

Cytokine Levels After Consumption of a Medicinal *Agaricus blazei* Murill-Based Mushroom Extract, AndoSan™, in Patients with Crohn's Disease and Ulcerative Colitis in a Randomized Single-Blinded Placebo-Controlled Study

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Abstract

Ingestion of the *Agaricus blazei* Murill-based mushroom extract AndoSan™ has been shown in randomized placebo-controlled studies to improve symptoms in Crohn's disease (CD) and ulcerative colitis (UC) and also fatigue and quality of life in the latter patients. The aim was to examine whether this clinical impact of AndoSan™ intake could be explained by influence on foremost pro-inflammatory cytokines in the patients. Fifty patients with symptomatic UC and CD were randomized and blinded for oral daily intake of AndoSan™ or placebo. Blood samples taken before (visit 1) and after 21 days' (visit 3) consumption were analysed for cytokines IL-1 β , IL-2, IL-4-8, IL-10, IL-12-13, IL-17, G-CSF, GM-CSF, IFN- γ , MCP-1, MIP-1 β and TNF- α . Baseline cytokine levels were similar in CD and UC. In CD, cytokine levels at visit 1 versus visit 3 were unaltered within the AndoSan™ and the placebo groups. Only IL-2 was significantly reduced at visit 3 in the AndoSan™ compared with the placebo group. However, when combining IL-1 β , IL-6 and G-CSF in the patients with CD, the cytokine levels were significantly lower in the AndoSan™ - versus the placebo group, visit 3. In UC, levels of IL-2, IL-5 and MIP-1 β were reduced within the AndoSan™ group. IL-5 was also reduced at visit 3 compared with placebo. Generally, the effect on reduction in systemic cytokine levels by consumption of AndoSan™ was limited and supported only marginally anti-inflammatory effects in these patients. Therefore, other explanations behind the clinical anti-inflammatory effects than the contribution of cytokines seem more pertinent, including anti-allergic and antioxidant activities.

Introduction

The mushroom *Agaricus blazei* Murill (AbM) of the *Basidiomycetes* family, naturally growing in the Piedade area outside São Paulo, Brazil, has for centuries been utilized as a food ingredient by the local population who had less prevalent diseases such as atherosclerosis, hyperlipidemia, diabetes and cancer than neighbouring populations [1], presumably owing to consumption of AbM.

The mushroom was introduced to the health food market in Japan in the 1960s and effects of AbM (*Himematsutake*, *jp*) and other *Basidiomycetes* mushrooms such as *Hericium erinaceus* (He) (*Yamabushitake*, *jp*) [2] and

Grifola frondosa (Gf) (*Maitake*, *jp*) [3] have received an increasing research effort.

AbM *per se* and the AbM-based mushroom extract, AndoSan™ (ACE Co. Ltd., Gifu-ken, Japan), composed of AbM (82%), He (15%) and Gf (3%), contain immunomodulatory β -glucans but also other biologically active substances like α -glucans [4], proteoglycans [5], lectins [6], ergosterol (provitamin D2) [7], agaritine [8], isoflavonoids [9], antioxidant substances [10], anti-inflammatory substances such as isolated alkaline and aqueous extracts [11] and the steroid 4-hydroxy-17-methylcisterol (4-HM) [12].

Both AbM and AndoSan™ exhibit as reviewed [13, 14] undoubtedly effects of antitumour, anti-allergic, pro-

inflammatory as well as of anti-inflammatory nature. *In vitro*, AndoSan™ stimulates mononuclear phagocytes to secrete pro-inflammatory cytokines IL-1 β , IL-6, IL-8 and TNF α [15] and, in addition, also the chemokine MIP-1 β [16] in monocyte-derived dendritic cells.

These effects are probably in part mediated by binding of glucans in the extract to Toll-like receptor 2 [17], the dectin-1 receptor [18], the lectin-binding site of CD11b/18 [19] and possibly CR4 CD11c/18 [20]. As, however, AndoSan™ is an extract of the mushrooms' mycelium rather than their fruit bodies, it contains less β -glucan than anticipated from the published data of β -glucan content in the fruit bodies [21]. Therefore, action also of other yet-not-identified immunomodulating substances in the extract must be considered to explain the observed effects. An example is an isolated fraction of AndoSan™ that was found to inhibit the production in macrophages of the tumour-associated and pro-inflammatory protease, legumain [22].

Microarray expression analysis supported the *in vitro* results above as AbM-stimulated promonocytic THP-1 tumour cells [23] demonstrated markedly upregulated genes for IL-1 β , IL-8, moderately for TLR-2 and MyD88, but not for TLR-4. In another *in vivo* study, however, daily AndoSan™ consumption for a week in patients with chronic hepatitis C [24] had no effect on expression of these genes in blood cells.

Stimulation *ex vivo* of whole blood with AndoSan™ resulted in a markedly release, mainly from monocytes, of different types of functional cytokines (pro-inflammatory IL-1 β , IL-6 and TNF α , anti-inflammatory IL-10, chemokines IL-8, MIP-1 β and MCP-1, leucocyte growth factors G-CSF and GM-CSF, pleiotropic IL-7, IL-17, Th-1, IFN γ , IL-2, IL-12, Th-2, IL-4, IL-5 and IL-13) [24]. However, after 12 days' consumption of AndoSan™ (60 ml/day) in 8 healthy volunteers in this study [25], there was a significant reduction in cytokine levels in plasma of IL-1 β , TNF α , IL-6, IL-2 and IL-17, which also indicated an anti-inflammatory effect *in vivo*, when given orally. In addition, in another pilot study [26] after oral consumption of the mushroom extract there was a reduction at day 12 of intracellular levels of reactive oxygen species (mainly superoxide ion) *in vivo* in granulocytes and monocytes, also supporting an anti-inflammatory effect.

In patients with CD and UC, increased mucosal levels have been demonstrated for MIP-1 β , MCP-1, IL-8 [27], IL-1 β [28], IL-6 and TNF α [29]. Cytokine levels in serum are less well studied, but increased levels have been reported for IL-6 [29] and TNF α [30] as well as MIP-1 β [31] also for the latter disease. Moreover, in a recent extensive review [32] persistently elevated cytokines in blood were considered to be IFN γ , IL-6, IL-7 and IL-8 in CD and GRO (chemokine), TNF α and IL-8 in UC, compared with findings in healthy individuals.

Similarly as for healthy individuals [24], in 11 patients with CD and 10 patients with UC who likewise consumed the mushroom extract [33], cytokine levels were reduced in both untreated and in LPS-stimulated blood *ex vivo*. For CD, respective reductions in cytokine levels were for IL-2, IL-8, IL-17 and IL-1 β , MIP-1 β , MCP-1, IL-8, IL-17, G-CSF and GM-CSF, whilst MCP-1 and MIP-1 β , IL-6, IL-1 β , IL-8, G-CSF, MCP-1, GM-CSF were reduced in UC.

On this background, we examined whether a potential decline in pathological levels of cytokines mediated by the mushroom extract *in vivo* could explain the observed beneficial clinical effect in these patients with IBD. In two recent single-blinded randomized placebo-controlled studies in which patients with UC [34] and CD [35] received this mushroom extract (AndoSan™) for 3 weeks, there were clinical improvements, in both diseases, as measured by reduction in symptom score, but also regarding fatigue and HRQoL in UC.

The reported potential AndoSan™ induced reduction in levels of pro-inflammatory cytokines and reactive oxygen species in granulocytes and monocytes from three pilot studies [24, 25, 33] needed further investigation. Therefore, the aim of this study was to ascertain whether cytokine levels were influenced by this mushroom extract in patients participating in these two placebo-controlled clinical studies.

Materials and methods

Inclusion of patients. About 173 patients with CD and 210 patients with UC were phone-interviewed and those with SCDAI- and CAI score of at least 3 were given the opportunity to join the study. At the first attendance, SCDAI and CAI was rerecorded and criteria for exclusion were pregnancy, biological treatment with antibodies to TNF α (adalimumab, infliximab), daily use of more than 5 mg of prednisolone, change in medication and/or consumption of mushroom products from 2 weeks before till end of the study.

Patient characteristics. In CD [35], the patients in the two groups were comparable in most aspects except from more ileal and ileocolic resections in the AndoSan™ group. Comorbidities, exclusively in the AndoSan™ group, were Mb Bechterew in three, diabetes mellitus in one and chronic obstructive lung disease in one patient(s). Combinations of 5-ASA, azathioprine and budesonide or prednisolone were consumed in three and two patients, respectively. Topical treatment with 5-ASA was used in three patients in the AndoSan™ group. In UC [34], the study groups were comparable with respect to details of demographic data and patient characteristics. Eight patients in the AndoSan™ group used several medications, azathioprine and 5-ASA in 4 and azathioprine and prednisolone in one patient(s), respectively. Three patients

used oral 5-ASA combined with rectal enema with budesonide, prednisolone and 5-ASA, respectively. In the placebo group, two patients used azathioprine and 5-ASA and five patients used 5-ASA by oral and rectal route. The medication was unaltered from baseline and throughout the study period, in both groups.

Patient's symptom scoring. The patient-reported symptom score was the simple Crohn's disease activity index (SCDAI) also denoted the Harvey–Bradshaw index [36]. The simple index is based on five graded items; general well-being (very well = 0, slightly below par = 1, poor = 2, very poor = 3, terrible = 4), abdominal pain (none = 0, mild = 1, moderate = 2, severe = 3), number of liquid stools per day (1 = 0, 2 = 1, 3–4 = 2, 5–6 = 3, 7–9 = 4, >9 = 5), abdominal mass (this item was not examined) and extra-intestinal manifestations (arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula, abscess (score 1 per item)). The symptom score ranges from 0 to 21. Scores 3–5 meant mild, 6–9 moderate and over 9 severe disease activity. A criterion for inclusion was a score beyond 2. Mean symptom score at baseline were 5.52 and 5.04, which after 3 weeks decreased to 4.08 ($P = 0.001$) and 4.68 ($P = 0.327$), in the AndoSan™ and placebo group, respectively [35]. In patients with UC, patient-reported symptom score was a modified version of the clinical activity index (CAI), including only the four clinical items and adding one item defining stool consistency (normal = 0, soft = 1, watery = 2) [34, 37]. The modified CAI contained four self-reported items concerning abdominal pain (score 0–3) and stool with regard to frequency (0–4), consistency (0–2) and blood (0–3). The fifth item evaluated by the physician dealt with general well-being (0–3) of the patient. The symptom score ranged from 0 to 15. Mean symptom score at baseline were 5.88 and 5.81, which after 3 weeks decreased to 4.50 ($P = 0.001$) and 5.27 ($P = 0.114$), in the AndoSan™ and placebo group, respectively [34].

Experimental design and randomization. This is a single-centre randomized two-armed patient-blinded study designed to determine in this setting whether daily oral intake of a mushroom extract, AndoSan™, in addition to improved clinical symptoms, fatigue and quality of life in patients with CD or UC during the 21 days' study period, influenced the level of 17 different cytokines, with emphasis on the pro-inflammatory cytokines. Patient evaluation and blood samples were taken before (visit 1, day 0), during (visit 2, day 14) and after (visit 3, day 21) consumption of AndoSan™ (30 ml twice daily), and cytokine analysis from visits 1 and 3 will herein be presented.

Block randomization was done [34, 35] after the phone interview, with uneven and even numbers given for AndoSan™ or placebo, respectively. Fifty patients with CD and UC were randomized, respectively, into 25 and 24 patients in the AndoSan™ group and 25 and 26 patients in

the placebo group. Prospective differences of 20% between the experimental and placebo groups and assumed standard deviation of 20% for the different parameters with a significant level of 5% and a power of 90% ($\beta = 0.10$) demand about 25 patients per randomized arm (calculated in cooperation Oslo Center for Biostatistics and Epidemiology, Oslo University Hospital).

Reagents. The mushroom extract AndoSan™ was provided by the company Immunopharma AS (organization no. 994924273), Oslo, Norway. It was stored at 4 °C in metal cans and used under sterile conditions *ex vivo* and kept sterile until taken by volunteers for *in vivo* experiments. This mushroom extract is a commercial product produced by the company ACE Co. Ltd., Gifu-ken, Japan, for Immunopharma AS. The AbM mixed powder contains per 100 g the following constituents: moisture 5.8 g, protein 2.6 g, fat 0.3 g, carbohydrates 89.4 g, of which β -glucan constitutes 2.8 g and ash 1.9 g. The AndoSan™ extract contains 82.4% of *Basidiomycetes* mushroom derived from AbM (Himematsutake, *jp*), 14.7% from He (Yamabushitake) [2] and 2.9% from Gf (Maitake) [3], and its final concentration was 340 g/l. The amount per litre of the extract was for sodium 11 mg, phosphorus 254 mg, calcium 35 mg, potassium 483 mg, magnesium 99 mg and zinc 60 mg. The LPS content of AndoSan™ was found, using the Limulus amoebocyte lysate test (COAMATIC Chromo-LAL; Chromogenix, Falmouth, MA, USA) with the detection limit 0.005 EU/ml (1 EU = 0.1 ng/ml), to be a miniscule concentration of <0.5 pg/ml. The results from tests for heavy metals were conformable with strict Japanese regulations for health foods. AndoSan™ had been heat-sterilized (124 °C for 1 h) by the producer. Potential radioactivity in the extract was not detected neither by examination by the Norwegian Food Safety Authorities nor during the routine quality control done by Meiji Co, Japan.

The placebo group received an equal volume of colour-like drink with ionized water containing 0.5 ml per litre of caramel colour (E150c) with salt.

Analyses. Blood was harvested from the antecubital vein into glass tubes containing 15 IU heparin per ml or 10 mmol EDTA per ml. The EDTA blood was each time (days 0 and 21) analysed for haemoglobin, haematocrit, mean cellular volume, mean cellular haemoglobin, reticulocytes, immature reticulocytes, leucocytes including a differential count of neutrophils, basophils, eosinophils, lymphocytes and monocytes, thrombocytes, C-reactive protein (CRP), urea, creatinine, bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ -glutamine transferase, alkaline phosphatase and pancreatic amylase. There were [34, 35] no alterations in these blood parameters both in patients with CD and UC throughout the experimental period.

We used the multiplex bead-based sandwich immunoassay performed using Bio-Plex xMAP technology (Bio-Rad, Austin, TX, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, CA, USA), powered using BIO-PLEX MANAGER (version 6.0.1) software (Bio-Rad Laboratories, Hercules, CA, USA) for the analysis of 17 different cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFN- γ , MCP-1, MIP-1 β and TNF α) following manufacturers' instructions and detailed elsewhere [38]. Briefly, samples were thawed on ice, vortexed and then spun down at 10 000 $\times g$ for 10 min at 4 °C before dilution and further processing. The STATLIA software package (ver. 3.2, Brendan Scientific Carlsbad, CA, USA), incorporating a weighted, five-parameter logistic curve-fitting method, was used to calculate sample cytokine concentrations. Controls were used to validate interassay variation. One test of each sample (visits 1 and 3) was run on the same plate, thereby avoiding intra-assay variation. To optimize the assay for low level detection, the cytokine analysis process included (1) a standard point in addition to the vendor's recommendation and (2) use of a magnetic plate washer to yield more reliable results. There was an interassay coefficient of variation, usually from 10 to 30%, but more than 50% in one plate in CD. Therefore, the results on within-group and intergroup cytokine levels were also presented as a relative index, using median and range values for each cytokine at visit 3 relative to visit 1 (baseline) where the index was defined as 1 for each cytokine from each participant. In addition, to validate the results the analyses on cytokine levels in patients with UC were performed a second time, yielding comparable results. Accordingly, we used the data from the initial cytokine analyses. Baseline for upper out of range levels were given as the highest measurable cytokine concentration, whilst undetectable levels were set to 0 pg/ml that excluded the possibility of comparing the results using relative indices. Number of cytokines in upper out of range levels at baseline for both CD ($n = 50$) and UC ($n = 50$) were 30% for MIP-1 β , but <2% for the remaining 16 cytokines.

Statistical analysis. Data are presented as median and range values. Wilcoxon test and Mann–Whitney U -test were used for within-group or intergroup analysis, both within and between each disease, respectively. P values below 0.05 were considered statistically significant. The SPSS statistical program for the social sciences, version 23 (IBM), was used in the analyses.

Ethical considerations. The study was approved on 8 April 2011, by the regional ethics committee (REC – South East Norway, ref. 2011/404) and followed the guidelines of the Helsinki declaration. The participants were informed and signed a written consent for participation, including the option of study withdrawal. The patients were recruited and followed up at the Department of Medicine, Oslo

University Hospital, Ullevål, Norway, in the period of June 2012 to May 2014. The study was registered with unique protocol ID AbM2012-IBD and clinical trials gov ID NCT 01496053 (15 December 2011). The authors confirm that all ongoing and related trials for this drug/intervention are registered.

Results

Plasma cytokine levels

When measuring baseline plasma cytokine levels at visit 1, before intake of AndoSan™ or placebo for 21 days in the patients with CD (Table 1) and UC (Table 2), there were comparable cytokine levels in the respective groups. In addition, baseline cytokine levels were comparable in these two inflammatory bowel diseases. As there was a considerable interassay variation in cytokine levels, we chose to use indices of cytokines at visit 3 presented as a relative index with the value 1 at visit 1 for each patient. Hence, such normalization of data by use of median and range relative indices for all patients at visit 3, differences in cytokine levels both within each group from visits 1 to 3 and between the groups at visit 3 could be accurately determined. In CD, there was only a reduction at visit 3 of IL-2 in the AndoSan™ group compared with the placebo group (Table 3). In UC, IL-2, IL-5 and MIP-1 β levels were slightly reduced in the AndoSan™ group from visits 1 to 3 (Table 4). However, in UC IL-5 was the only cytokine also being reduced at visit 3 in the AndoSan™ compared with the placebo group (Table 4).

Table 1 Baseline cytokine values for 50 patients with CD in the AndoSan™ and placebo groups.

CD	AndoSan™ ($n = 25$)	Placebo ($n = 25$)
Analyte	V1	V1
IL-1 β	9.66 (0–67.4)	8.81 (2.6–142.4)
IL-2	35.6 (0–166.1)	16.1 (0–345.3)
IL-4	2.15 (0.23–5.98)	1.90 (0.65–15.5)
IL-5	0.70 (0–17.6)	0.57 (0–6.18)
IL-6	105.1 (11.8–1331.7)	118.2 (23.8–8464.5)
IL-7	4.18 (0.57–41.0)	2.37 (0–23.7)
IL-8	539.5 (49.1–7114.1)	622.7 (214.9–7114.1)
IL-10	21.8 (0–864.1)	14.9 (0–678.1)
IL-12	17.6 (6.35–1426.2)	21.3 (5.5–12671.5)
IL-13	3.15 (0–52.6)	2.57 (0–17.4)
IL-17	175.1 (35.6–1193.8)	162.2 (71.6–252.7)
G-CSF	112.9 (9.2–2008.7)	85.1 (50.3–434.3)
GM-CSF	124.8 (0–420.5)	116.7 (0–285.8)
IFN- γ	85.9 (0–377.9)	52.4 (17.9–397.6)
MCP-1	224.7 (31.6–1359.0)	225.1 (31.2–2366.9)
MIP-1 β	2354.9 (169.5–5622.2)	2669.2 (398.7–5622.2)
TNF- α	53.0 (7.9–219.8)	42.6 (11.8–1434.4)

Median (range) cytokine levels are given as pg/ml.

Moreover, as in CD a combination of all pro-inflammatory, chemokine and growth factor analytes tended to decrease when comparing levels at visit 3 for AndoSan™ and placebo groups, combinations of 8–3 of these analytes were further compared (Table 5). Exclusively for IL-1 β , IL-6 and G-CSF, there was a significantly reduced level ($P = 0.048$) in the AndoSan™ (0.79 (0.04–22.2)) relative to the placebo group (1.19 (0.02–35.6)).

Table 2 Baseline cytokine values for 50 patients with UC in the AndoSan™ and placebo groups.

UC	AndoSan™ ($n = 24$)	Placebo ($n = 26$)
Analyte	V1	V1
IL-1 β	10.2 (1.98–482.2)	13.3 (0–348.0)
IL-2	23.2 (0–243.7)	12.9 (0–339.1)
IL-4	1.79 (0.52–6.25)	1.85 (0–5.41)
IL-5	0.52 (0–3.43)	0.62 (0–3.02)
IL-6	162.8 (28.5–8574.4)	320.4 (18.1–8574.4)
IL-7	2.77 (0–10.3)	4.00 (0–26.5)
IL-8	761.2 (91.6–5235.9)	1063.7 (71.6–3231.6)
IL-10	22.9 (1.79–421.2)	27.8 (0–73.5)
IL-12	18.8 (2.74–398.7)	21.1 (6.94–166.0)
IL-13	2.25 (0–13.5)	3.17 (0–8.45)
IL-17	153.1 (33.5–355.2)	154.8 (7.61–306.3)
G-CSF	131.2 (0–329.4)	114.2 (0–274.0)
GM-CSF	87.6 (12.0–295.7)	115.5 (0–362.4)
IFN- γ	49.0 (11.1–292.0)	69.6 (17.7–191.3)
MCP-1	295.3 (83.0–7840.0)	395.5 (0–3337.0)
MIP-1 β	2669.2 (381.9–5901.0)	2759.5 (95.1–5900.9)
TNF- α	41.5 (20.0–4748.6)	60.5 (6.27–3294.4)

Median (range) cytokine levels are given as pg/ml.

Subgroup analyses

When the patients at baseline were dichotomized into symptom scores ≤ 5 (CD: $n = 30$, UC: $n = 27$) and ≥ 6 (CD: $n = 20$, UC: $n = 23$) for both diseases and compared with cytokine levels in these two groups, neither positive nor negative correlation was found between degree of symptom score and cytokine levels (data not shown).

The disease locations for CD patients were ileal (8 versus 11), ileocolic (12 versus 9) and colic (5 versus 5) in the placebo and AndoSan™ groups, respectively. In the placebo group, we found significant differences upon disease locations for the relative cytokine values (at visit 3) for IL-6, IL-8, IL-10, IL-17, GM-CSF, IFN- γ and MCP-1. No such changes were found in the AndoSan™ group. When comparing these subgroups on behalf of intervention, we found significant lower relative values in the AndoSan™ group for the cytokines IL-2 ($P = 0.028$), IL-5 ($P = 0.026$), IL-6 ($P = 0.020$), IL-10 ($P = 0.036$) and IFN- γ ($P = 0.020$), for the ileal disease location. No significant changes were found in the other two disease locations (ileocolic and colic), neither within nor between the study groups.

Corresponding distribution for UC were rectosigmoidal (7 versus 5), left colon (10 versus 6) and entire colon (9 versus 13), respectively. We found no significant differences for the relative cytokine levels at visit 3 when comparing the different disease locations, and neither also when comparing the subgroups regarding intervention (placebo versus AndoSan™).

In CD, 17 and 14 patients had inflammatory and 8 and 11 patients had stenotic disease presentation in the placebo

Table 3 Index values in patients with CD comparing cytokine levels at visits 1 and 3 within and at visit 3 between the study groups.

CD Analyte	AndoSan™			Placebo			
	No	Index V3	P_{V1V3}	No	Index V3	P_{V1V3}	$P_{AvsP V3}$
IL-1b	23	0.70 (0–10.8)	0.951	25	1.10 (0.06–12.0)	0.192	0.227
IL-2	23	0.79 (0–11.7)	0.068	19	1.37 (0–6.43)	0.070	0.025
IL-4	25	0.92 (0.08–8.52)	0.300	25	0.99 (0–3.66)	0.798	0.607
IL-5	19	0.92 (0–1.58)	0.309	22	1.14 (0–3.81)	0.833	0.388
IL-6	25	0.75 (0.08–22.2)	0.510	25	1.19 (0.02–35.6)	0.143	0.190
IL-7	25	1.04 (0–5.18)	0.904	22	1.17 (0–2.55)	0.123	0.291
IL-8	25	0.81 (0.03–3.30)	0.619	25	0.85 (0.12–5.58)	0.798	0.900
IL-10	20	0.80 (0–2.48)	0.502	23	1.43 (0–8.73)	0.128	0.301
IL-12	25	1.01 (0.08–4.78)	0.968	25	1.00 (0.30–5.23)	0.841	0.930
IL-13	24	0.93 (0–3.76)	0.808	24	1.05 (0–3.16)	0.549	0.726
IL-17	25	0.93 (0.07–5.71)	0.619	25	1.03 (0–2.29)	0.946	0.900
G-CSF	25	0.87 (0.05–15.0)	0.527	25	1.07 (0–2.15)	0.677	0.594
GM-CSF	21	0.95 (0–1.58)	0.289	23	0.86 (0–1.78)	0.073	0.630
IFN- γ	24	0.91 (0.11–6.13)	0.121	25	1.13 (0.05–3.73)	0.459	0.308
MCP-1	25	0.65 (0.08–3.89)	0.737	25	0.81 (0.10–9.69)	0.527	0.946
MIP-1b	25	0.82 (0.22–9.15)	0.758	25	1.00 (0.07–2.98)	0.911	0.712
TNF-a	25	0.82 (0.06–5.67)	0.946	25	0.97 (0.04–4.16)	0.819	0.734

No = number of patients with detectable analytes included.

Table 4 Index values in patients with UC comparing cytokine levels at visits 1 and 3 within and at visit 3 between the study groups.

UC Analyte	AndoSan™			Placebo			
	No	Index V3	P_{V1V3}	No	Index V3	P_{V1V3}	$P_{AvsP V3}$
IL-1 β	24	0.76 (0.02–39.2)	0.587	25	0.52 (0.05–20.9)	0.778	0.509
IL-2	19	0.45 (0–3.37)	0.048	19	0.84 (0–2.81)	0.322	0.145
IL-4	24	1.05 (0.28–3.48)	0.424	25	0.87 (0.56–1.56)	0.376	0.312
IL-5	15	0.55 (0–2.17)	0.041	20	1.18 (0–3.32)	0.145	0.017
IL-6	24	0.68 (0.01–71.8)	0.855	26	0.60 (0.03–19.3)	0.316	0.641
IL-7	22	0.95 (0.23–2.76)	0.408	23	0.86 (0–6.35)	0.709	0.716
IL-8	24	1.04 (0.11–6.43)	0.503	26	0.77 (0.20–3.90)	0.732	0.449
IL-10	24	0.90 (0.17–6.69)	0.440	25	0.85 (0.24–3.26)	0.459	0.603
IL-12	24	1.00 (0.38–5.30)	0.607	26	1.13 (0.46–2.54)	0.101	0.600
IL-13	22	1.06 (0–3.28)	0.601	25	1.16 (0–3.65)	0.158	0.529
IL-17	24	1.01 (0.37–4.53)	0.511	26	1.01 (0.55–11.8)	0.292	0.900
G-CSF	23	0.85 (0.19–5.19)	0.987	25	1.09 (0.33–3.29)	0.158	0.489
GM-CSF	24	0.87 (0.14–3.52)	0.753	24	0.88 (0–2.16)	0.209	0.592
IFN- γ	24	0.93 (0–5.49)	0.886	26	0.87 (0.46–3.37)	0.258	0.846
MCP-1	24	0.94 (0.06–12.6)	0.607	25	0.73 (0.15–3.13)	0.061	0.289
MIP-1 β	24	1.00 (0.20–6.49)	0.043	26	1.00 (0.36–6.75)	0.123	0.435
TNF- α	24	1.03 (0.01–11.6)	0.909	24	0.78 (0.02–7.73)	0.819	0.207

No = number of patients with detectable analytes included.

Table 5 Comparison of combinations of cytokines between the AndoSan and placebo groups in CD.

Combinations of cytokines	Relative index at visit 3 AndoSan™	Placebo	P_{AvsP}
IL-1 β , IL-6, IL-8, TNF α + G-CSF, GM-CSF, MCP-1, MIP-1 β ($n = 8$)	0.82 (0.04–22.2) [$n = 192$]	1.00 (0.02–35.6) [$n = 192$]	0.105
IL-1 β , IL-6, IL-8, TNF α + MCP-1, MIP-1 β ($n = 6$)	0.80 (0.04–22.2) [$n = 147$]	1.00 (0.02–35.6) [$n = 150$]	0.162
IL-1 β , IL-6, G-CSF ($n = 3$)	0.79 (0.04–22.2) [$n = 72$]	1.19 (0.02–35.6) [$n = 73$]	0.048

and AndoSan™ groups, respectively. When comparing the relative cytokine values at visit 3, it was only for IL-6 in the placebo group a difference ($P = 0.049$) between inflammatory and stenotic disease (1.65 versus 0.76). It was only for IL-2 with inflammatory disease presentation a difference (1.37 versus 0.68) in favour of the AndoSan™ group ($P = 0.019$).

For the CD patients, 14 versus 11 in the placebo group and 18 versus 7 in the AndoSan™ group used medication versus no medication, respectively. Numbers of patients with bowel resections were six in the placebo group and 9 in the AndoSan™ group. There were no significant differences, within or between the study groups, when comparing the relative cytokine values at visit 3 upon resection or medication status.

For UC, only three out of 50 patients, two in the placebo group and one in the AndoSan™ group did not use any medication during the study period, and thereby no analyses were done.

Discussion

Recently, the *Agaricus blazei* Murill-based mushroom extract AndoSan™ [14] has been shown to exert a clinical beneficiary effect in especially UC but also CD. The main finding in the current cytokine examination in this

placebo-controlled patient-blinded prospective study was that AndoSan™ did largely not influence the plasma levels of the 17 different cytokines analysed throughout the experimental period. At best, a marginal systemic anti-inflammatory effect for patients consuming AndoSan™ may be interpreted from the results.

The baseline cytokine levels between the two inflammatory diseases were actually quite comparable and for both conditions pro-inflammatory analytes were increased when further compared with normative data [30, 32, 33].

The 17 cytokines analysed covered a wide functionality being of Th1-type (IL-2, IFN- γ , IL-12), Th2-type (IL-4, IL-5, IL-13), chemokines (MCP-1, MIP-1 β , IL-8), pro-inflammatory (IL-1 β , IL-6, IL-8, TNF α), leucocyte growth factors (G-CSF, GM-CSF), anti-inflammatory (IL-10) as well as Th17-type (IL-17) responses. Due to variations in interassay coefficients both within and between the groups, cytokine levels were compared using a relative index that eliminated these variations and thereby secured the validity of the results.

Because we used the same sample-dilution analysing the 17 different cytokines of a wide concentration range (e.g. 0.5–2669 pg/ml), measurements of high and low cytokine concentrations became less accurate. However, except from IL-2 (20%) and IL-5 (24%) in the lower range, and MIP-1b (30%) in the upper range, this inaccuracy must have been

limited as remaining cytokines were detectable in about 98% of the measurements. Reduced compliance in carrying out the study, with missing or incorrect oral intake of AndoSan™ or placebo, may be a possible source of error, even though this was not the impression in conversation with patients during and after the study period of 3 weeks.

There was an all-over trend in CD of lower levels for pro-inflammatory, chemokine and growth factor analytes after AndoSan™ compared with consumption of placebo. However, only when combining alterations in IL-1 β , IL-6 and G-CSF from visit 1 to visit 3 in CD, there was a significant reduction in the AndoSan™ group, implying a marginal anti-inflammatory effect. With the exception of IL-2 in CD and IL-2, IL-5 and the chemokine MIP-1 β in UC, all other within or intergroup comparisons were insignificant, also when comparing between the diseases. However, contrary to the AndoSan™ group, in subgroup analysis in CD patients of the placebo group, there was an increased level of seven cytokines (IL-6, IL-8, IL-10, IL-17, GM-CSF, IFN- γ and MCP-1) in ileal disease compared with colic disease and ileocolic disease. We also found significantly lower cytokine levels in the AndoSan™ group for ileal disease for IL-2, IL-5, IL-6, IL-10 and IFN- γ compared with placebo. It can be inferred from these results that AndoSan™ exerts an anti-inflammatory effect.

In this study, we did not compare cytokine levels between patients with IBD and healthy individuals. However, elevated levels in 10 out of these 17 cytokines in patients with IBD (IL-2, IL-6, IL-8, IL-12, IL-17, TNF α , IFN- γ , MCP-1, GM-CSF and MIP-1 β) have been reported [33]. Interestingly, the reduction in elevated levels of IL-2, MIP-1 β and IL-6 combined with MIP-1 β and G-CSF (Table 5) may contribute to the pathogenesis in the patients with IBD. Despite the fact that a marginal anti-inflammatory effect was present in CD, the more pronounced clinical effect, comprising symptoms, fatigue and HRQoL, was found in the patients with UC. Thus, we were not able to demonstrate same degree of anti-inflammatory effect as previously indicated by the decline in plasma cytokines in the two similar pilot studies [24, 33] without control groups.

In a randomized placebo-controlled clinical study in 57 elderly females, there was no difference in level of the chosen cytokines IL-6, TNF- α and IFN- γ after daily consumption of the AbM dry extract (900 mg) or placebo (600 mg) for 60 days [39]. Thus, AbM had no modulating effect on the levels of these classical pro-inflammatory cytokines, which accords with our data regarding these cytokines. In conclusion, at best, a potential marginal systemic anti-inflammatory effect in CD and UC cannot be ruled out as the aforementioned reduction in cytokine levels in CD and UC exclusively was in the patient group consuming AndoSan™. Moreover, there was neither a positive nor a negative correlation between dichotomized symptom scores (≤ 5 versus ≥ 6) and cytokine levels. This

supported that the anti-inflammatory effect in UC and CD, translated into the reported clinical effects in these IBD patients [34, 35], also must be explained by other mechanisms: Firstly, although antioxidant effect was not particularly demonstrated in patients with UC and CD (data not shown), levels of ROS (mainly superoxide ion/ O_2^-) in granulocytes and monocytes harvested from eight healthy volunteers consuming AndoSan™ (60 ml/day) for 12 days were reduced, supporting such an effect [25]. Secondly, several *in vitro* and *in vivo* studies in rodents [14] have concluded that an anti-inflammatory effect also can be explained by other parameters than cytokines. The inhibitory effect of an isolated carbohydrate fraction of AndoSan™ [22] on the tissue degrading pro-inflammatory and tumour-associated enzyme, legumain (asparaginyl endopeptidase) *in vitro*, which probably activates proMMP and processing of cathepsins, may also be valid *in vivo* and also contribute to less pro-inflammatory activity in the patients with IBD. Alkaline and aqueous substances isolated from AbM [11] had, when given orally to rats for 1–2 weeks, several anti-inflammatory effects. These included improved healing of stress-induced ulcers, reductions in paw oedema in the presence of nystatin or Freund's adjuvant, as well as reduced neutrophil migration to the peritoneal cavity, that in part was supposedly related to downregulating the immune system by interactions with β -glucans of the extract. In a recent study [40], a water-soluble polysaccharide isolated from AbM that was given orally for 8 weeks to ovariectomized and osteopaenic rats, markedly decreased ICAM-1, cyclooxygenase-2, inducible nitric oxide synthetase and total antioxidant status. Moreover, AbM contains absorbable low molecular weight antioxidant substances [10], which downregulate the levels of reactive oxygen species (ROS) *in vitro*.

There also was an anti-allergic effect in mice sensitized to ovalbumin (OVA), as demonstrated by reduction in specific anti-OVA IgE antibodies, both when AndoSan™ was given before or after the OVA immunization [41]. Additionally, in this allergy model there was an increase in Th1 relative to Th2 cytokines in spleen cell cultures *ex vivo* obtained from the animals treated with AndoSan™.

In agreement with our findings, it has been shown in mice with colitis, induced by dextran sulphate, that dietary intake of the mushroom *Agaricus bisporus* [42], rich in flavonoids, as well as β -glucans from some yeasts [43], can have strong modulating effect on intestinal inflammation.

Although it has been speculated that CD is Th1 (IL-2, IFN γ , IL-12) and that UC is a Th2 (IL-4, IL-5, IL-13)-related disease based on the Th1/Th2 dichotomy [26], the cytokine data presented in this investigation actually show comparable baseline cytokine levels, thereby not clearly supporting this paradigm. This is also supported by previous [33] cytokine measurements where the same cytokines were elevated (TNF α , IFN γ , IL-2, IL-6, IL-8, IL-12, IL-17, MCP-1, GM-CSF, MIP-1 β) or reduced (IL-7) in

CD and UC compared with healthy individuals. On the other hand, regarding the Th1/Th2 profile, there actually was a reduction, only, in the Th1 cytokine IL-2 in CD and the Th2 cytokine IL-5 in UC in the AndoSan™ group, respectively. Since 3 weeks of AndoSan™ in fact helped ameliorate symptoms, the Th1/Th2 dichotomy may eventually have merit in IBD. A possible follow-up of symptom scoring of the patients with IBD included in the current clinical study could tell whether the mushroom extract supplementation gave prolonged clinical effects.

In conclusion, in this placebo-controlled clinical study consumption of AndoSan™ influenced cytokine levels, marginally for UC and moderately for CD, supporting a weak systemic anti-inflammatory effect. Therefore, the reported beneficiary clinical effects mediated by AndoSan™ for these patients probably also involve other anti-inflammatory compounds, not examined.

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Author contributions

SPT participated in recruitment of patients, cytokine analysis, draft and writing of manuscript. EJ participated in experimental design, draft and writing of manuscript. GH participated in experimental design and cytokine analysis. IL participated in basic clinical recruitment of patients and review of manuscript. TL participated in cytokine analysis and review of manuscript.

Declaration of commercial interest

Two of the authors (GH and EJ) have patent/patent applications and financial interests relating to material (AndoSan™) pertinent to this article: (1) WO2005065063 A2, Appl. No.: 10/585600, NO- and PCT-filed January 2004 and January 2005, respectively, by inventor Hetland, Geir, and (2) NO20090003383, Appl. No.: 20090003383 20091119, by inventors Hetland Geir and Johnson Egil and filed by Applicant Immunopharma AS in November 2009 and financial interest of Geir Hetland as shareholder in Immunopharma AS of Norway, commercializing AndoSan™. This does not alter the authors' adherence to the Scandinavian Journal of Immunology policies on sharing data and materials.

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