mcg	37.5	150
Magnesium	mg	100	400
Zinc	mg	5	20
Selenium	mcg	17.5	70
Copper	mg	0.5	2.0
Manganese	mg	0.5	2.0
Chromium	mcg	30	120
Molybdenum	mcg	18.8	75.2
Chloride	mg (mEq)	215 (6)	860 (24)
Choline	mg	125	500
L-Carnitine	mg	35	140
Taurine	mg	35	140
Water	mL	202	808

*Includes amino acids as a source of protein

**Includes 10% vitamin A activity from beta carotene

mcg = µg

Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include: buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; hydrophilic polymers such as polyvinylpyrrolidone; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counter ions such as sodium; and/or non-ionic surfactants such as TWEEN, PLURONICS or PEG.

Excipients may be, for example: inert diluents, such as calcium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, such as corn starch or alginic acid; binding agents, such as starch or acacia; and lubricating agents, such as magnesium stearate, stearic acid or talc.

Disclosed herein is use of at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid in the manufacture of a pharmaconutrient composition or a nutritional formulation for treating intestinal inflammation in a subject.

Also disclosed is a method of treating intestinal inflammation in a subject, the method comprising administering to the subject at least two pharmaconutrients having similar mechanisms of pharmacological activity.

Further disclosed is a method of treating intestinal inflammation in a subject, the method comprising administering to the subject a pharmaconutrient composition or a nutritional formulation comprising at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.

Also disclosed is a pharmaconutrient composition or a nutritional formulation comprising at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid for use in a method of treating intestinal inflammation in a subject.

In one embodiment of the use and/or method disclosed above, the pharmaconutrient composition is the pharmaconutrient composition of the present disclosure or the nutritional formulation is the nutritional formulation of the present disclosure.

As used herein, "treating" or "treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the aim is to prevent, ameliorate, reduce or slow down (lessen) the occurrence of a condition, disease, disorder, or phenotype, including an abnormality or symptom, in particular intestinal inflammation.

"Preventing", "prevention", "preventative" or "prophylactic" refers to keeping from occurring, or to hinder, defend from, or protect from the occurrence of a condition, disease, disorder, or phenotype, including an abnormality or symptom, in particular intestinal inflammation. A subject in need of prevention may be prone to develop the condition. Successful prevention can be demonstrated in a study involving multiple subjects in which a group that receives a therapeutic agent has either fewer incidences or delayed incidences of the condition, disease, disorder, or phenotype compared to a similar control group that receives only placebo.

The term “ameliorate” or “amelioration” refers to a decrease, reduction or elimination of a condition, disease, disorder, or phenotype, including an abnormality or symptom, in particular intestinal inflammation. A subject in need of treatment may already have the condition, or may be prone to have the condition or may be in whom the condition is to be prevented.

It flows that as used herein, “intestinal inflammation” may be due to an inflammatory bowel disease (IBD), an intestinal cancer, necrotising enterocolitis, cystic fibrosis, or surgery, or may be in a subject requiring intensive care. The intestinal inflammation may be due to dysregulation of NF- κ B signalling. The IBD may be Crohn’s disease (CD), ulcerative colitis (UC), or inflammatory bowel disease unclassified (IBDU). Furthermore, treating intestinal inflammation extends to any of the symptoms, complications, or co-indications of intestinal inflammation as described herein.

“Intestinal inflammation” describes a cascade of biochemical events that generally results in the stimulation and release of inflammatory mediators. These mediators then act on cells of the vasculature, immune and mucosal systems generally resulting in increased blood flow, increased heat, increased flow of fluid, protein and cells from the circulation to the tissue, increased pain and tissue necrosis. This response can occur in any tissue of the intestine. Inflammation is generally associated with activation of the NF- κ B pathway. Proxy markers of NF- κ B activation, including IL-8, are accepted as measures of the inflammatory response.

In one embodiment, “treating” may comprise inducing remission or maintaining remission of the intestinal inflammation. In another embodiment, “treating” comprises reducing a 12 month relapse rate compared with subjects treated similarly but in the absence of the pharmaconutrient composition or the nutritional formulation

For inducing remission of intestinal inflammation, the subject may be treated for about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12 weeks, or greater than 12 weeks with the pharmaconutrient composition or the nutritional formulation. Alternatively, the subject may be treated for up to 1, up to 2, up to 3, up to 4, up to 5, up to 6, up to 7, up to 8, up to 9, up to 10, up to 11, or up to 12 weeks with the pharmaconutrient composition or the nutritional formulation.

In one embodiment, “treating” intestinal inflammation results in about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% reduction in intestinal inflammation in a subject compared with a subject not treated according to the present disclosure. Reduction in intestinal inflammation may be quantified by measuring any number of:

serum based markers including C reactive protein, Erythrocyte Sedimentation rate, serum albumin, serum platelet count and serum cytokines;

faecal markers including faecal calprotectin, faecal S100A12 and faecal cytokines; and

disease activity scores including Physicians Global Assessment (PGA), Crohn's Disease Activity Index (CDAI) and Paediatric Crohn's Disease Activity Index (PCDAI).

The person skilled in the art will understand the methods available quantification of intestinal inflammation such as the examples of the preceding paragraph. Such methods may include, for example, IL-8, which may be measured by ELISA, for example.

In one embodiment, treating comprises inducing remission and administering the pharmaconutrient composition or the nutritional formulation for about 8 weeks or up to about 12 weeks.

The "subject" to be treated may be a mammal. The mammal may be a human, or may be a domestic, zoo, or companion animal. While it is particularly contemplated that the subject to be treated according to the invention is human, the invention is also applicable to veterinary treatment, including treatment of companion animals such as dogs and cats, and domestic animals such as horses, cattle and sheep, or zoo animals such as felids, canids, bovids, and ungulates.

The subject may be a paediatric subject or an adult subject. The paediatric subject may be from about 5 to about 17, about 6 to about 16, about 7 to about 15, about 8 to about 14, about 9 to about 13, or about 10 to about 12 years. Alternatively, the paediatric subject may be about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, or about 18 years.

Also disclosed is a kit comprising the pharmaconutrient composition or the nutritional formulation of the present disclosure. The kit may be used according to the method of treating intestinal inflammation in a subject disclosed above.

The pharmaconutrient composition may comprise a "carrier" that refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaconutrients are administered. Such carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

The pharmaconutrient composition may be in the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, granulates, gels including hydrogels, pastes, ointments, creams, delivery devices, sustained-release formulations, suppositories, injectables, implants, sprays, drops, aerosols and the like.

Administration of a pharmaconutrient composition may be by any suitable means that results in an amount of pharmaconutrient that is effective for the treatment or prevention of intestinal inflammation.

The pharmaconutrient composition may be provided in a dosage form that is suitable for

oral, enteral or parenteral administration.

The pharmaconutrient composition can be formulated as neutral or salt forms.

Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

Pharmaceutically acceptable salts include sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, camphorsulfonate, pamoate, phenyl acetate, trifluoroacetate, acrylate, chloro benzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, o-acetoxybenzoate, naphthalene-2-benzoate, isobutyrate, phenylbutyrate, .alpha.-hydroxybutyrate, butyne-1,4-dicarboxylate, hexyne-1,4-dicarboxylate, caprate, caprylate, cinnamate, glycollate, heptanoate, hippurate, malate, hydroxymaleate, malonate, mandelate, mesylate, nicotinate, phthalate, teraphthalate, propiolate, propionate, phenylpropionate, sebacate, suberate, p-bromobenzenesulfonate, chlorobenzenesulfonate, ethylsulfonate, 2-hydroxyethylsulfonate, methylsulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, naphthalene-1,5-sulfonate, xylenesulfonate, and tartarate salts.

The term "pharmaceutically acceptable salt" also refers to a salt of a pharmaconutrient having an acidic functional group, such as a carboxylic acid functional group, and a base. Suitable bases include, but are not limited to, hydroxides of alkali metals such as sodium, potassium, and lithium; hydroxides of alkaline earth metal such as calcium and magnesium; hydroxides of other metals, such as aluminum and zinc; ammonia, and organic amines, such as unsubstituted or hydroxy-substituted mono-, di-, or tri-alkylamines, dicyclohexylamine; tributyl amine; pyridine; N-methyl, N-ethylamine; diethylamine; triethylamine; mono-, bis-, or tris-(2-OH-lower alkylamines), such as mono-, bis-, or tris-(2-hydroxyethyl)amine, 2-hydroxy-tert-butylamine, or tris(hydroxymethyl)methylamine, N,N-di-lower alkyl-N(hydroxyl-lower alkyl)-amines, such as N,N-dimethyl-N-(2-hydroxyethyl)amine or tri-(2-hydroxyethyl)amine; N-methyl-D-glucamine; and amino acids such as arginine, lysine, and the like.

The term "pharmaceutically acceptable salt" also includes a hydrate of a compound of the invention.

The pharmaconutrient compositions may be administered orally, rectally, enterally, or parenterally. Formulations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. A variety of aqueous carriers can be used, e.g., water, buffered water, saline, and the like. Examples of other suitable vehicles include polypropylene glycol, polyethylene glycol, vegetable oils, gelatin, hydrogels, hydrogenated naphthalenes, and injectable organic esters, such as ethyl oleate. Such formulations may also contain auxiliary substances, such as

preserving, wetting, buffering, emulsifying, and/or dispersing agents. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the pharmaconutrients.

Alternatively, the compositions can be administered orally or rectally. Compositions intended for oral or rectal use can be prepared in solid or liquid forms, according to any method known to the art for the manufacture of compositions for oral or rectal administration. For oral administration, the pharmaconutrients or pharmaconutrient composition may be administered as a food.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. Generally, these contain pharmaconutrients admixed with non-toxic pharmaceutically acceptable excipients. These include, for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, sucrose, glucose, mannitol, cellulose, starch, calcium phosphate, sodium phosphate, kaolin and the like. Binding agents, buffering agents, and/or lubricating agents (e.g., magnesium stearate) may also be used. Tablets and pills can additionally be prepared with enteric coatings. The pharmaconutrient composition may optionally contain sweetening, flavouring, colouring, perfuming, and preserving agents in order to provide a more palatable preparation.

Liquid dosage forms for oral administration can include emulsions, solutions, suspensions, syrups, and soft gelatin capsules. These forms can contain inert diluents commonly used in the art, such as water or an oil medium, and can also include adjuvants, such as wetting agents, emulsifying agents, and suspending agents.

The pharmaconutrients may be admixed in a tablet or other vehicle, or may be partitioned. In one example, the pharmaconutrient is contained on the inside of the tablet, and an additional active agent is on the outside, such that a substantial portion of the additional active agent is released prior to the release of the pharmaconutrient.

In one embodiment, pharmaconutrient compositions may comprise one or more pharmaceutically acceptable excipients. In one embodiment, such excipients include, but are not limited to, buffering agents, non-ionic surfactants, preservatives, tonicity agents, amino acids, sugars and pH-adjusting agents. Suitable buffering agents include, but are not limited to, monobasic sodium phosphate, dibasic sodium phosphate, and sodium acetate. Suitable non-ionic surfactants include, but are not limited to, polyoxyethylene sorbitan fatty acid esters such as polysorbate 20 and polysorbate 80. Suitable preservatives include, but are not limited to, benzyl alcohol. Suitable tonicity agents include, but are not limited to sodium chloride, mannitol, and sorbitol. Suitable sugars include, but are not limited to, α,α -trehalose dehydrate. Suitable amino acids include, but are not limited to glycine and histidine. Suitable pH-adjusting agents include, but are not limited to, hydrochloric acid, acetic acid, and sodium hydroxide. In one embodiment, the pH-adjusting agent or agents are present in an amount effective to provide a pH of about 3 to about 8, about 4 to about 7, about 5 to about 6, about 6 to about 7, or about 7 to about 7.5.

Some natural products, such as veegum, alginates, xanthan gum, gelatin, acacia and tragacanth, may also be used to increase the viscosity of a solution.

Stabilizers may also be used such as, for example, chelating agents, e.g., EDTA. Antioxidants may also be used, e.g., sodium bisulfite, sodium thiosulfite, 8-hydroxy quinoline or ascorbic acid.

The amount of the pharmaconutrient composition that will be effective for its intended therapeutic use can be determined by standard clinical techniques based on the present description. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The amount of pharmaconutrient that is admixed with the carrier materials to produce a single dosage can vary depending upon the mammal being treated and the particular mode of administration.

The dosage of the pharmaconutrient composition can depend on several factors including the severity of the condition, whether the condition is to be treated or prevented, and the age, weight, and health of the subject to be treated. Additionally, pharmacogenomic (the effect of genotype on the pharmacokinetic, pharmacodynamic or efficacy profile of a therapeutic) information about a particular subject may affect dosage used. Furthermore, the exact individual dosages can be adjusted somewhat depending on a variety of factors, including the specific combination therapies being administered, the time of administration, the route of administration, the nature of the formulation, the rate of excretion, the particular disease being treated, the severity of the disorder, and the anatomical location of the disorder. Some variations in the dosage can be expected.

For systemic administration, a therapeutically effective dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC₅₀ as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Initial dosages can also be estimated from *in vivo* data, e.g., animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually to provide plasma levels of the pharmaconutrients that are sufficient to maintain therapeutic effect. In cases of local administration or selective uptake, the effective local concentration of the compounds may not be related to plasma concentration. One having skill in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

The amount of pharmaconutrient composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration, and the judgment of the prescribing physician. The therapy may be repeated intermittently while symptoms are detectable or even when they are not detectable. The therapy may be provided alone or in combination with other drugs.

Administration of the pharmaconutrient composition, may independently, be one to four times daily, one to four times per week, one to four times per month, one to six times per year or once

every two, three, four or five years. Administration can be for the duration of one day, one week, or one month, two months, three months, six months, one year, two years, three years, and may even be for the life of the subject. Chronic, long term administration may be indicated. The dosage may be administered as a single dose or divided into multiple doses.

5 In addition to treating pre-existing intestinal inflammation, the pharmaconutrient composition can be administered prophylactically in order to prevent or slow the onset of intestinal inflammation. In prophylactic applications, the pharmaconutrient composition can be administered to a subject susceptible to or otherwise at risk of intestinal inflammation.

10 Pharmaconutrient compositions according to the invention may be formulated to release the pharmaconutrients substantially immediately upon administration or at any predetermined time period after administration, using controlled release formulations. For example, a pharmaconutrient composition can be provided in sustained-release form. The use of immediate or sustained release compositions depends on the nature of the intestinal inflammation being treated. If the intestinal inflammation is acute, treatment with an immediate release form can be utilized over a prolonged
15 release composition. For certain preventative or long-term treatments, a sustained released composition can also be appropriate.

Administration of the pharmaconutrient composition in controlled release formulations can be useful where the pharmaconutrient composition has (i) a narrow therapeutic index (e.g., the difference between the plasma concentration leading to harmful side effects or toxic reactions and the
20 plasma concentration leading to a therapeutic effect is small; generally, the therapeutic index, TI, is defined as the ratio of median lethal dose (LD₅₀) to median effective dose (ED₅₀)); (ii) a narrow absorption window in the gastro-intestinal tract; or (iii) a short biological half-life, so that frequent dosing during a day is required in order to sustain the plasma level at a therapeutic level.

Many strategies can be pursued to obtain controlled release in which the rate of release
25 outweighs the rate of degradation or metabolism of the active components. For example, controlled release can be obtained by the appropriate selection of formulation parameters and ingredients, including, e.g., appropriate controlled release compositions and coatings. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions, emulsions, microcapsules, microspheres, nanoparticles, patches, and liposomes. Methods for preparing such sustained or
30 controlled release formulations are well known in the art.

The pharmaconutrient composition can also be delivered using a drug-delivery device such as an implant. As used herein, the term "implant" refers to any material that does not significantly migrate from the insertion site following implantation. An implant can be biodegradable, non-biodegradable, or composed of both biodegradable and non-biodegradable materials. A non-
35 biodegradable implant can include, if desired, a refillable reservoir. Implants useful in the methods of the invention include, for example, patches, particles, sheets, plaques, microcapsules and the like, and can be of any shape and size compatible with the selected site of insertion. It is understood that an

implant useful in the invention generally releases the implanted pharmaconutrient composition at an effective dosage to the intestines of the subject over an extended period of time.

The implant comprising the pharmaconutrient composition may be dispersed in a biodegradable polymer matrix. The matrix can comprise PLGA (polylactic acid-polyglycolic acid copolymer), an ester-end capped polymer, an acid end-capped polymer, or a mixture thereof. In another embodiment, the implant may comprise a surfactant and a lipophilic compound. The lipophilic compound can be present in an amount of about 80-99% by weight of the implant. Suitable lipophilic compounds include, but are not limited to, glyceryl palmitostearate, diethylene glycol monostearate, propylene glycol monostearate, glyceryl monostearate, glyceryl monolinoleate, glyceryl monooleate, glyceryl monopalmitate, glyceryl monolaurate, glyceryl dilaurate, glyceryl monomyristate, glyceryl dimyristate, glyceryl monopalmitate, glyceryl dipalmitate, glyceryl monostearate, glyceryl distearate, glyceryl monooleate, glyceryl dioleate, glyceryl monolinoleate, glyceryl dilinoleate, glyceryl monoarachidate, glyceryl diarachidate, glyceryl monobehenate, glyceryl dibehenate, and mixtures thereof. In another embodiment, the implant may comprise the pharmaconutrient composition housed within a hollow sleeve. The pharmaconutrient composition may be delivered to the intestine by inserting the sleeve into the intestine, releasing the implant from the sleeve into the intestine, and then removing the sleeve from the intestine.

It must also be noted that, as used in the subject specification, the singular forms “a”, “an” and “the” include plural aspects unless the context clearly dictates otherwise.

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word “comprise” or variations such as “comprises” or “comprising” is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

EXAMPLES

Cell culture experiments

Background

The luminal surface of the human gut is lined with a highly polarized and self-renewal epithelium. The primary function of these cells is absorbing nutrients. However, because they are in direct contact with gut lumen through its apical surface, intestinal epithelial cells form a protective barrier against different invasive infectious agents, toxins and antigenic factors. The epithelial cells are also in contact with cytokines secreted by other cells in the intestinal mucosa at the basolateral surface, as such epithelial cells are implicated in homeostasis of the complex mucosal immune

response. The mucosal immune responses play an imperative role in epithelial defensive mechanism against invasive pathogens. However, in intestinal inflammation, the mucosal immune response is altered and intestinal epithelial cells start secreting excessively number of cytokines. It has been reported that the pro-inflammatory cytokines TNF- α , IL-1 α , IL-1 β and IL-8 are produced largely in the inflamed intestinal mucosa of IBD subjects. The released cytokines are responsible for extensive histological damage in the intestinal epithelium of IBD subjects. This suggests that the intestinal epithelial cells are involved in and play an important role in the control of tissue injury in IBD. Thus, intestinal epithelial cells have been used as an *in vitro* model of intestinal inflammation.

Three cell lines were used in this work: HT29; Caco2; and INT407 cells. HT29 and Caco-2 cell lines have characteristics of normal intestinal epithelium including epithelial polarity, presence of the actin binding protein villin and the occurrence of enterocytes differentiation. HT29 cells undergo and exhibit enterocyte differentiation when replicated *in vitro*. Caco-2 cells also display the morphologic and functional properties of normal intestinal enterocytes after 2 weeks of seeding onto culture plate. Therefore, experiments involving Caco-2 were conducted 14 days after seeding. HT29 cells reach confluence within 5 days; therefore experiments involving HT29 were carried out after 5 days of cell culture. HT29 and Caco2 cells were cultured as an *in vitro* model of intestinal inflammation in various studies: for investigating pathogenesis of IBD, for better understanding the intestinal epithelial barrier function and its disruption in IBD and for assessing efficacy of medications used for IBD.

Materials

HT29 (ATCC HTB-38), Caco2 (ATCC HTB-37) and INT407 (ATCC CCL6) cell lines were obtained from American Tissue Culture Type and stored at -180 °C. In all experimentations the cells were used between passages 40 to 50.

Osmolite is a PF from Abbott Nutrition. n-3 fatty acids were supplied as canola oil, which is rich in α -linolenic acid. L-Glutamine, L-Arginine, Vitamin D3, curcumin were from Sigma-Aldrich.

Cell culture

Cells were seeded in 24-well plates at concentration of 5×10^5 cells per well and maintained in culture media. Cells were incubated at 37°C with 5% CO₂ with media changed every alternative day. HT29 cells were cultured in McCoy's 5A medium containing 10% FBS and 100U/mL penicillin/streptomycin. INT407 cells were maintained in BME containing 10% FBS and 100U/ml penicillin/streptomycin whilst Caco2 cells were maintained in MEM supplemented with 20% FBS, 1% Penicillin/Streptomycin, 1% Na pyruvate, 1% Na bicarbonate and 1% MEM EAA. Experiments were conducted following 5 days incubation or if 90% confluence was reached for HT29 and INT407 cell lines or conducted after 14 days for Caco2 cells.

Induction of inflammation

Inflammation was induced by exposing cells to 50ng/ml TNF- α and incubating HT29 cells for 6 hours or INT407 cells for 24 hours. Inflammation was induced in Caco2 cells using a mixture of

50 ng/ml of TNF- α , 50 ng/ml of INF- γ , 25ng/ml IL-1 β and 1ug/ml LPS for 24 hours.

Treatment protocol

Glutamine, arginine, alpha-linolenic acid (ALA) and PF were dissolved directly in the media. Vitamin D3 was diluted in 100% ethanol first and then added to the cells to give ethanol final concentration of 0.1% v/v to the media whereas curcumin was first dissolved in DMSO and later mixed with the culture medium with a final DMSO concentration of 0.1% v/v in culture media. Because PF was given at a concentration of 1:5 to the media, concentrations of glutamine, arginine, vitamin D3 and ALA that were used in some experiments as PF candidates also were given 1/5 of their respective concentration in PF. This calculation was based on the fact that 2 litres of PF given a day to subjects with IBD will be diluted 5 times with the 8 litres of daily intestinal fluid turnover of GIT secretions before it reaches the intestinal epithelium and exerts its effect.

Cell viability

Following experimentation, cell viability was determined by Trypan Blue exclusion and/or by MTT assay.

Enzyme-linked immunosorbent assay

Background

In the present work IL-8 was measured as an inflammatory marker of the *in vitro* model of intestinal inflammation to assess the response to the given supplements (treatment). IL-8 is a member of chemokine family which is a group of proteins produced during the inflammation by many different cell types including epithelial cells. Resting cells usually secrete very low levels of chemokines, however, upon inflammation the secretion of IL-8 is up-regulated. The up-regulation is mediated through activation of NF- κ B pathway of infiltrating immunocytes and tissue cells in response to pro-inflammatory cytokines TNF- α , INF- γ and IL-1 β . Chemokines are responsible for recruitment and activation of immunocytes, a character of chronic inflammation as in IBD.

Specifically, IL-8 plays role as a powerful neutrophil chemoattractant and activator that is accounting for perpetuation of inflammation in CD. In a study involving intestinal mucosal samples collected from normal subjects and from subjects with CD, IL-8 was among of the pro-inflammatory cytokines that were significantly high in CD subjects compared to controls. Additionally, in the laboratories, researchers were able to enhance IL-8 production from colonic epithelial cells replicated in culture media and treated with recombinant human cytokines. Therefore, IL-8 is often used as inflammatory marker for the *in vitro* models of intestinal inflammation involving cultured epithelial monolayer. In parallel with these studies, in this work intestinal epithelial cells (HT29, Caco2 and INT407) also exhibited a strong inflammatory response to recombinant cytokines when cultured and treated with TNF- α (HT29 and INT407) cells. We have therefore established IL-8 level as an approximate inflammatory marker for the used *in vitro* model.

Materials

IL-8 was assayed using IL-8 Human Antibody Pair, Novex® IL-8 ELISA Kits from

Invitrogen. ELISA was conducted in Nunc-Immuno™ Micro Well™ 96 well solid plates from Maxisorp Nunc. Plates were coated with monoclonal IL-8 antibody. Coating buffer A comprised 8g NaCl, 1.13g Na₂HPO₄, 0.2g KCl to 1 litre distilled water, and was adjusted to pH 7.4. Assay buffer comprised 8g NaCl, 1.13g Na₂HPO₄, 0.2g KCl, 5g bovine serum albumin, 1ml Tween 20 to 1 litre distilled water, and was adjusted to pH 7.4. Washing buffer comprised 250µl Tween 20 in 500mL PBS. Following incubation and washing, signals were detected with biotinylated secondary antibody and HRP conjugate. Soluble 3,3',5,5'-tetramethylbenzidine (TMB) substrate was from Thermo-Fischer Scientific. Stop solution comprised 1.8N H₂SO₄. Absorbance was read at 450nm and then converted to picograms per millilitre based on the standard curve obtained with the recombinant cytokine.

Method

The concentration of IL-8 in culture supernatant was measured using ELISA kit according to manufacturer's protocol. The lower limit of detection was between 15.6pg/ml and 31.5 pg/ml.

Western Blot

Materials

Primary Antibodies were rabbit anti-human polyclonal anti-Iκκ, anti-phosphorylated Iκκ, anti-IκB and anti-phosphorylated IκB (Abcam, Cambridge, UK). Secondary antibodies were goat anti-rabbit (BIO-RAD Co). Detection was by Immune-Star HRP chemiluminescent kit, visualized by GelDoc (Bio-Rad).

Methods

For Western blot experiments, cells were seeded in a 6-well plate at a concentration 10⁶ cells/well and grown until confluence then used for experimentation. Following experimentation, cells were lysed on ice. Protein was assayed using bicinchoninic acid (BCA).

Equal volumes of cell lysate and 2X Laemmli loading buffer were mixed to give a 40µg concentration of protein. The mixture of lysate and the loading buffer was heated at 100°C for 5 minutes for denaturing the protein. Samples and 5µl of Precision Plus Protein™ WesternC™ were then loaded into wells of the SDS-PAGE gel. The gel was run at 100 V for 15 minutes at then increased to 200V for 45 minutes. Subsequently, protein was transferred from the gel to a PDVF membrane.

The membrane was blocked for 1 hour at room temperature. Membranes were probed with primary antibodies diluted in TBST buffer according to the manufacturer's protocol overnight at 4°C. After washing, membranes were incubated with secondary antibodies conjugated with HRP for 1 hour at room temperature. Membranes were then incubated in a mixture of luminol and peroxide buffer in a 1:1 ratio for 3 to 5 minutes and visualized.

Kinase assay

The kinase assay was conducted as per the manufacturer's guidelines (CycLex Iκκ α and β Assay/Inhibitor Screening Kit; CycLex Co., Ltd, Japan). In brief, glutamine and arginine were

dissolved directly in the kinase buffer. Wells with no enzyme (negative control) and K252a (Sigma-Aldrich), the synthetic inhibitor of I κ B, were also included. Curcumin and K252a were dissolved first in DMSO then added to the kinase buffer to give 0.5% v/v concentration of DMSO in the reaction buffer. Additional solvent control (0.5% DMSO) was also included. The reaction was started by pipetting the β -subunit of I κ B to all I κ B α pre-coated wells, except for the no enzyme control wells. This was followed by addition of kinase reaction buffer (kinase buffer and 20x ATP at ratio of 1:20). Anti-Phospho-I κ B α S32 antibody was then pipetted into each well. The plate was washed and HRP-conjugated anti-mouse IgG added. The plate was washed again and substrate was loaded into all wells. Colour reaction was stopped by adding stop solution and the absorbance, reflecting the amount of generated phosphorylated I κ B α , read at 450nm.

Real-time polymerase chain reaction (RT-PCR)

For measuring mRNA expression of IL-8, RNA was extracted from HT29 cells using TRIzol reagent (Invitrogen). Extracted RNA was reverse-transcribed to complementary DNA (cDNA) using SuperScript[®] VILO[™] cDNA Synthesis Kit (Life Technologies, USA). cDNA was amplified using Realplex Mastercycler (Eppendorf, Barkhausenweg, Hamburg, Germany) and SYBR-Green fluorescence detection system: (iQ[™] SYBR[®] Green Supermix; Bio-Rad). Average threshold cycle (C_T) was measured and gene expression quantified relative to housekeeping gene β 2M.

Example 1 - Investigating the active anti-inflammatory ingredients of PF

Introduction

Nutritional problems are often associated with IBD, most notably in the paediatric population with underweight and stunting common features at presentation. Importantly, nutritional therapy is becoming an increasingly viable therapeutic option to treat IBD, especially CD, where this therapy is employed to, in part, address the nutritional complications of the disease. Enteral nutrition utilizing PF or EF given as the sole nutritional source (EEN) is now the preferred option for induction and maintenance of young subjects with CD.

While not wishing to be bound by theory, it is thought that direct anti-inflammatory effects, enhancing mucosal healing, resting inflamed bowel and/or modifications of intestinal micro-flora play roles in induction of remission consequent to PF treatment resulting in a fall of pro-inflammatory cytokines and correction of inflammatory indices. Further, the anti-inflammatory effect of PF occurs long before any detectable improvement in the nutritional parameters of participants.

Standard PF comprises a mixture of protein, carbohydrate, fat and water as well as vitamins and trace minerals. Despite proven efficacy in clinical practice, it has not been yet investigated which component of PF can fully or partially explain its role in inducing remission in subjects with CD. Nevertheless, in various studies glutamine, arginine, vitamin D and n-3 fatty acids supplements, which are present in PF, are independently shown to possess immuno-modulating effects and are capable of modulating the inflammatory response. Therefore, the inventors sought to further investigate the anti-inflammatory properties of these nutrients on cultured intestinal epithelium as an

in vitro model of intestinal inflammation.

Results

Glutamine, arginine and vitamin D3, but not ALA at their concentrations of PF significantly reduced IL-8 production from HT29 cell line in response to TNF- α and that effect was magnified with their combination.

HT29 cells were grown to confluence then exposed to 50 ng/mL TNF- α . Cells were exposed to 12.7 mM glutamine, 1.8 mM arginine, 3.8 nM vitamin D3 and 0.72 mM ALA individually or in combination. After 6 hours incubation, supernatants were collected for measuring IL-8 levels. In the positive control, IL-8 was increased to around 160 times its level in un-stimulated cells ($P < 0.0001$) (Figure 2). In the presence of 12.7 mM glutamine, IL-8 production was significantly decreased from 16555 to 11688 pg/ml ($P = 0.0163$) (Figure 2). Arginine (at 1.8 mM) also suppressed IL-8 production in TNF- α stimulated HT29 cells. IL-8 level was substantially reduced by arginine treatment from 16555 pg/ml to 9471 pg/ml ($P = 0.0010$) (Figure 2). Further, 3.8 nM vitamin D3 attenuated IL-8 secretion from TNF- α exposed cells with reduction from 16555 pg/ml to 9447 pg/ml ($P = 0.0010$) (Figure 2). ALA 0.72 mM had no effect on IL-8 production from TNF- α stimulated cell ($P = 0.1264$) (Figure 2). The combination of glutamine 12.7 mM, arginine 1.8 mM, vitamin D3 3.8 nM and ALA 0.72 mM further reduced IL-8 production from HT29 cells in response to TNF- α stimulation (16555 vs 6605 pg/ml, $P < 0.0001$) (Figure 2).

Standard PF was superior to combination of glutamine, arginine, vitamin D3, and ALA at their concentrations of PF in attenuating IL-8 level.

HT29 cells were treated with either 12.7 mM glutamine, 1.8 mM arginine, 3.8 nM vitamin D3 and 0.72 mM ALA (combination), or with standard PF at concentration of 1:5 to media. Cells were then incubated with 50 ng/ml TNF- α for 6 hours.

PF treatment prompted a substantial reduction in IL-8 response to TNF- α exposure. Expression of IL-8 was reduced from 20237 pg/ml in TNF- α stimulated cells to 4283 pg/ml after PF treatment ($P < 0.0001$) (Figure 3). In the combination treated group, IL-8 was attenuated from 20237 pg/ml in TNF- α stimulated cells to 8888 pg/ml in the treated cells ($P < 0.0001$) (Figure 3). Cell viability was maintained above 95% in all 7 experimental groups (Figure 4).

Glutamine, arginine and vitamin D3, but not ALA attenuated IL-8 secretion from HT29 in response to TNF- α in a dose dependent manner without any detrimental effect on the cell viability.

HT29 cells were exposed to 50 ng/ml TNF- α for 5 days. Thereafter, cells were treated with a range of concentrations of glutamine (0.5, 1, 2.5, 5, 7.5, 10, 15, 50, 120 and 240 mM) or arginine (0.5, 2, 5, 10 and 50 mM). Alternatively, cells were pre-incubated with vitamin D3 at 1, 10, 30 and 100 nM for 1 hour before being exposed to TNF- α , or cells were pre-incubated with ALA at 0.3, 0.72, 1.44, 3.6, 7.2 mM for 2 days before TNF- α was added on day five.

Glutamine inhibited IL-8 secretion from TNF- α stimulated HT29 cells in a dose dependent manner (Figure 5). The inhibitory effect started at a concentration of 10 mM when IL-8 levels were

reduced from 22344 pg/ml to 17616 pg/ml, however, this decrease is not statistically significant compared to positive control group ($P < 0.09$). Statistical significance was achieved at a concentration of 15 mM ($P < 0.0023$) (Figure 5). Further, greater inhibition of IL-8 production was evident with increasing glutamine concentration as seen with 50 and 120 mM glutamine ($P < 0.0003$ and 0.0002 , respectively) and 240 mM glutamine where IL-8 reduced by 5 fold compared to the positive control group (22344 to 4273 pg/ml; $P < 0.0001$) (Figure 5). Cell viability of glutamine treated group remained above 95% even with high concentrations of glutamine ($P > 0.05$ in glutamine treated groups vs untreated group) (Figure 6).

Arginine also had a dose dependent effect on IL-8 production. The activity started at 2.5 mM arginine when IL-8 levels were attenuated from 16465pg/ml after TNF- α exposure to 12397pg/ml ($P = 0.0452$ versus positive control group) (Figure 7). Increasing arginine concentration resulted in greater reduction in IL-8 level ($P = 0.0022$ for 5 mM, $P = 0.0011$ for 10 mM and $P < 0.0001$ for 20 and 40mM arginine versus positive control group) (Figure 7). Further, cells treated with arginine at 50mM produced six-fold less IL-8 compared to the positive control cells (from 16465 to 2459 pg/ml, $P < 0.0001$), and was significantly lower than the 20 mM arginine treated group ($P = 0.0474$) (Figure 7). Cell viability remained high at all arginine concentrations ($P > 0.05$ in arginine treated groups vs untreated group) (Figure 8).

Vitamin D3 exhibited a strong anti-inflammatory effect in a dose dependent manner (Figure 9). In the presence of 1 nM vitamin D3, IL-8 levels were significantly attenuated compared to the positive control group (from 15351 to 10061 pg/ml, $P = 0.0021$) (Figure 9). The greatest suppression was seen with the highest tested concentration of vitamin D3 (100nM) where IL-8 was reduced 2-fold compared the positive control group (from 15351 to 7406pg/ml, $P < 0.0001$) (Figure 9). Cell viability remained above 90% throughout ($P > 0.05$ in vitamin D3 treated groups vs untreated group) (Figure 10).

ALA appeared to have no anti-inflammatory effect at the highest concentration tested (Figure 11). Further, no detectible anti-inflammatory effect of ALA was seen when added simultaneously with TNF- α (not shown).

Discussion

These *in vitro* investigations have demonstrated that glutamine, arginine and vitamin D3, but not ALA, at concentrations equivalent to their concentrations in standard PF, can prevent the TNF- α mediated production of the pro-inflammatory cytokine IL-8 in human intestinal epithelial cells. This inhibition of IL-8 production was amplified when these four nutrients were combined and added to the cells. Further, in the present study glutamine, arginine and vitamin D3 all had strong dose-dependent anti-inflammatory effects with increasing concentrations of glutamine, arginine and vitamin D3 resulting in a considerable and significant reduction in IL-8 production from TNF- α exposed HT29 cells. However, in this model of intestinal inflammation, n-3 fatty acids had no detectable anti-inflammatory effect at the concentrations tested.

Whilst not wishing to be bound by theory, the inventors consider that n-3 fatty acids, for example other than ALA such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) or at higher concentrations, are yet encompassed by the invention.

The present study is the first to test *in vitro* the anti-inflammatory effect of combining glutamine, arginine, vitamin D3 and n-3 fatty acids. Here, supplements other than n-3 fatty acids at equivalent concentrations to their concentrations in PF showed an effect on IL-8 production from TNF exposed intestinal epithelial cells. Dose response of increased vitamin D3 concentrations on inflammatory response of intestinal inflammation *in vitro* has not been shown before.

Example 2 - Investigating the effect of combined glutamine and arginine on pro-inflammatory cytokine production from activated intestinal epithelial cells and elucidating mechanisms of action.

Introduction

Glutamine is the most abundant amino acid in the human body. Glutamine is utilized at a very high rate by intestinal epithelial cells and immunocytes. Further, systemic and mucosal glutamine is markedly depleted in inflammatory conditions including CD. Mucosal atrophy, disrupted intestinal barrier function, increased bacterial translocation and reduced glutathione synthesis are consequences of glutamine deprivation.

Arginine is an amino acid with multiple metabolic and immunological functions. Diet, endogenous synthesis and turnover of body proteins are the three main sources of free arginine in the body. Approximately 40% of dietary arginine is catabolized by the intestine before entering the circulation. The gut is also implicated in endogenous arginine synthesis that involves the intestinal-renal axis, in which the citrulline synthesized from glutamine in the small intestine is converted into arginine in the kidney. During catabolic conditions, de novo synthesis of arginine fails to meet the increased demand resulting in disrupted body arginine homeostasis, thus arginine becomes an (conditional) essential amino acid. It has been reported that arginine deficiency in preterm babies results in severe metabolic derangements and multiple organ failure including intestinal dysfunction.

Glutamine and arginine together offer several benefits to the intestinal epithelium, they are classified as immunonutrients, are depleted together in conditions of stress, and are closely linked by metabolism. Also, glutamine serves as a precursor for the de novo production of arginine.

Results

Intestinal epithelial cells can tolerate high glutamine and arginine

Colonic epithelial cells (HT29 and Caco2) were incubated with increasing glutamine (1, 10, 50, 100, 200 and 240 mM) and arginine (0.5, 1, 2.5, 5, 10, 20 and 50 mM) for 24 hours. At the end of incubation, cell viability was determined. There was no effect on viability of the two cell lines with any of the concentrations with no significant drop in viability compared to the control ($P > 0.05$ for all treated groups) (Figure 12, Figure 13 and Figure 14).

Combined glutamine and arginine attenuates proinflammatory cytokine production in intestinal epithelial cells in response to TNF- α

HT29 and Caco2 were seeded in a 24-well plate and grown until confluence. Inflammation was induced in HT29 cells with TNF- α 50 ng/mL and in Caco2 cells with TNF- α 50 ng/mL, LPS 1 μ g/mL, IL-1 β 25 ng/mL and INF- γ 25 ng/mL. Cells were then incubated further with either 240 mM glutamine or 50 mM arginine individually or in combination.

Glutamine treated HT29 cells produced 3-fold less IL-8 compared to the positive control cells (from 13659 to 3847 pg/ml, $p < 0.0001$) (Figure 15A and 15B). Arginine treatment also exhibited a strong anti-inflammatory activity that resulted in a significant decline in TNF- α induced IL-8 production (from 13659 to 3000 pg/ml, $p < 0.0001$) (Figure 15A and 15B). Moreover, by combining glutamine and arginine there was a further significant reduction in IL-8 compared to positive control group (from 13659 to 151 pg/ml, $p < 0.0001$) (Figure 15A and 15B). IL-8 level in combined glutamine and arginine treated group remained significantly lower than in groups treated with either glutamine (A $P = 0.0051$, B $P < 0.05$) or arginine (A $P = 0.025$, B $P < 0.05$) alone. The combination of glutamine and arginine, but not glutamine alone nor arginine, completely abrogated IL-8 production from HT29 in response to TNF- α exposure ($P = 0.0058$ for glutamine, $P = 0.0283$ for arginine and $P = 0.9536$ for the combination versus negative control group) (Figure 15A).

Confluent HT29 cells were co-supplemented with 240mM glutamine and 50mM arginine together incubated with TNF- α (50 ng/ml) for 5 hours. Positive (cells exposed to TNF- α) and negative controls (just confluent cells) were included. IL-8 mRNA expression was determined by real-time PCR (Figure 15C). TNF- α exposure promoted more than 100-fold increase in the IL-8 mRNA ($P < 0.05$; Figure 15C). However, with glutamine and arginine co-supplementation, the expression significantly dropped 4-fold compared to the positive control ($P < 0.05$; Figure 15C). However, IL-8 mRNA expression remained 25-fold higher than the negative control ($P < 0.05$; Figure 15C).

Results similar to HT29 cells were seen in Caco2 cells. A mixture of TNF- α , LPS, IL-1 β and INF- γ induced Caco2 cells to produce 124.4pg/ml of IL-8 compared to an undetectable level of IL-8 in unstimulated cells. In response to the inflammatory stimuli, both glutamine and arginine amino acids significantly ameliorated IL-8 production from Caco2 cells. IL-8 production declined from 124.4 pg/ml to 71.9 pg/ml in the presence of glutamine ($P = 0.001$) (Figure 16) and to 69.8 pg/ml in the presence of arginine ($P = 0.0007$) (Figure 16). Further, when glutamine and arginine were combined, IL-8 levels were further reduced (from 124.4 to 47.60pg/ml, $P < 0.0001$), although IL-8 levels remained significantly higher than un-stimulated cells ($P = 0.0024$ vs negative control group) (Figure 16).

Glutamine inhibits TNF induced I κ k expression (activation) whilst arginine enhanced the expression.

Four groups of HT29 cells were exposed to TNF- α (100ng/ml) for 5, 15, or 30 minutes. In the third and four groups, in addition to TNF- α treatment, cells were incubated with either glutamine

240 mM or arginine 50 mM for the indicated points of time. Proteins of interest were probed by Western blot with anti-I κ B primary rabbit antibodies (1:1000 dilutions) or anti-phosphorylated I κ B primary rabbit antibodies (1:1000 dilutions). Secondary goat anti-rabbit IgG antibodies were utilized at 1:25,000 dilutions.

TNF- α caused overexpression of I κ B as evident by appearance of a strong band of I κ B at 5 minute which corresponded to a visualised faint band of phosphorylated I κ B (Figure 17). By 15 minutes most I κ B was phosphorylated, consistent with drop in the level of I κ B with complete loss of band at 30 minutes (Figure 17). In parallel, a strong blot of phosphorylated I κ B was visualised at 15 minutes of TNF- α exposure before returning to the control level by 30 minutes (Figure 17). In the presence of glutamine, faint bands of I κ B and its phosphorylated form were visualised in all time points (Figure 17). Further, in arginine treated groups, there was an over expression of I κ B protein where strong blots of I κ B were detected at all time points (Figure 17). Correspondingly, a strong band of phosphorylated I κ B was only evident after 30 minutes (Figure 17).

Glutamine inhibits TNF induced I κ B degradation and phosphorylation whereas arginine enhanced the I κ B degradation.

HT29 cells were exposed to 100 ng/ml of TNF- α . Simultaneously, cells were treated with either glutamine 240 mM or arginine 50 mM and incubated further with TNF- α for 5, 15, 30 and 60 minutes. Membranes were probed overnight at 4°C with the rabbit primary anti-I κ B and anti-phosphorylated I κ B antibodies (1:1000 dilution). Subsequently, blots were incubated with secondary antibodies goat anti-rabbit IgG (1: 25000 dilution) and bands were visualized. In response to TNF- α , cells showed initial partial drop in the level of I κ B within 5 minutes which was consistent with early appearance of phosphorylated I κ B band that remained detected over 30 minutes (Figure 18). After the early drop, I κ B accumulated at 15 minutes and more at 30 minutes of TNF- α exposure before normalising by 60 minutes that was consistent with disappearance of phosphorylated I κ B band (Figure 18). Glutamine treatment prevented the rapid, initial loss of I κ B seen after TNF- α exposure and, instead induced a more gradual rise in protein levels over 1 hour (Figure 18). In accordance with these observations, phosphorylated I κ B bands were not visualized in presence of glutamine (Figure 18). In the presence of arginine, the TNF- α -induced early drop in the level of I κ B was less evident, but after 30 minutes most of I κ B was degraded with complete loss of band at 60 minutes.

Discussion

These *in vitro* investigations using two different human intestinal epithelial cell lines have demonstrated that glutamine and arginine significantly decreased production of IL-8, a pro-inflammatory cytokine, in response to induced inflammation. In both cell lines, when glutamine and arginine were combined, the inhibitory effect on IL-8 production was further amplified. Further, we were able to show that the anti-inflammatory effects of glutamine were associated with blockade of I κ B phosphorylation and thereby a decrease in I κ B degradation by inhibition of up-regulation of members of the I κ B complex. In contrast, arginine's effect in down regulating the inflammatory

response appeared not to be mediated through this NF- κ B transcription factor.

To our knowledge this is the first *in vitro* study involving intestinal epithelial cells to demonstrate that glutamine and arginine produce additive immunomodulating effects. Here, the individual administration of either glutamine or arginine led to significant, but partial, reduction in the TNF- α mediated production of the pro-inflammatory cytokine IL-8. However, when glutamine and arginine were combined, IL-8 was markedly reduced in the both HT29 and Caco2 cell lines; in fact completely abrogation in HT29 cells.

During the inflammatory process, such as in IBD, various cytokines are circulating leading to NF- κ B activation and thereby promotion of pro-inflammatory cytokine gene expression. The NF- κ B signal transduction pathway is an important regulator of cytokine transcription in intestinal epithelial cells. Dimeric NF- κ B transcription factor belongs to the Rel family of DNA-binding proteins and plays a critical role in the immune and inflammatory responses. NF- κ B dimers are kept in the cytoplasm through interaction with the inhibitory protein I κ B. In response to cell stimulation with pro-inflammatory cytokines (IL-1, TNF- α and LPS), I κ B subunits undergo rapid phosphorylation, which targets it for rapid polyubiquitination and thereby degradation through the 26S proteasome. Degradation of I κ B leads to liberation of NF- κ B dimers (P50/P65) that are then able to move into the nucleus and bind DNA. In the present work, immediately after adding TNF- α , NF- κ B was activated as evident by phosphorylation and early partial drop in the level of I κ B. Glutamine blocked the TNF- α induced phosphorylation of I κ B and thus prevented its early partial degradation. Further, glutamine prompted a rise in the level of I κ B. Thus, these data indicate that glutamine mediates its anti-inflammatory effects by influencing NF- κ B pathway activation by blocking I κ B complex activation, thereby inhibiting phosphorylation and degradation of I κ B.

Example 3 - Developing a novel nutritional therapy with enhanced anti-inflammatory properties for CD by introducing curcumin to an amino acids-enriched PF using an in vitro model of intestinal inflammation.

Introduction

Nutritional therapy is becoming an increasingly attractive treatment option for managing IBD. The European Society of Parenteral and Enteral Nutrition recommend EEN as a first choice in children with active CD. However, EEN remains underutilized in clinical practice due in part to poor subject compliance and poor palatability as result of prolonged duration of treatment. Further, EEN is thought to have lower efficacy in adult subjects. Three meta-analyses have shown that steroids are superior to EEN as primary treatment in adult CD subjects. Thus, there is a need for improved therapeutic nutritional regimen with enhanced efficacy, by magnifying the anti-inflammatory properties of the conventional nutritional formulas is essential.

As shown herein, manipulating glutamine and arginine, two candidates within the standard PF composition, enhances their anti-inflammatory activities. However, if glutamine and arginine are supplemented in a formulation at such high concentrations (240 mM glutamine, 50 mM arginine), the

high protein content could compromise the safety of the therapy.

Standard PF's prescription is based upon the subject's daily estimated energy requirement . The Schofield equation, which estimates basal metabolic rate from weight, is the commonest and the best utilized equation for calculation the estimated energy requirement. Using standard PF, the requirements are translated to 40 ml (1.76 g protein) per kg per day of Osmolite formulation (Abbott-Nutrition) to give 35-40 kcal/kg body weight a day. Further, it has been reported that protein intake can be increased safely up to 4-5 g per kg in subjects receiving 2300 Kcal daily. Based on these calculations, the 40ml of Osmolite received per kg a day can be further fortified with glutamine and arginine until the upper safe limit of protein (4-5 g) is reached, which is equal to 14mmol of combined glutamine and arginine concentrations per 40 ml Osmolite. Therefore, in creating a safe formula, both glutamine and arginine concentrations together should not exceed 350 mM (60 g per litre Osmolite), the highest tolerable concentration, which is equivalent to 70 mM *in vitro* experiment, as standard PF in the established *in vitro* protocol is utilized at 1 to 5 dilution to the culture media. Based on these calculations, the highest tolerable concentrations of glutamine and arginine in the nutritional formulation are 250 mM glutamine and 100 mM arginine, or accounting for intestinal dilution 50 mM glutamine and 20 mM arginine.

However, results shown herein indicate these low concentrations are sub-optimal in reducing the inflammatory response. Thus, for development of a novel formulation, the addition of glutamine and arginine at the desirable concentrations may also be combined with additional pharmaconutrients, to ensure a formulation with maximal efficacy and optimum safety. One of the proposed nutrients is curcumin, which has potential therapeutic implications in IBD.

Turmeric (the common name for *Curcuma longa*) is an Indian spice that belongs to the ginger family. In ancient time, turmeric powder was utilized as a traditional and natural remedy for various health conditions such as joint pain, ulcers, liver disease, wounds and skin diseases. The active ingredient of turmeric is curcumin with the chemical name of diferuloylmethane. Curcumin exhibits anti-microbial, anti-inflammatory, anti-oxidant and anti-neoplastic properties, and has been extensively investigated for its proposed benefits in managing chronic inflammatory conditions. Curcumin is considered safe and inexpensive.

Standard PF is being currently prescribed for CD subjects in either acute or chronic disease stages, as an induction therapy for the active disease (utilized as EEN) or as a maintenance therapy for the recurrent cases (utilized as EN). The inflammation suppressing properties of curcumin during active inflammation are not well defined. We therefore sought to ascertain and compare the anti-inflammatory properties of curcumin when added at differing times to an inflammatory stimulus. We then investigated whether adding curcumin to new PF enriched with tolerable glutamine and arginine concentrations further influenced the inflammatory response, in an *in vitro* model of intestinal inflammation.

Results

Curcumin reduces epithelial cell viability and activity at high concentrations

HT29 and INT407 cells were treated with increasing concentrations of curcumin (0, 10, 25, 50, 75 or 100 μ M) in DMSO for 24 hours. The final concentration of DMSO for all experiments remained constant at 0.1% v/v, which did not have an effect on cell viability (Figure 20). Increasing concentrations of curcumin (up to 50 μ M) had no significant effect on cell viability, with viability remaining above 90% for both cell lines ($P > 0.05$) (Figure 20). However, curcumin concentrations of 75 μ M and above significantly decreased cell viability ($P < 0.0001$ 75 μ M and 100 μ M curcumin treated groups in both cell lines) (Figures 20 and 21). Therefore, for further experimentation, curcumin concentrations of up to 50 μ M were used.

Curcumin blocks IL-8 production from TNF- α exposed intestinal epithelial cell

HT29 and INT407 cells were treated with curcumin at 10, 25 and 50 μ M for the duration of TNF- α incubation (6 or 24 hours). To examine preventive and therapeutic effects, 10, 25 and 50 μ M of curcumin were administered starting 24, 6 and 1 hour prior to or at the same time as addition of TNF- α . Cell supernatants were then analysed for IL-8 by ELISA. Curcumin exhibited a strong anti-inflammatory effect on intestinal epithelial cells in response to TNF- α exposure.

Curcumin-treated HT29 cells showed a considerable reduction in IL-8 level in a dose dependent fashion (Figure 22). Increasing curcumin concentration showed significant reduction in IL-8 production from cells compared to the positive control with 50 μ M curcumin having the greatest effect on repressing IL-8 levels (Figure 22). Interestingly, 1 hour pre-incubation and no pre-incubation with curcumin had the greatest effect on repressing IL-8 production but only at high curcumin concentrations (Figure 22). At the lowest curcumin concentration (10 μ M), pre-incubating for 24-hours, had a greater effect on reducing IL-8 production compared to 1 hour or no pre-incubation (Figure 22). In INT407 cells, low concentrations of curcumin (10 and 25 μ M) with long pre-incubation (24 hours) and no pre-incubation increased the amount of IL-8 production (Figure 22). However, 50 μ M curcumin with or without pre-incubation had similar activity to that observed in HT29 cells, with almost complete blocking of IL-8 production in response to TNF- α (Figure 22).

Curcumin suppresses I κ B degradation in intestinal epithelial cell

HT29 and INT407 cells were seeded in 6-well plates at a concentration 10^6 cells/well and grown for 5 days before being treated with 50 μ M curcumin for 1 hour before or at the same time as administration of 100 ng/ml TNF- α . Curcumin-treated cells were then incubated with TNF- α for 5, 15 and 30 minutes. Membranes were probed with the rabbit anti-I κ B and anti-phosphorylated I κ B primary antibodies (1:1000 dilution) overnight 4°C. Subsequently, blots were incubated with secondary antibodies goat anti-rabbit IgG (1: 25000 dilution). When TNF- α was added to HT29 cells there was rapid degradation of I κ B at 5 and 15 minutes before returning to normal after 30 minutes of TNF- α exposure (Figure 23). This is consistent with appearance of phosphorylated I κ B as early as 5 minutes, which is subsequently degraded by proteasome activity (Figure 23). In curcumin-treated

cells (1 hour or no pre-incubation) there was no evidence of the initial loss of I κ B; instead treated cells showed an accumulation of I κ B that corresponded with a lack of phosphorylated I κ B (Figure 23). Similar observations were also made with INT407 cells. Once exposed to TNF- α , control cells exhibited a reduction in I κ B that was consistent appearance of phosphorylated I κ B (Figure 24). In contrast, curcumin treated cells (1 hour or no pre-incubation) showed an increase in the I κ B level in conjunction with a lack of phosphorylated I κ B in response to TNF- α stimulation (Figure 24).

Novel formulation completely blocks IL-8 production in response to TNF- α stimulation

HT29 cells were treated with increasing concentrations of glutamine and arginine components of PF (glutamine/ arginine: 50/2, 50/10, 50/15, 50/20, 50/25, 50/30 and 240/50 mM/mM). PF was given at concentration 1 to 5 to the original culture media as was calculated before. Therefore, glutamine and arginine also were added at 1/5 of their concentrations to PF diluted 1/5 to arrive at the new formula. In the next experiment, cells were treated with either: standard PF or curcumin alone or in combination with glutamine and arginine-enriched PF (formulation was allocated from previous experiment based on the lowest safe glutamine and arginine concentrations required for achieving best significance in blocking IL-8 production). Curcumin here was dissolved directly in PF and then added to the media to give a final concentration of 50 μ M in the culture media. After 6 hours incubation, supernatants were collected and analysed for IL-8 by ELISA. Standard PF considerably ameliorated IL-8 production from HT29 cells in response to TNF- α stimulation ($P < 0.0001$ as compared to positive control group) (Figure 25). Further, increasing glutamine and arginine concentrations (glutamine to arginine: 50/15, 50/20, 50/25 and 50/30) enhanced the PF activity in reducing IL-8 levels ($P = 0.0339, 0.0038, 0.0037, 0.0028$ and 0.0006 , respectively as compared to standard PF) (Figure 25).

However, at the highest tolerable glutamine and arginine concentrations of PF (glutamine/ arginine 50/20 mM/mM) IL-8 remained significantly higher than in negative control group ($P = 0.0026$ versus negative control group) (Figure 25). Next, when curcumin was added to the enriched-PF (glutamine to arginine: 50/20) IL-8 levels were completely abrogated such that there was no significant difference to the control group ($P = 0.1914$) (Figure 26). In contrast, in curcumin-only or enriched PF treated groups, although IL-8 production was considerably attenuated as compared to positive control group, IL-8 levels remained significantly higher than negative control ($P = 0.0004$ and 0.0099 , respectively) (Figure 26).

PF supplemented with Glutamine, Arginine and Curcumin maintains the viability of intestinal epithelial cells

HT29 cells were treated with either standard PF (glutamine 12.7 mM and arginine 1.8 mM) or PF fortified with glutamine and arginine at different concentrations (glutamine/ arginine: 50/1.8, 50/10, 50/15, 50/20, 50/25, 50/30 and 240/50 mM/ mM) for 24 hours. Cell viability remained higher than 90% (Figure 27). In the next experiment, HT29 cells were treated for 24 hours with standard PF, glutamine and arginine enriched-PF, PF supplemented with glutamine, arginine, and curcumin, or

with curcumin only. Cell viability remained comparable to the untreated group ($P > 0.05$) (Figures 27 and 28).

Discussion

These *in vitro* investigations have demonstrated that curcumin can prevent the TNF- α mediated production of the pro-inflammatory cytokine IL-8 in human intestinal epithelial cells. Further, this inhibition of IL-8 is similar whether curcumin was introduced prior to or at the same time as TNF- α stimulation. These results also indicated that curcumin exerts these anti-inflammatory activities by impairing the degradation of I κ B, thereby modulating the NF- κ B signal transduction pathway. Further, this work also has shown that altering glutamine and arginine components of standard PF enhances the anti-inflammatory activity of enteral diet utilizing PF. Glutamine and arginine-enriched PF was more efficient than standard PF in preventing the TNF- α mediated production of the pro-inflammatory cytokine IL-8 in human intestinal epithelial cells. Moreover, addition of curcumin to glutamine and arginine-enriched PF further enhanced the anti-inflammatory activity and resulted in complete inhibition of IL-8 production. Importantly, PF supplemented with arginine, glutamine and curcumin at these concentrations completely abrogated the inflammatory response and has no detrimental effects on the activity and/or viability of intestinal epithelial cells. However, it is important to note that these experiments suggest that curcumin may have a narrow safety window. Cells treated with curcumin concentrations greater than 50 μ M had a significant drop in cell viability. Nevertheless, curcumin may both induce and maintain remission of intestinal inflammation.

Example 4

To develop a supplementation protocol, glutamine (Figure 29A), arginine (Figure 29B) and curcumin (Figure 29C) were added to cell culture media for culture with HT29 cells. In the presence of TNF- α , glutamine, arginine and curcumin displayed dose-response characteristics with reduced IL-8 levels in response to increasing supplement concentrations. To ensure this was not due to cell death, cell viability was measured. There was no loss of cell viability for any of the supplements at any concentration.

To develop a formulation for consumption, nutritional guidelines must be considered. Existing PF contains 40 g/L and to stay within recommended daily protein intake we cannot exceed 80 g/L supplemented PF. Therefore, 250 mM glutamine and 50 mM arginine were selected as suitable target concentrations to achieve a supplemented PF with optimal anti-inflammatory properties while staying within the recommended daily intake limit (although the calculated maximum tolerable concentration may allow arginine to be increased to 100 mM). The arrows (Figure 29A and 29B) indicate glutamine and arginine concentrations in standard PF, with a circle indicating our target supplemented concentrations. The target concentrations represent a significant reduction in the IL-8 response compared to the standard concentrations. When PF was supplemented with the target concentrations of glutamine, arginine and curcumin, the IL-8 response to TNF- α was abrogated

(Figure 29D).

Example 5

The NF- κ B signal transduction pathway is an important regulator of cytokine transcription in intestinal epithelial cells. The key step in its activation is I κ B phosphorylation by the protein kinase complex I κ k. The kinase assay reported here shows that the anti-inflammatory property of supplemented PF is mediated through inhibition of NF- κ B pathway.

In this example, confluent HT29 cells were differentially exposed to TNF- α (50ng/ml) glutamine (240mM) or arginine (50mM) and incubated for 5, 15, or 30 minutes before cytosolic and nuclear cell lysates were collected for investigation of I κ k activation. Membranes were probed with: anti-I κ k or anti-ph I κ k antibodies (A); anti-I κ B or anti-ph I κ B antibodies (B); anti-P65 antibodies (C); or anti-P38 antibodies or anti-ph P38 antibodies (D). B-actin was included as a loading control and protein bands were visualised by chemiluminescence.

As shown in Figure 30, TNF- α immediately activated I κ k, as evident by the early appearance of a strong band of phosphorylated I κ k 5 minutes following TNF- α exposure. Correspondingly, I κ B levels were reduced at 5 and 15 minutes, which is consistent with the appearance of phosphorylated I κ B (Figure 30). Subsequently, P65 translocated into the nucleus 15 minutes following TNF- α exposure, with the majority of P65 appearing to have migrated into the nucleus at 60 minutes (Figure 30). In the presence of glutamine or arginine, phosphorylated-I κ k bands were reduced in intensity (Figure 30). Correspondingly, the phosphorylated I κ B bands were also reduced in intensity resulting in reduced P65 nuclear bands at 15 minutes with loss of the band at 30 minutes and at 60 minutes with arginine supplementation (Figure 30).

P38 was activated as early as 15 minutes after TNF- α exposure as indicated by appearance of the phosphorylated-P38 band at this time (Figure 30). In contrast, the phosphorylated-P38 band was reduced in intensity in cells pre-incubated with either glutamine or arginine (Figure 30). There appeared to be no change in the intensity of the total P38 band with any of the treatments (Figure 30). This demonstrated that both glutamine and arginine blocked TNF- α induced P38 MAPK phosphorylation.

Also in this example, cells were grown on glass slides for 3 days then analysed by immunohistochemistry for expression and nuclear migration of P65 subunit of NF- κ B. HT29 cells were unstimulated (A) or stimulated with TNF- α (50ng/ml) for 1 hour (B), or pre-incubated with either 240mM glutamine (C) or 50mM arginine (D) for 1 hour then stimulated with TNF- α (50ng/ml) for another 1 hour. Slides were then incubated with rabbit polyclonal anti-human P65 antibody and detected using 488 Alexa (green) secondary goat anti-rabbit antibodies. Nuclei were counter stained with DAPI fluorescence (blue). The slides were visualized by Axioplan 2 immunofluorescent (40x magnification) illustrating epithelial monolayer histology (1), P65 expression (2) and P65 expression with nuclei counterstaining (3).

Consistently, in the control monolayers the NF- κ B P65 subunit was abundantly present in

the cytoplasm (green fluorescence) with minimal localization in the nucleus (blue fluorescence) (Figure 31). Upon TNF- α stimulation, a significant number of P65 subunits translocated into the nucleus (yellowish white colour) (Figure 31). P65 trafficking was partially prevented by glutamine and almost completely prevented by arginine that was accompanied by a corresponding significant reduction in the expression of P65 in the cytoplasm (Figure 31).

This example demonstrated that glutamine and arginine inhibited TNF- α induced I κ B phosphorylation and thereby prevented I κ B phosphorylation-degradation and P65 nuclear migration.

Example 6

In this example, increasing concentrations of glutamine (10, 50, 100 or 240mM) or arginine (10, 20 or 50mM) were added to HT29 cells to investigate I κ B kinase activity in response to glutamine or arginine.

The addition of the fractionated I κ B β subunit to I κ B coated wells resulted in a colour reaction, which represented I κ B phosphorylation and translated to 100% I κ B kinase activity (Figure 32). The synthetic inhibitor, K252a, completely inhibited I κ B kinase activity as the O.D. was not different to the no enzyme control ($P > 0.05$; Figure 32). In the presence of glutamine there was significant inhibition of I κ B kinase activity, beginning with the lowest glutamine concentration (10mM) and increasing with proportionally to glutamine concentration (50 and 100mM; Figure 32) and ending with complete abolition of enzyme activity at the highest glutamine concentration tested (240mM; $P > 0.05$ vs. K252a; Figure 32). Similarly, arginine showed a significant dose dependent attenuation in the I κ B kinase activity (Figure 32) with maximal inhibition reached at 50mM arginine when the I κ B kinase activity was equivalent to K252a ($P > 0.05$; Figure 32).

This example demonstrated that both glutamine and arginine directly inhibited I κ B kinase activity.

Example 7

To determine if inhibition of IL-8 by the supplemented PF was a direct result of suppressing the NF- κ B pathway in HT29 cells, the effect of the combination of glutamine, arginine and curcumin on I κ B kinase was assayed. Glutamine and arginine were dissolved directly in kinase buffer and then added to the reaction buffer to give a final concentration of 12.7mM (Glu 1) or 50mM (Glu 2) glutamine, or 1.8mM (Arg 1) or 20mM (Arg 2) arginine. K252a and curcumin were added to the kinase buffer to produce a final concentrations of 10mM and 50 μ M, respectively, when added to the reaction buffer. Glutamine and arginine at all concentrations significantly reduced I κ B kinase activity (Figure 33). However, curcumin at 50 μ M suppressed I κ B kinase activity the most compared to the supplements individually. Importantly, the combination of glutamine 50mM, arginine 20mM and curcumin 50 μ M showed complete suppression in the I κ B complex activity ($P = 0.66$ vs. K252a; $P = 0.10$ vs. no I κ B kinase control; Figure 33).

This example demonstrated that co-supplementation with glutamine, arginine and curcumin completely abrogated IL-8 production by HT29 cells in response to stimulation with TNF- α .

Example 8 - Using ex vivo cultured colonic biopsies from subjects with Crohn's disease to investigate the effect of the nutritional formulation in attenuating intestinal inflammation

Introduction

The human gut mucosa comprises intestinal epithelial cells (mainly enterocytes) and supporting lamina propria hosting numerous inflammatory cells. There is cross talk and a complex interaction between the gut microbiota and cells of the gut mucosa, which requires tight regulation to control the mucosal immune response and maintain homeostasis. Disruption of intestinal homeostasis is a hallmark of IBD and results in increased production of proinflammatory cytokines. Immune cells from the lamina propria, including monocytes, neutrophils, macrophages and lymphocytes, and to lesser extent epithelial cells, are the source of these cytokines and largely contribute to mucosal and systemic concentrations of inflammatory mediators. This intricacy and multiplicity of gut responses becomes even more complex during pathology.

To further investigate the nutritional formulation's efficacy on the inflamed human gut mucosa, experiments using an *ex vivo* system was used. Collected tissue specimens retain their histological structure and maintain cellular function if essential nutrients are provided. Human colonic biopsies can be successfully cultured *in vitro* for up to 48 hours without detrimental effects on differentiation or metabolic activity. Further, intestinal biopsies collected from patients with CD spontaneously simultaneously release proinflammatory cytokines including TNF- α and IL-6 as well as other mediators. Therefore, in this example the anti-inflammatory properties of the nutritional formulation were further examined by utilizing *ex vivo* cultured colonic biopsies collected from paediatric subjects with active CD.

The inventors hypothesised that the nutritional formulation would attenuate inflammation to a greater extent than standard PF, in a tissue culture model that utilizes colonic biopsies collected from subjects with active CD

The inventors aimed to examine the extent to which the nutritional formulation, in comparison to standard PF, reduces cytokine release from *ex vivo* cultured biopsies collected from inflamed regions of the gut mucosa of CD subjects, and to assess the effect of the nutritional formulation on tissue viability of *ex vivo* cultured intestinal mucosa.

Results

Nutritional formulation has no detrimental effect on tissue culture of colonic biopsies from CD subjects

Forty tissue culture experiments were included in this study. Biopsies were collected from 10 normal subjects and 10 subjects with confirmed CD. Tissue specimens of normal subjects were cultured with media only (negative control). Colonic tissues of CD subjects were cultured with media only (positive control), standard PF, or the nutritional formulation comprising glutamine 50 mM, arginine 20mM and curcumin 50 μ M . Tissue viability was assessed by assaying lactate dehydrogenase (LDH) activity. LDH activity was 0.5 ± 0.1 U/mg of tissue for all biopsies collected

(Figure 34A) LDH release was not significantly different between controls or any of the treatment groups ($P>0.05$; Figure 34B).

Nutritional formulation completely inhibited pro-inflammatory cytokine and chemokine production from the cultured intestinal mucosa of active CD subjects

Following tissue culture, concentrations of TNF- α , IL-6, and IL-8 were measured in the culture media using ELISA cytoset kits. The level of TNF- α was higher in supernatant collected from CD subject biopsies (45 ± 5 pg/mg tissue) compared to negative control (1.5 ± 0.3 pg/mg tissue) ($P<0.05$; Figure 7.2A). TNF- α concentration decreased by approximately 5 pg/mg tissue in CD subject biopsies incubated with the nutritional formulation ($P<0.05$ vs. positive control; Figure 35A), resulting in a TNF- α supernatant concentration identical to that of the negative control ($P>0.05$; Figure 35A). A trend toward lower TNF- α produced by standard PF treated CD subject biopsies (15 ± 2 pg/mg tissue) relative to positive control was observed ($P>0.05$ vs. positive control; Figure 35A).

IL-8 released into the supernatant in negative control biopsies was approximately 200 ± 20 pg/mg tissue but was significantly higher at 2200 ± 45 pg/mg tissue in the culture media of CD subject biopsies ($P<0.05$; Figure 35B). IL-8 levels were equal to negative control (180 ± 10 pg/mg tissue) with the nutritional formulation ($P>0.05$ vs. negative control; Figure 35B), and approximately 500 ± 13 pg/mg tissue with standard PF treatment, which was not significantly different to the positive control ($P>0.05$ vs. positive control; Figure 35B).

IL-6 production was approximately 5 times higher in CD subject biopsies compared to the negative control biopsies ($P<0.05$ vs. negative control; Figure 35C). However, IL-6 levels were considerably decreased by the nutritional formulation compared with the positive control ($P<0.05$ vs. positive control; Figure 35C). Standard PF treatment also resulted in a reduction in IL-6 production compared to positive control, although it did not reach significance ($P=0.07$ vs. positive control; Figure 35C).

Discussion

The nutritional formulation ameliorated gut inflammation in biopsies collected from children with active CD. The effect of the nutritional formulation on *ex vivo* cultured colonic biopsies was manifested by a decrease in the release of the key proinflammatory cytokines TNF- α and IL-6 and the chemokine IL-8. The nutritional formulation was superior to standard PF in attenuating release of inflammatory mediators, resulting in levels equivalent to that of normal non-inflamed cultured mucosa. Further, suppression of cytokines and chemokines was achieved without altering LDH tissue release, indicating that the biopsies remained viable.

Consistent with previous reports in the literature, concentrations of released proinflammatory mediators is higher in tissue cultures of biopsies from the inflamed mucosa compared to biopsies from the non-inflamed bowel. What is unique in the current study is that novel formula prompted a strong anti-inflammatory response in the inflamed gut mucosa from CD patients. Following incubation with the nutritional formulation, concentrations of cytokines in the supernatant

was equivalent to that of the normal non-inflamed mucosa. Although levels were lower in standard PF treated biopsies compared to un-treated biopsies from CD patients, this difference was not significant.

TNF- α , IL-8 and IL-6 are considered master pro-inflammatory mediators serving vital functions in IBD pathogenesis, and are derived from mononuclear cells of the lamina propria, most notably from macrophages. The results of this example indicate that the nutritional formulation has a strong direct anti-inflammatory effect that likely targets all the intestinal cells, including epithelial and non-epithelial types, involved in the inflammatory process. Further, this effect is manifested by the reduction of key inflammatory cytokines involved in the ongoing inflammatory response, and indicates that the nutritional formulation has superior therapeutic benefits in the treatment of CD compared with standard PF.

Conclusion

The nutritional formulation, but not standard PF, suppressed inflammation in biopsies from subjects with active CD with no adverse effects on tissue viability. Further, cytokine production by cultured colonic biopsies was completely abrogated by treatment with the nutritional formulation. Thus, manipulating conventional nutritional treatment of CD can result in a more effective and safe therapy with enhanced immune effects.

Example 9

We have tested the supplemented formulation in the BALB/c TNBS model of colitis. Eight week old mice were injected with 2.5mg TNBS in 45% ethanol per rectal and then fed for 7 days with standard PF or PF supplemented with glutamine (250 mM), arginine (50 mM), and curcumin (50 μ M). The mice tolerated the supplemented formulation and showed improved weight gain at 7 days compared to standard (Figure 36). Inflammatory markers from these investigations are being analysed.

In further work with this rodent model of IBD, four groups will be used. One negative control group will comprise of mice that will be fed a normal diet. One positive control group will comprise mice that will be subjected to a rectal administration of TNBS injection for induction of inflammation. The third and fourth groups, in addition to TNBS injected rectally, mice will be treated with either standard PF or PF supplemented with glutamine, arginine and curcumin. Colonic expression (mRNA) of LIX, TNF- α and IL-1 β pro-inflammatory cytokines will be quantified using Real-Time PCR to confirm the anti-inflammatory effects of the PF supplemented with glutamine, arginine and curcumin.

Example 10

Aim

We will use a double blinded, randomised and placebo controlled clinical trial of glutamine/arginine/curcumin supplemented nutritional formula, with standard nutritional formulation as comparison, to induce and maintain disease remission in children with newly diagnosed CD. The primary aim of this example is to reduce 12 month relapse rates by 50% in children with newly

diagnosed CD who receive exclusive enteral nutrition as their primary treatment.

Recruitment cohort

One hundred and twenty (120) children with newly diagnosed active CD.

Inclusion criteria

- 5 • Male and female children aged between 5 and 17 years.
- Newly diagnosed with CD. Diagnosis of CD will be based on serology, endoscopic and histological findings.
- Physician's decision to treat with exclusive enteral nutrition.
- Parental and participant consent.

10 Exclusion criteria

- Existing gastrointestinal (other than CD) or neurological disorders.
- Unable or unwilling to attend for follow-up.

Study design and randomisation

15 Recruited children will be randomly assigned to one of two treatment arms. Randomisation will be conducted by a research pharmacist, who will retain the randomisation records until completion of the study. Randomisation will be achieved using a random number generating computer software.

Treatment Arms

Arm 1: Placebo control group.

20 Sixty (60) children will be randomised to this arm. These children will receive standard PF plus an additional placebo sachet containing starch. These children will undergo the standard exclusive enteral nutrition (EEN) protocol currently in place at Sydney Children's Hospital which consists of 8 weeks of EEN. The children will be instructed to add 1 sachet to 250ml PF (with mixing) prior to consumption. Following EEN children will receive standard of care therapy, which will
25 include nutritional therapy.

Arm 2: Glutamine, arginine and curcumin supplemented group

 Sixty (60) children will be randomised to this arm. These children will receive standard PF plus an additional sachet containing glutamine, arginine and curcumin. These children will undergo the standard EEN protocol as detailed above. The children will be instructed to add 1 sachet to 250ml
30 PF (with mixing) prior to consumption. Following EEN, children will receive standard of care therapy, which will include supplemental nutritional therapy.

Sample collection

 Week 0 is designated as the week children receive their diagnosis and begin EEN. A timeline of therapy received with samples collected is below. At each time point children will have
35 both serum and stool samples collected, height and weight measured with PCDAI and PGA calculated. At the designated time points they will be asked to complete a quality of life questionnaire (IMPACT 3).

Weeks	0	1	2	4	6	8	16	26	52
Therapy	EEN	EEN	EEN	EEN	EEN	EEN	SoC	SoC	SoC
QOL	Yes	no	no	Yes	No	Yes	Yes	Yes	Yes
Samples	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ht/Wt	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
PCDAI	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
PGA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

EEN- Exclusive enteral nutrition; SoC - standard of care therapy; QOL - quality of life questionnaire (IMPACT 3); Samples - serum and stools samples; Ht/Wt – height and weight measured; PCDAI – Pediatric Crohn’s Disease Activity Index; PGA – Physician’s Global Assessment.

- 5 An additional time point will be included if children suffer a relapse episode. A relapse episode will be classified as entering remission (PCDAI <15) followed by reoccurrence / worsening symptoms with a PCDAI of ≥ 15 .

Assays

- 10 All serum samples will be assayed for liver chemistry, creatinine, erythrocyte sedimentation rate, C-reactive protein, hematocrit, albumin, platelets and serum inflammatory proteins (IL-1, TNF- α , IL-6, IL-10, IL-12, IL-23 & TGF- β). Stool will be assayed for calprotectin, S100A12, osteoprotegerin and defensins.

Primary outcome measure

- 15 The remission rate at 12 months will be the primary outcome measure. Remission rate at 12 months will be classified as entering remission (PCDAI <15) at any time through the study period and not suffering a relapse event (PCDAI ≥ 15) within the 52 week study period.

Secondary outcome measures

- 20 Single secondary outcome measured will be remission at week 8 (PCDAI < 15), time to relapse (weeks), number of relapse events at 12 months and additional maintenance medications. Secondary measures that will be assessed at each time point are; reduction in PCDAI, reduction in PGA, weight gain, height gain, decreased blood and stool inflammatory markers and improved quality of life. :

- 25 This clinical trial will take place over three sites, Sydney Children’s Hospital, Royal Brisbane Hospital and Christchurch Hospital. Based on 2012 data it is expected that at least 70 children per year will be diagnosed with CD amongst the three sites. Previously we have achieved recruitment rates of approximately 80% and therefore we expect to recruit 56 subjects per year into this study. Recruitment is expected to continue for two and a half years. We conservatively estimate we will recruit a minimum of 120 children into the study over the recruitment period.

Recruitment justification and power calculation:

- 30 Data previously published on EEN at Sydney Children’s Hospital identified that the 12 month relapse rate was 61% when EEN was used as the initial induction therapy³. The primary aim of this proposal is to reduce 12 month relapse rates by 50%, which equals a 12 month relapse rate of

30.5%. To calculate whether this study has sufficient power, we used an $\alpha=0.05$, power $(1-\beta)=0.80$ with group size of 60 and calculated the Arm 2 proportion must be 0.377 or less to achieve statistical significance. Therefore this study will return a significant result for the primary outcome if Arm 2 12 month relapse rates are 37.7% or less, which indicates that the study is sufficiently powered to show whether the primary aim has been achieved.

Outcomes

This study will reduce the number and frequency of relapse events that occur in the first year following diagnosis. By reducing the number and frequency of inflammatory relapses in the bowel, we will assist in limiting damage to the bowel and promote bowel healing in the early stage of disease. Maintaining a healthy bowel early on in the disease will provide benefits not only for the year the child is taking the supplements, but should also lead to improved longer-term outcomes as a healed bowel will allow for better disease control while they live with this incurable disease.

CLAIMS

1. A pharmaconutrient composition for treating intestinal inflammation in a subject, the pharmaconutrient composition comprising at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.
2. The pharmaconutrient composition of claim 1 comprising curcumin and at least one of arginine, glutamine, vitamin D3, and an n-3 fatty acid.
3. The pharmaconutrient composition of claim 1 comprising three of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.
4. The pharmaconutrient composition of claim 3 comprising curcumin and at least two of arginine, glutamine, vitamin D3, and an n-3 fatty acid.
5. The pharmaconutrient composition of any one of claims 1 to 4 comprising arginine, glutamine, and curcumin.
6. The pharmaconutrient composition of any one of claims 1 to 5 comprising about 1 mM to about 600 mM arginine, about 1 mM to about 1200 mM glutamine, about 1 μ M to about 100 μ M curcumin, about 1 nM to about 100 nM vitamin D3, or about 0.1 mM to about 10 mM of an n-3 fatty acid, or comprising arginine, glutamine, curcumin, vitamin D3, or an n-3 fatty acid sufficient to produce about 1 mM to about 600 mM arginine, about 1 mM to about 1200 mM glutamine, about 1 μ M to about 100 μ M curcumin, about 1 nM to about 100 nM vitamin D3, or about 0.1 mM to about 10 mM of an n-3 fatty acid.
7. The pharmaconutrient composition of any one of claims 1 to 6 comprising about 50 mM or about 100 mM arginine, about 240 mM or about 250 mM glutamine, about 50 μ M or about 54 μ M curcumin, about 100 nM vitamin D3, or about 7 mM of an n-3 fatty acid, or comprising arginine, glutamine, curcumin, vitamin D3, or an n-3 fatty acid sufficient to produce about 50 mM or about 100 mM arginine, about 240 mM or about 250 mM glutamine, about 50 μ M or about 54 μ M curcumin, about 100 nM vitamin D3, or about 7 mM of an n-3 fatty acid.
8. The pharmaconutrient composition of any one of claims 1 to 6 comprising greater than 94 mM arginine, greater than 103 mM glutamine, greater than 52 nM vitamin D3, or greater than 8.6 mM of an n-3 fatty acid, or comprising arginine, glutamine, vitamin D3, or an n-3 fatty acid sufficient to produce greater than 94 mM arginine, greater than 103 mM glutamine, greater than 52 nM vitamin D3, or greater than 8.6 mM of an n-3 fatty acid.
9. The pharmaconutrient composition of any one claims 1 to 8 in unit dosage.
10. The pharmaconutrient composition of claim 9, the unit dosage comprising about 2.2 g arginine, about 9.1 g glutamine, and about 5 mg curcumin.
11. The pharmaconutrient composition of claim 9 or claim 10, wherein the unit dosage is contained in a sachet.

12. A nutritional formulation for treating intestinal inflammation in a subject, the nutritional formulation comprising the pharmaconutrient composition of any one of claims 1 to 11.
13. The nutritional formulation of claim 12 comprising about 40 g/L to about 80 g/L protein.
14. The nutritional formulation of claim 12 comprising greater than 78 g/L protein.
- 5 15. The nutritional formulation of any one of claims 12 to 14, which is an enteral nutritional formulation.
16. The nutritional formulation of claim 15, which is an exclusive enteral nutrition (EEN) formulation.
17. The nutritional formulation of any one of claims 12 to 16, which is an elemental nutritional
10 formulation or a polymeric nutritional formulation.
18. A method for producing a nutritional formulation that treats intestinal inflammation in a subject, the method comprising supplementing a nutritional formulation with the pharmaconutrient composition of any one of claims 1 to 11, or supplementing a nutritional
15 formulation with at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid, or supplementing a nutritional formulation comprising one of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid with at least one other of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.
19. A nutritional formulation produced by the method of claim 18.
20. A method of treating intestinal inflammation in a subject, the method comprising administering
20 to the subject at least two pharmaconutrients having similar mechanisms of pharmacological activity.
21. The method of claim 20, wherein the at least two pharmaconutrients are selected from arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.
22. The method of claim 20 or claim 21, wherein the at least two pharmaconutrients are
25 administered simultaneously or sequentially.
23. The method of any one of claims 20 to 22, wherein the at least two pharmaconutrients are administered by more than one route or in more than one form.
24. The method of any one of claims 20 to 23, wherein the at least two pharmaconutrients are administered orally, rectally, enterally, or parenterally.
- 30 25. The method of any one of claims 20 to 24, comprising administering the at least two pharmaconutrients in a pharmaconutrient composition or a nutritional formulation.
26. A method of treating intestinal inflammation in a subject, the method comprising administering to the subject a pharmaconutrient composition or a nutritional formulation comprising at least two of arginine, glutamine, curcumin, vitamin D3, and an n-3 fatty acid.
- 35 27. The method of claim 26, wherein the pharmaconutrient composition is the pharmaconutrient composition of any one of claims 2 to 11 or the nutritional formulation is the nutritional formulation of any one of claims 12 to 17 or 19.

28. The method of any one of claims 20 to 27, wherein the intestinal inflammation is due to an inflammatory bowel disease (IBD), an intestinal cancer, necrotising enterocolitis, cystic fibrosis, or surgery, or is in a subject requiring intensive care.
29. The method of any one of claims 20 to 28, wherein the intestinal inflammation is due to dysregulation of NF- κ B signalling.
30. The method of claim 28 or claim 29, wherein the IBD is Crohn's disease (CD) or ulcerative colitis (UC) or inflammatory bowel disease unclassified (IBDU).
31. The method of any one of claims 20 to 30, wherein the subject is a paediatric subject or an adult subject.
32. The method of claim 31, wherein the paediatric subject is from 5 years to 17 years.
33. The method of any one of claims 20 to 32, wherein treating comprises inducing remission or maintaining remission of the intestinal inflammation.
34. The method of claim 33, wherein treating comprises inducing remission and administering the pharmaconutrient composition or the nutritional formulation for about 8 weeks or up to about 12 weeks.
35. The method of any one of claims 20 to 34, wherein treating comprises reducing a 12 month relapse rate compared with subjects treated similarly but in the absence of the pharmaconutrient composition or the nutritional formulation.
36. A kit comprising the pharmaconutrient composition of any one of claims 1 to 11 or the nutritional formulation of any one of claims 12 to 17 or 19.

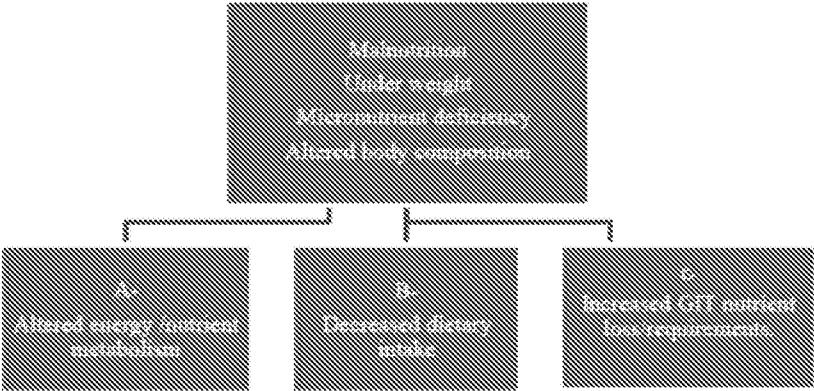


Figure 1.

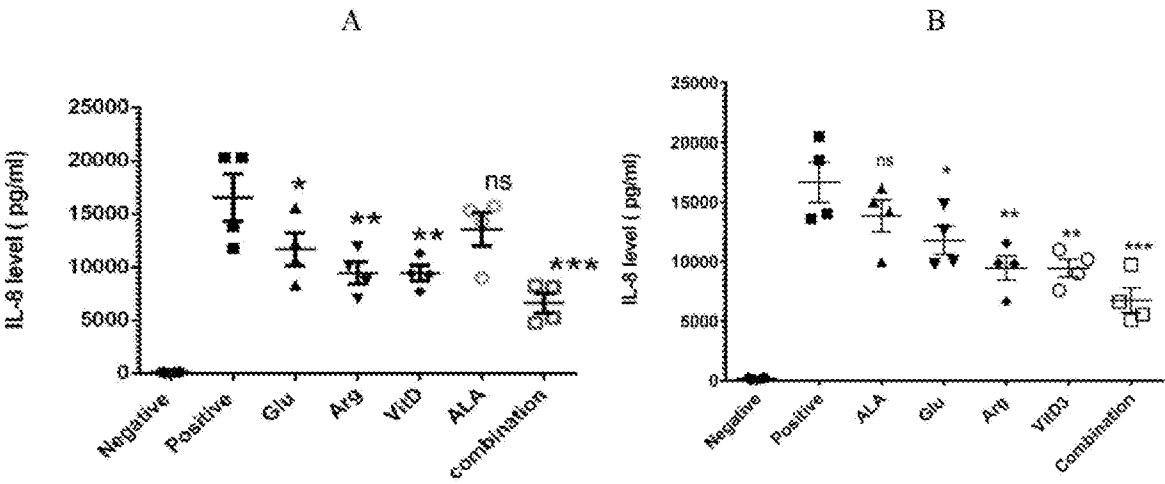


Figure 2.

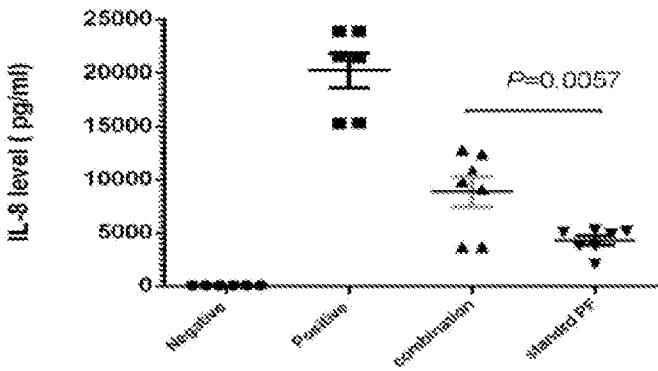


Figure 3.

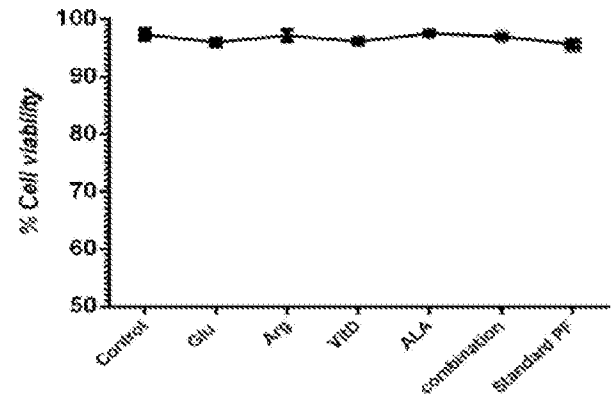


Figure 4.

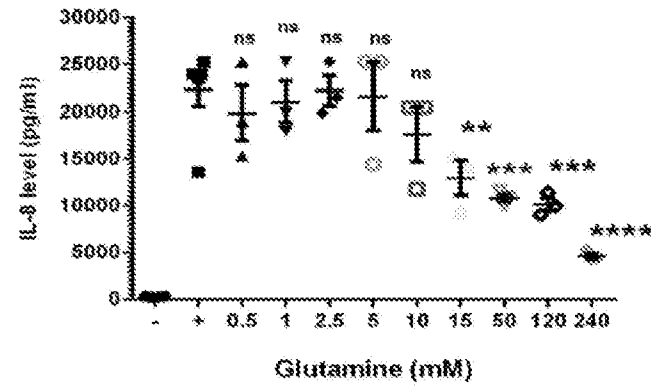


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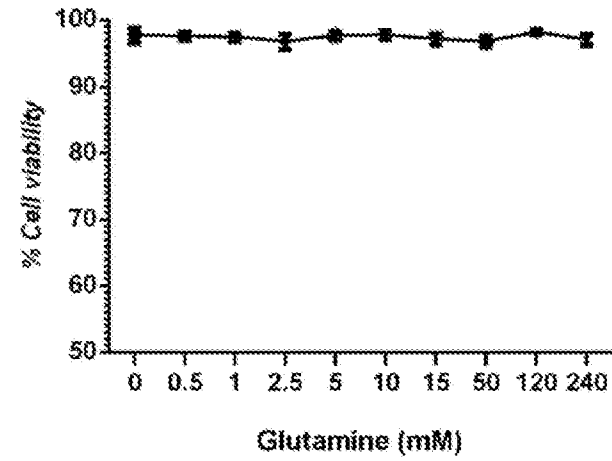


Figure 6.

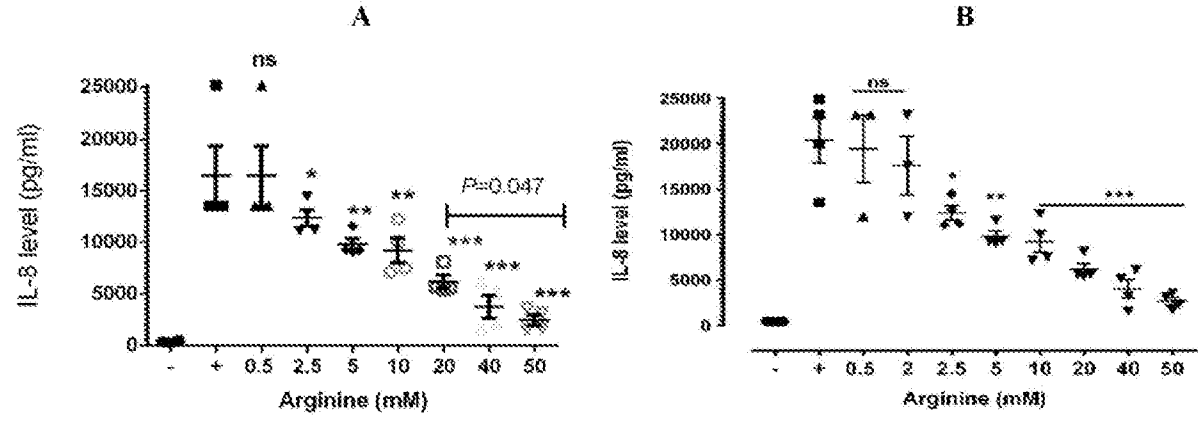


Figure 7.

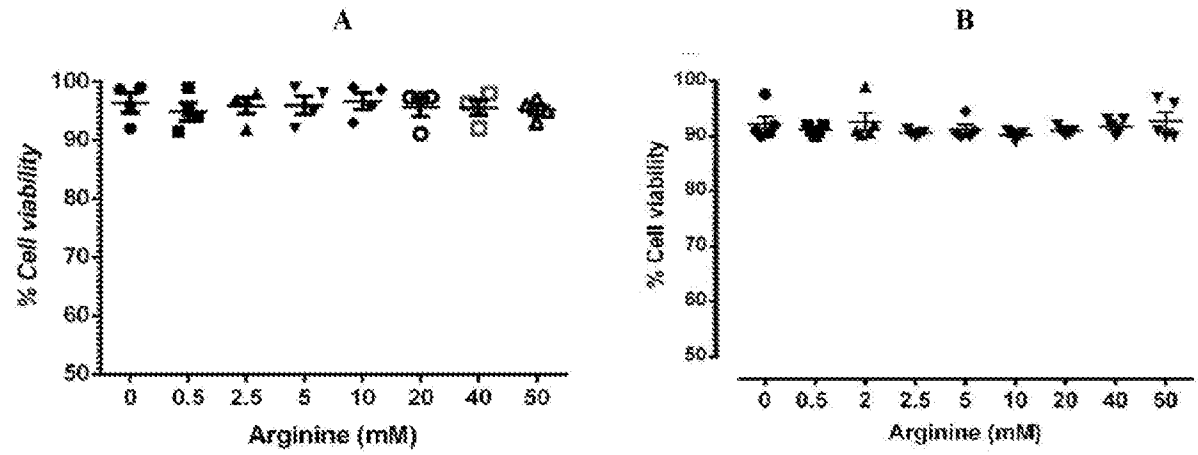


Figure 8.

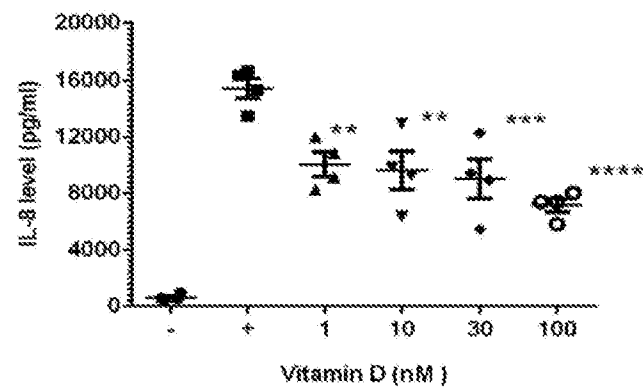


Figure 9.

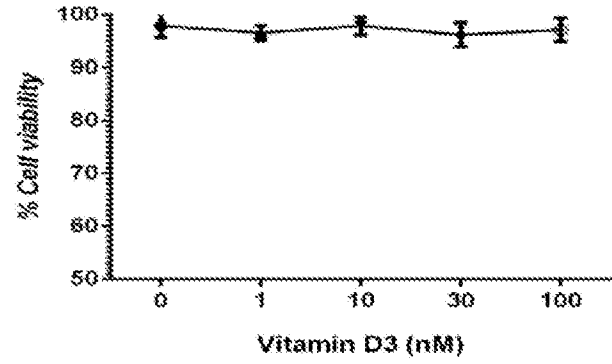


Figure 10.

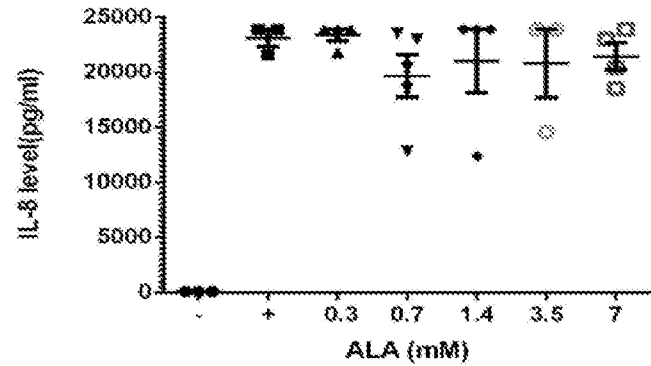


Figure 11.

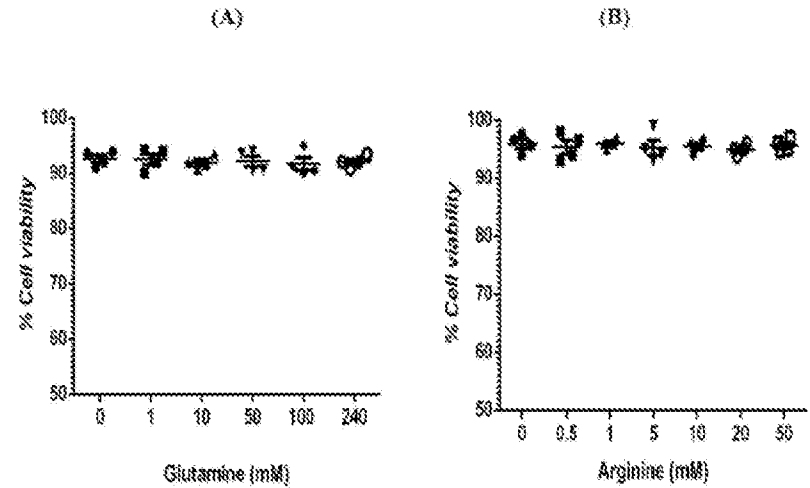


Figure 12.

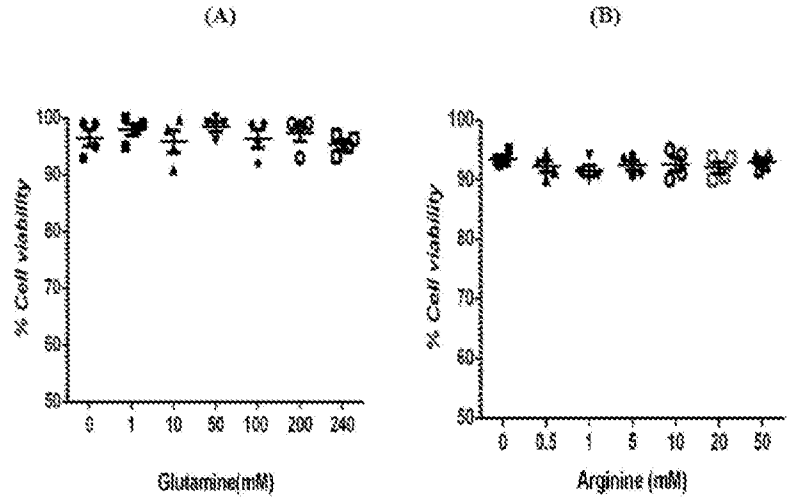


Figure 13.

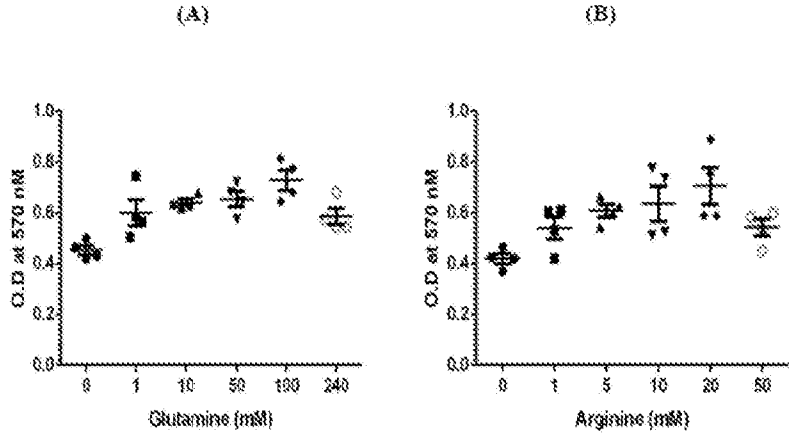


Figure 14.

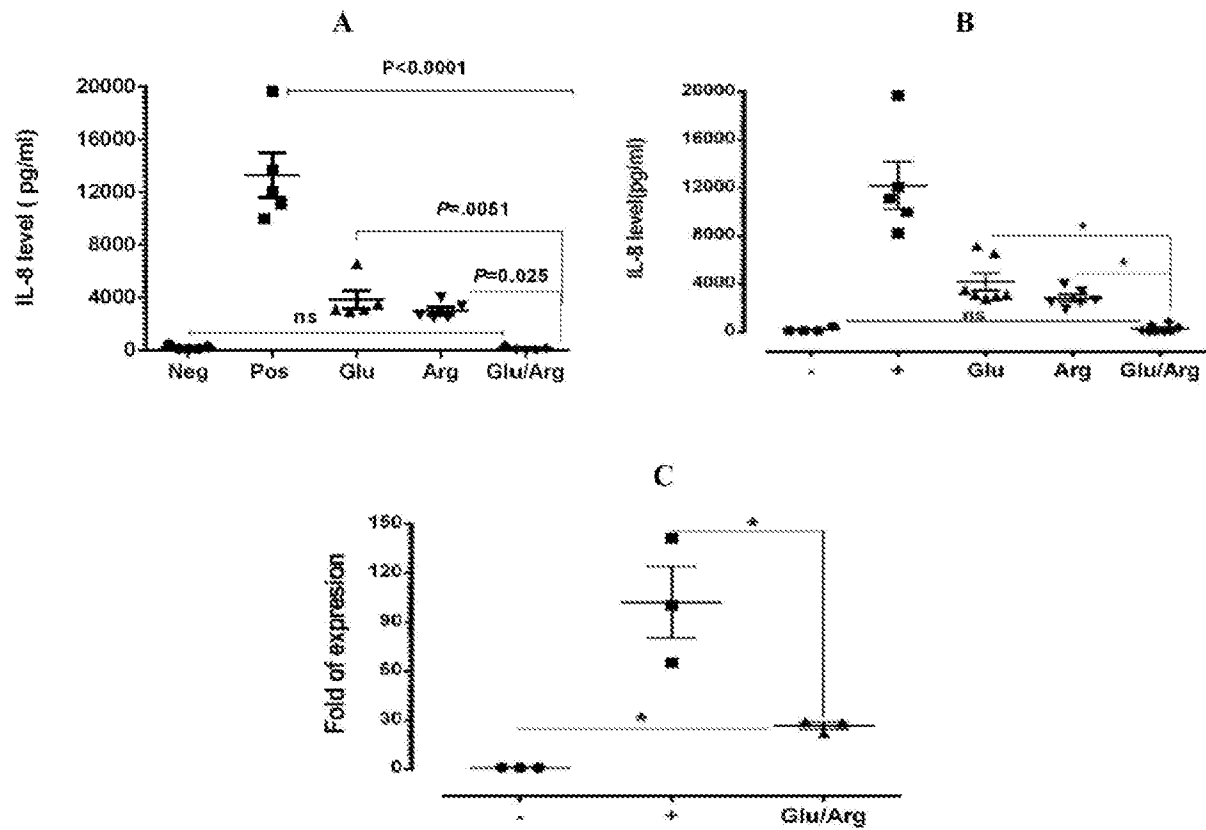


Figure 15.

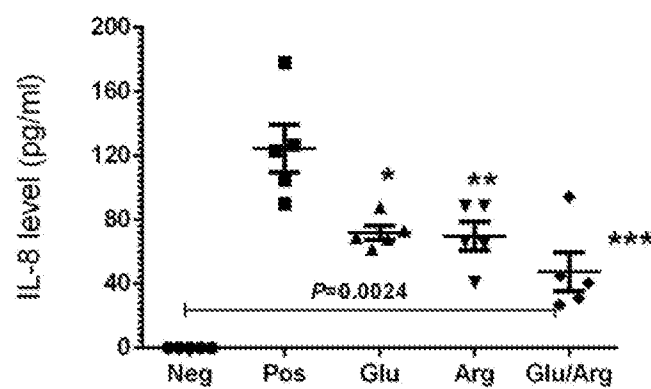


Figure 16.

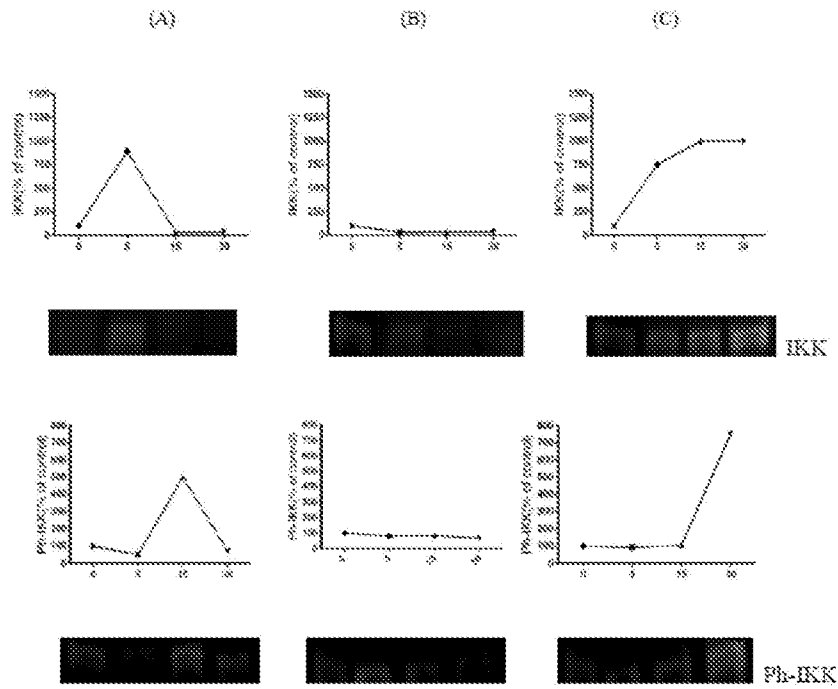


Figure 17.

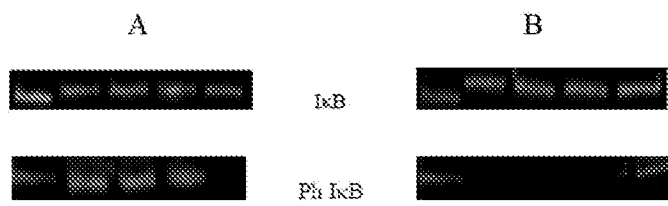


Figure 18.

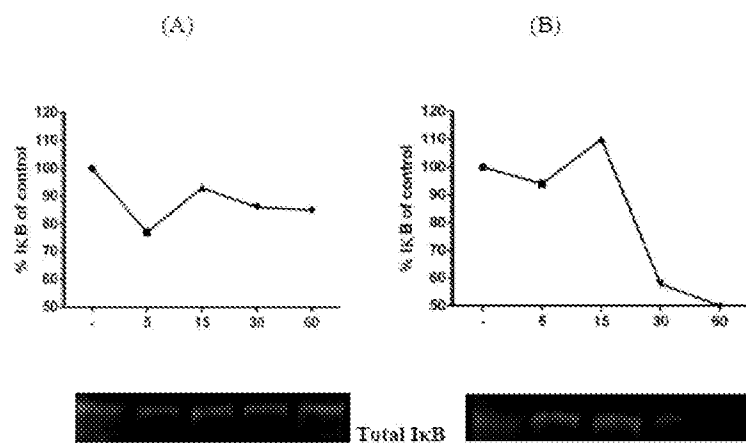


Figure 19.

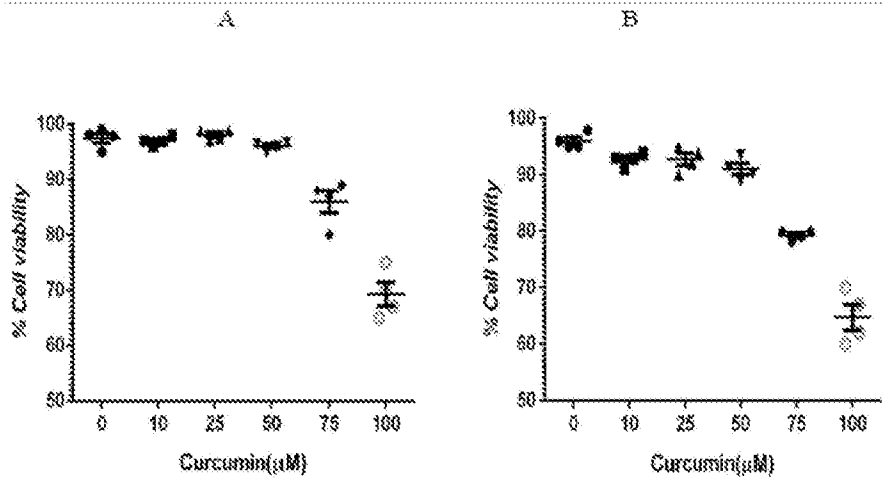


Figure 20.

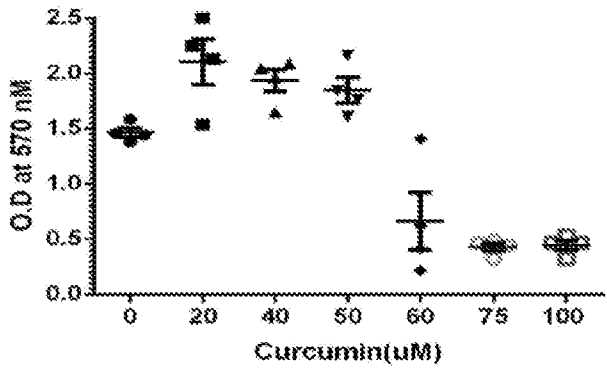


Figure 21.

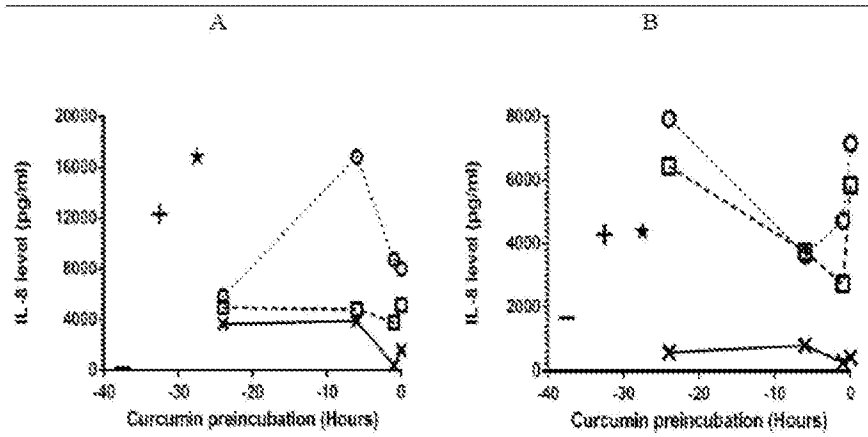


Figure 22.

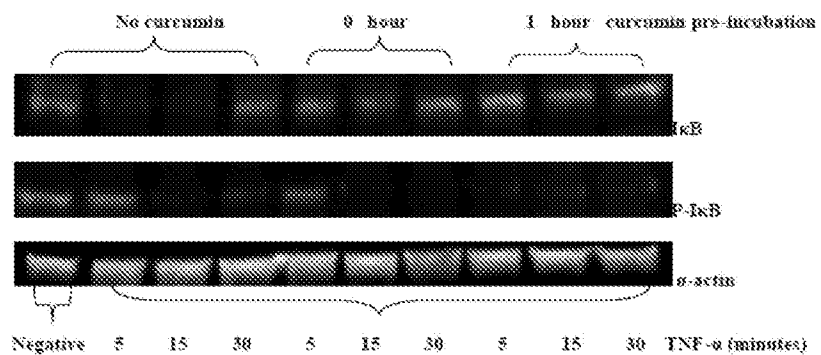


Figure 23.

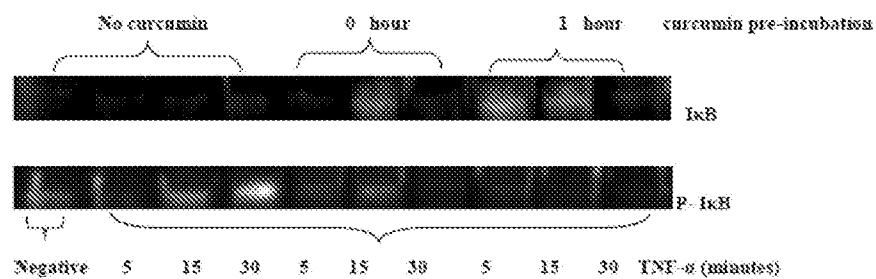


Figure 24.

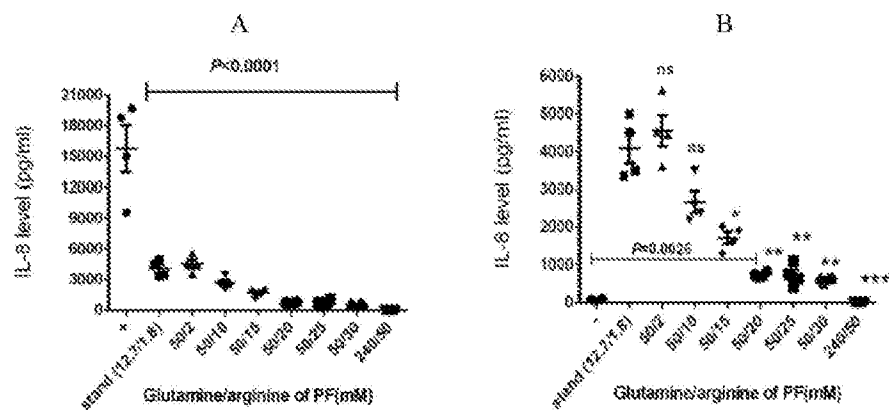


Figure 25.

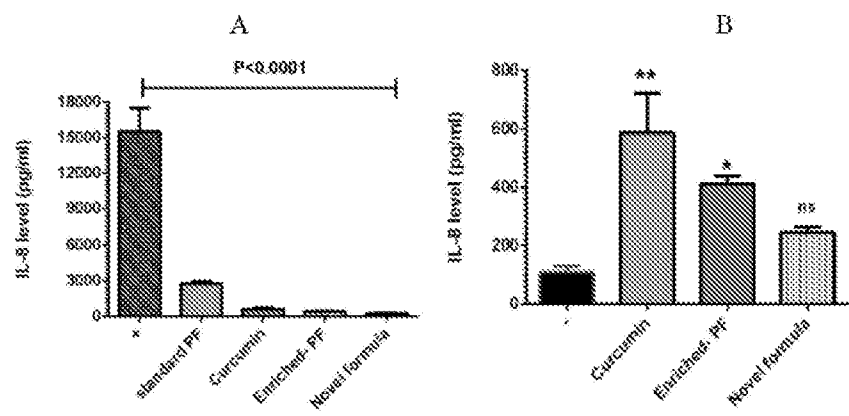


Figure 26.

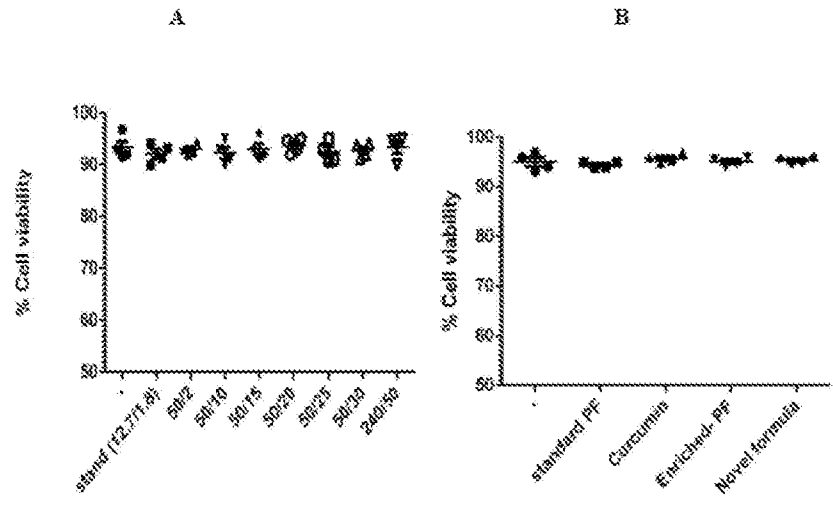


Figure 27.

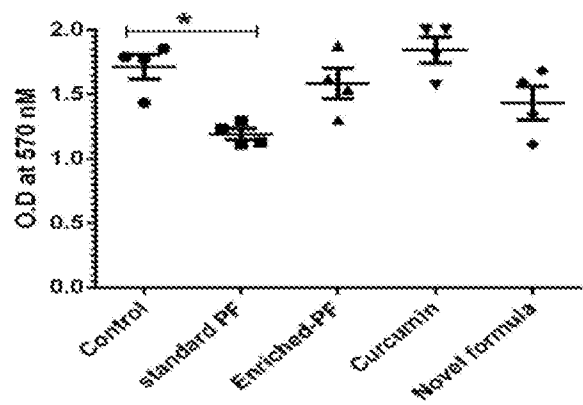


Figure 28.

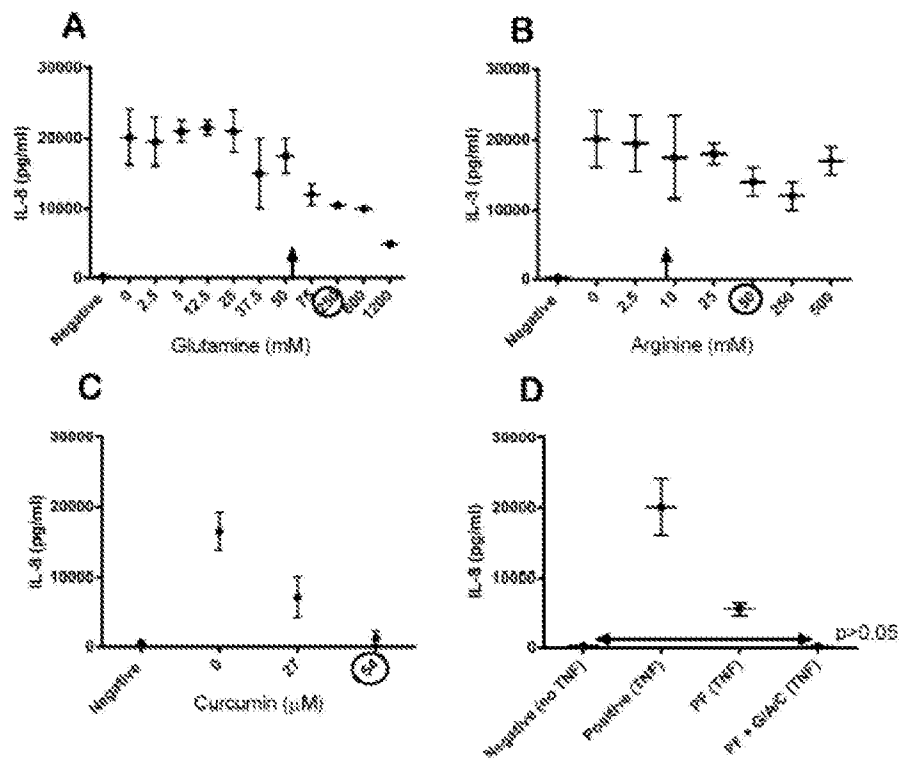


Figure 29.

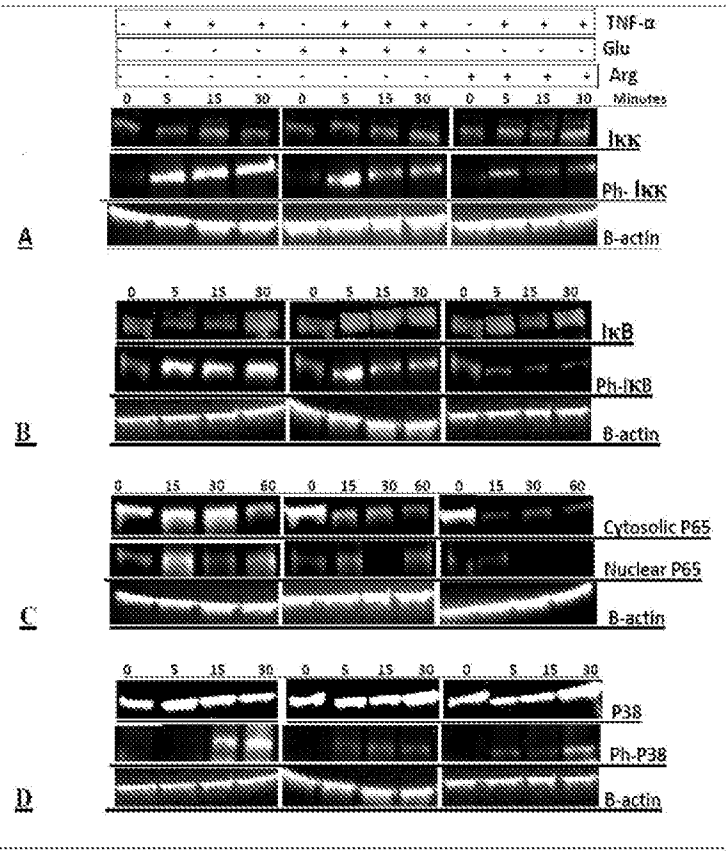


Figure 30.

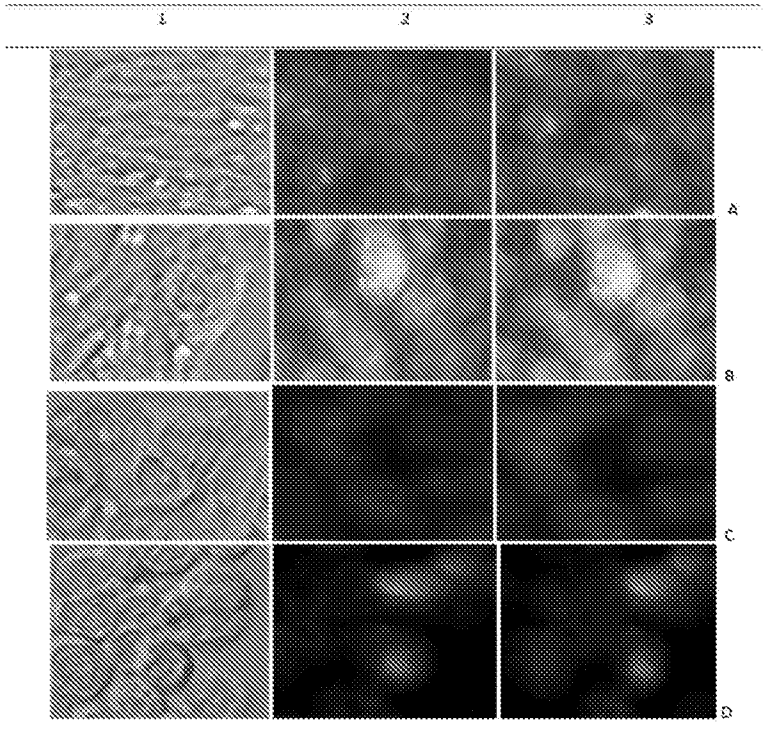


Figure 31.

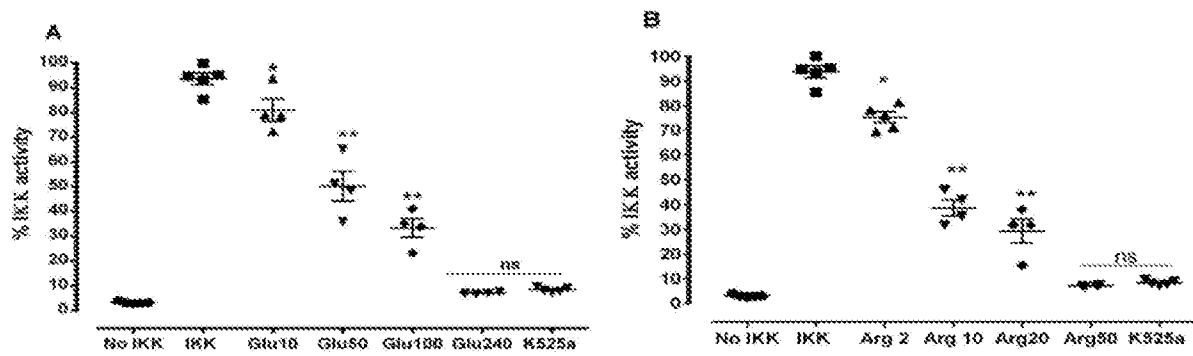


Figure 32.

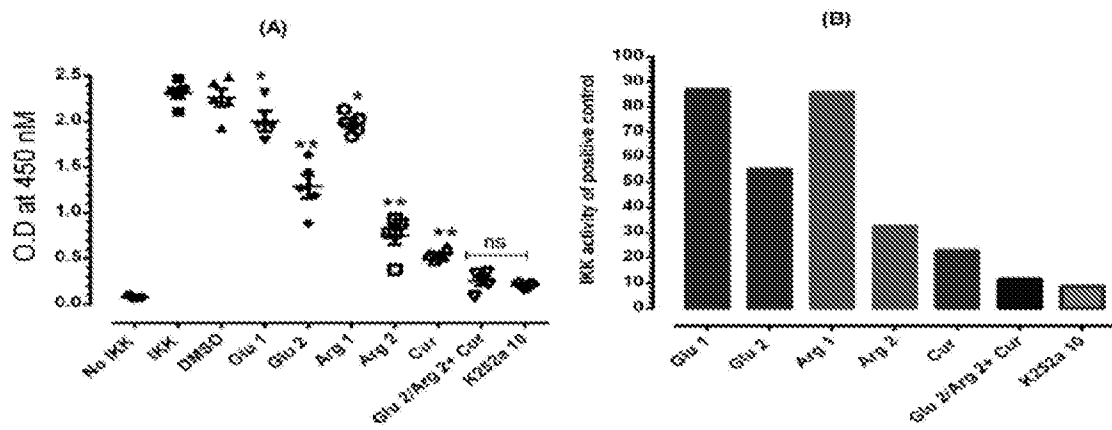


Figure 33.

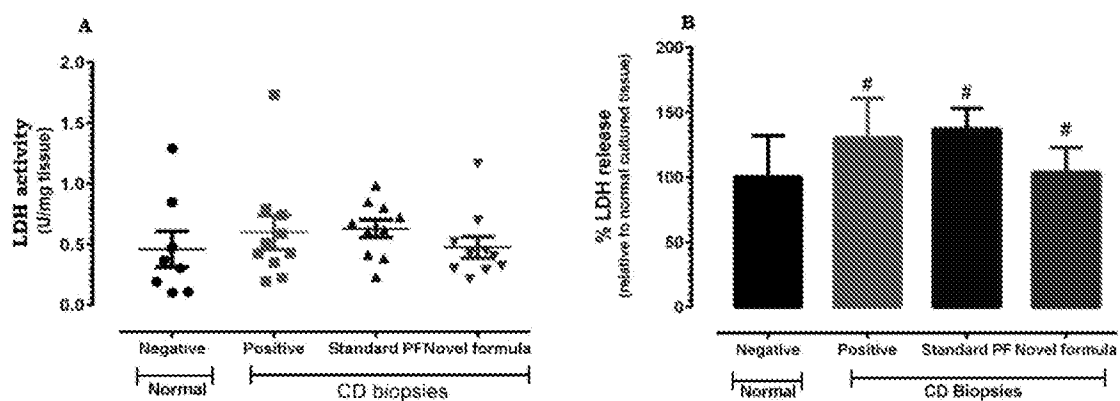


Figure 34.

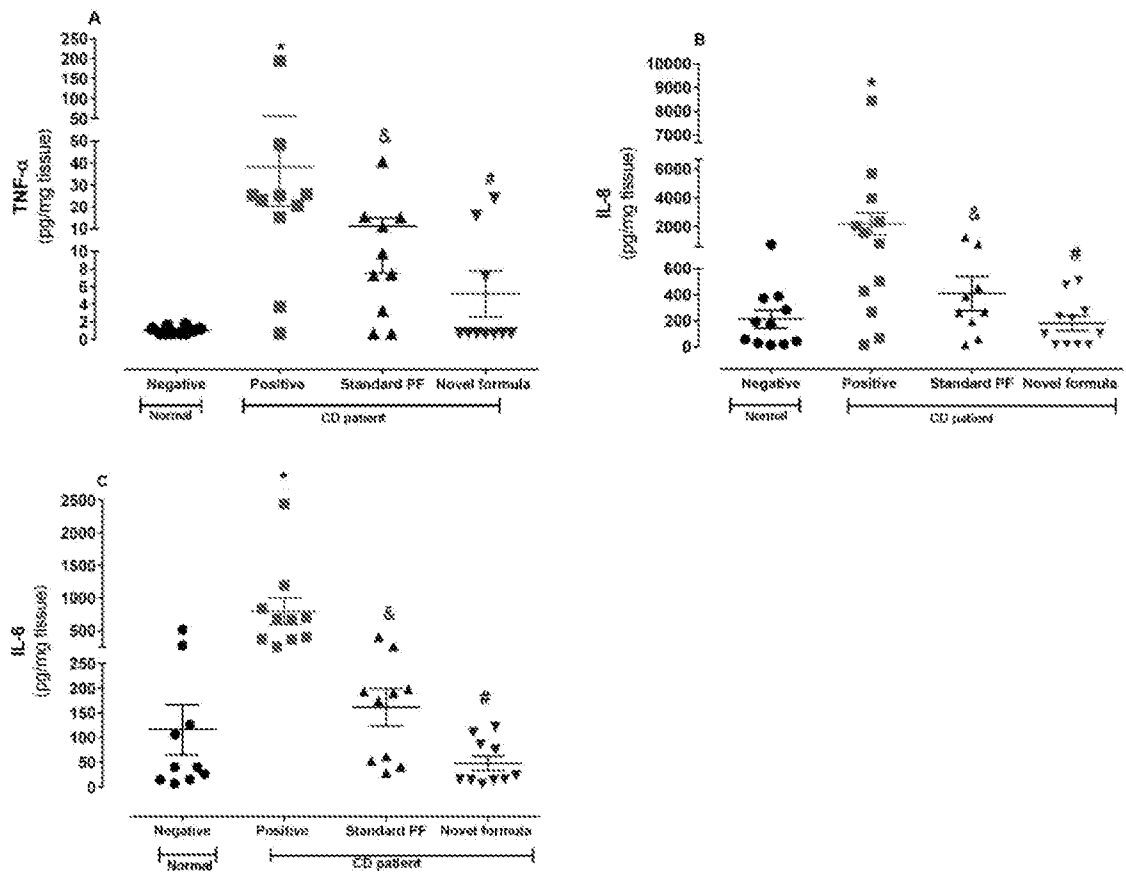


Figure 35.

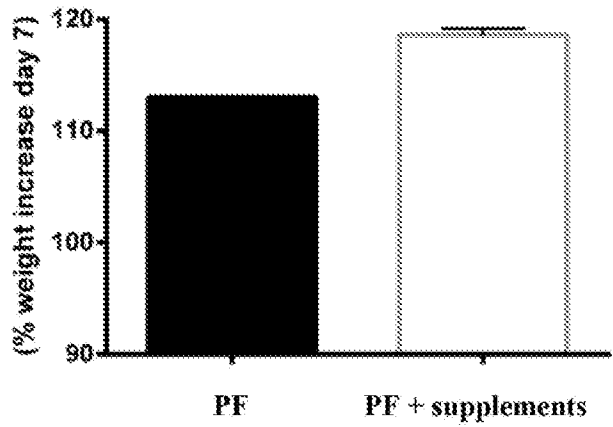


Figure 36.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/001117

A. CLASSIFICATION OF SUBJECT MATTER

A23L 1/29 (2006.01) A23L 1/305 (2006.01) A61P 1/00 (2006.01) A61P 3/02 (2006.01) A61K 31/593 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPOQUE; WPI, MEDLINE, EPODOC. PUBMED. Arginine, Glutamine, curcumin, Vitamin D, n-3 Fatty acids, enteral, nutritional, Crohn's , Inflammatory Bowel, cancer. colitis and the like

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
16 March 2015

Date of mailing of the international search report
16 March 2015

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2014/001117
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5719134 A (SCHMIDL et al) 17 February 1998 Columns 1, 2, 4, examples & claims.	1, 3, 6-9, 11-33, 36
X	US 2008/0268095 A1 (HERZOG) 30 October 2008 Paragraphs [0046]-[0052], [0071], Examples, Claims.	1-9, 11-19, 36
X	US 2009/0304827 A1 (KIM) 10 December 2009 Whole document, Table 1, Claims.	1-4, 6-9, 11-12, 15, 17-28, 30-36
X	EP 0689835 A2 (AJINOMOTO CO. INC.) 03 January 1996 Page 2-3, Examples and Claims.	1, 2, 6-9, 11-12, 15-28, 30-31, 33-36
X	WO 2002/069964 A1 (ERNEST) 12 August 2002 Pages 2-6, 8, Examples and Claims.	1,2, 6-9, 11-36
X	WO 2008/001086 A1 (SHS INTERNATIONAL LTD) 03 January 2008 Pages 1-8, examples	1,2, 6-9, 11-36

Form PCT/ISA/210 (fifth sheet) (July 2009)

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2014/001117	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
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		EP 2034854 A1	18 Mar 2009
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End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			