Clinical Effects and Cytokine Responses from Ingestion of AndoSan™
in Patients with Ulcerative Colitis and Crohn’s Disease
A Randomized Placebo Controlled Study

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Preface and acknowledgments

The present work was performed between 2012 and 2017 at the Departments of Gastrointestinal and Pediatric Surgery, Medicine, and Medical Biochemistry, Oslo University Hospital, Ullevål. During this period I was a research fellow from 2012–14 at Institute of Clinical Medicine, University of Oslo, followed by a position as registrar from 2014–17 at the Department of Gastrointestinal and Pediatric Surgery, Oslo University Hospital, Ullevål, where my work was equally divided between research and patient care.

This thesis builds upon the work of many others. Dr. Bernardshaw (2005) and Dr. Førland (2011) did their thesis on the same medicinal mushroom *Agaricus blazei* Murill and the mushroom extract AndoSan™, and their thorough research forms the basis for my work.

Professor Egil Johnson has been my supervisor. I am forever grateful for his advice, corrections, and constructive feedback throughout the research process. Without his support and patience, this project would not have been completed. It has been a pleasure having him as a mentor with all his academic and surgical experience, together with his enthusiasm and good mood.

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I would also like to thank Dr. Bjørn Atle Bjørnbeth, the previous head of the Department of Gastrointestinal and Pediatric Surgery, and Dr. Tom Glomsaker the present head of our Department, for all the support in my academic and surgical career.

And most importantly, special thanks to my dear family - Jannicke, Ida, Silje and Kaja, for continuously reminding me of the most important things in life.
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Oslo, April 2017

Stig Palm Therkelsen
List of papers

Effect of a Medicinal Agaricus blazei Murill-Based Mushroom Extract, AndoSan™, on Symptoms, Fatigue and Quality of Life in Patients with Ulcerative Colitis in a Randomized Single-Blinded Placebo Controlled Study.

Effect of the Medicinal Agaricus blazei Murill-Based Mushroom Extract, AndoSan™, on Symptoms, Fatigue and Quality of Life in Patients with Crohn's Disease in a Randomized Single-Blinded Placebo Controlled Study.

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.
Abbreviations

AMP: antimicrobial peptide
APC: antigen-presenting cell
CLR: C-type lectin receptor
CR: complement receptor
DAMP: damage-associated molecular patterns
DC: dendritic cell
Dectin-1: dendritic-cell-associated C-type lectin-1
EWAS: epigenome-wide association studies
G-CSF: granulocyte colony-stimulating factor
GALT: gut-associated lymphoid tissue
GM-CSF: granulocyte-monocyte colony-stimulating factor
GWAS: genome-wide association studies
IEC: intestinal epithelial cell
IFNγ: interferon γ
IL: interleukin
ILC: innate lymphoid cell
M cell: microfold cells
MAMP: microbe-associated molecular patterns
MCP-1: monocyte chemotactic protein-1
MHC: major histocompatibility complex
MIP-1β: macrophage inflammatory protein - 1β
MMP: matrix metalloproteinase
NLR: NOD-like receptor
NOD: nucleotide-binding oligomerization domain
NF-κB: nuclear transcription factor – kappa B
PAMP: pathogen-associated molecular patterns
PRR: pattern recognition receptor
ROS: reactive oxygen species
TCR: T cell receptor
TLR: Toll-like receptor
TNFα: tumor necrosis factor α
UPR: unfolded protein response
General introduction

Mushrooms are macrofungi with a distinctive fruiting body and are large enough to be seen with the naked eye. Most of the macrofungi belong to the class Basidiomycetes, but there are also others from the class Ascomycetes. The number of existing mushroom species in nature is estimated at approximately 10,000, from 550 genera and 80 families, of which about 10% are likely to be edible, and perhaps only 10% of the named species are known to science [1-4]. Out of these, approximately 700 species have been found to be medicinally useful [5, 6].

Humans have used mushrooms in their food since ancient times, with the oldest archaeological record dating from 3500 BC. For many centuries mushrooms were used as nutrients in the human diet, as agents of fermentation in the production of food and drink, and finally as medicine [5]. Mushrooms also have a nutritional value as a potential source of carbohydrates, proteins, amino acids, and minerals.

Medicinal mushrooms and fungi are thought to possess approximately 130 medicinal functions, including immunomodulation, antioxidant, radical scavenging, cardiovascular, antihypercholesterolemic, antiviral, antibacterial, antiparasitic, antifungal, detoxification, hepatoprotective, and antidiabetic effects [7]. Many, if not all, higher Basidiomycetes mushrooms contain biologically active compounds in fruiting bodies, cultured mycelium, and cultured broth. Special attention has been paid to the mushrooms’ bioactive polysaccharides and polysaccharide-protein complexes described to enhance innate and cell-mediated immune responses in animals and humans [7]. Modern clinical practice in Japan, China, Korea, Russia, and several other countries rely on mushroom-derived preparations in the treatment of patients [8-10]. In Japan, *Agaricus blazei* Murill (AbM) is used by an estimated 500,000 people and is the most popular complementary and alternative medicine taken by cancer patients [11]. Nowadays, medicinal mushrooms are used as dietary food and as dietary supplement products. The world mushroom production was 30 million metric tons in 2012 [12, 13], and the market of medicinal mushrooms as dietary supplement products is quickly growing. It has a value of more than 18 billion US dollars per year [14], including use as “mushroom pharmaceuticals”, natural bio-control agents in plant protection, and in cosmeceutical industry. Mushrooms are currently evaluated for their nutritional value and acceptability, as well as for their pharmacological properties. In particular, and
most importantly for modern medicine, medicinal mushrooms represent an unlimited source of polysaccharides (especially β-glucans) and polysaccharide-protein complexes with immunomodulating properties [10, 15]. Furthermore, higher Basidiomycetes mushrooms also contain biological high- and low-molecular-weight compounds (triterpenes, lactones, alkaloids, and other compounds) in fruiting bodies, cultured mycelia, and cultured broth [10, 15, 16].

Historically, it is a fact that substances for medicinal use have emerged from components extracted from mushrooms. Prominent examples are the immunosuppressive drug, cyclosporine A, isolated from the fungi Tolypocladium inflatum and penicillin isolated from Penicillium notatum [17, 18]. One of the natural compounds with immunomodulating properties that have attracted considerable interest are β-glucans, a group of branched glucose polymers [19].

AndoSan™ is an extract prepared from edible, medicinal Basidiomycetes mushrooms, mainly Agaricus blazei Murill, but it also contains Hericium erinaceus and Grifola frondosa, all of which have immunomodulating properties. The commercial dietary supplement AndoSan™ that we used in this study is a sterilized mixture from the mycelia of these three mushrooms as described in the following.

Hericium erinaceus (He) (Lion’s Mane Mushroom, Bearded Tooth Mushroom, Hedgehog Mushroom, Satyr’s Beard, Bearded Hedgehog Mushroom, pom pom mushroom, Bearded Tooth Fungus, and Yamabushitake (jp.)) has a long history of use in traditional Chinese medicine, an edible delicacy that is one of the famous four dishes in China. Recent studies demonstrates antibiotic properties (against MRSA [20], Salmonella typhimurium [21], Helicobacter pylori [22]), antioxidant [23], immunoregulatory [24, 25] and anticancer effects [26]. He contains a number of polysaccharides, such as β-glucan, heteroglucans, heteroxylans, as well as several cyanthane derivate triterpens known as hericenone and erinacine [27]. He is a good source of exogenous antioxidants with promising results on oxidative stress-related neurological diseases, such as Alzheimer’s disease. Erinacines and hericenones stimulate the release of nerve growth factor in rat brains and cultured nerve brain tissue [28]. He has free-radical-scavenging activity, including reducing power ability, chelating effects on ferrous ions, 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) free-radical-scavenging activity, β-carotene bleaching, and inhibition of lipid peroxidation [29, 30].

Grifola frondosa (Gf) (Hen of the Woods, Ram’s Head, Sheep’s Head, signorina, and Maitake (jp.)) is one of the most popular mushrooms in traditional Chinese medicine,
where it among others has been used as a remedy for pain and inflammation [31].

Extracts from the fruiting body or liquid-cultured mycelium of *Gf* has been shown to exert antitumor, antimutagenic, antihypertensive, antidiabetic, hypolipidemic effects, as well as increased synthesis of collagen in mice [32-35]. The fruiting body of this mushroom is rich in β-glucans. In a recent *in vivo* rat model of inflammatory bowel disease (IBD), oral administration of a water extract of *Gf* (GFW) for five days (1g/kg per day) demonstrated suppressed production of TNFα as well as its signaling through NF-κB leading to the expression of inflammatory chemokines, MCP-1 and IL-8. The results from this study also indicated that GFW contains strong antioxidant components that inhibit production of reactive oxygen species (ROS) by TNFα, and thus, ultimately suppress the TNFα-induced recruitment of leukocytes to epithelial cells, thereby suggesting GFW as an alternative medicine for IBD [36].

*Agaricus blazei* Murill (AbM) (fig. 1) is an edible mushroom naturally growing in the coastal Piedade rain forest area near the city of São Paulo, Brazil, and has for centuries been utilized as a food ingredient in the normal diet by the local population who were found to have less prevalent diseases such as atherosclerosis, hepatitis, hyperlipidemia, diabetes, viral infections and cancer than did neighbouring populations, presumably owing to constant consumption of AbM in their normal diet [6]. AbM was found to be particularly rich in different forms of β-glucans, such as β-(1→3), β-(1→4)-, and β-(1→6)-glucans [37, 38]. These glucans, which are an integral part of the cell wall of mushrooms, exhibit immunomodulatory effects on monocytes, macrophages, and natural killer (NK) cells [39-41]. In addition to β-glucans, the mushroom’s effect on the immune system is believed to be due to other biologically active substances like α-glucans [42], proteogluclans [37], lectins [43], ergosterol (provitamin D2) [44], riboglucan [45], glucomannan [45], sodium pyroglutamate [46], blazein [47], agaritine [48], isoflavonoids [49], antioxidant substances [50], anti-inflammatory substances such as isolated alkaline and aqueous extracts [51], and the steroid 4-hydroxy-17-methylincisterol (4-HM) [52]. Cellular and animal research has shown that AbM may stimulate the production of cytokines, such as interferons and interleukins [41]. AbM is known to have antiviral properties in cell culture [53, 54]. However, except for a brief pilot study in patients with chronic hepatitis C virus (HCV) infection where AbM (AndoSan™) had a small but non-significant reduction in serum HCV levels, the ability to inhibit viruses in the human body has not been studied [55]. Additional research suggests that the mushroom has a beneficial effect on cholesterol [56], hyperglycemia
and improvement of insulin resistance [56-58], and as an adjuvant for vaccines [54, 59-61] as well as inhibition of tumor growth and angiogenesis [46, 62].

Taxonomy and origin of AbM have for many years raised controversies among researchers (Wasser et al. 2002 [63] and 2014 [7] (Agaricus brasiliensis), Kerrigan 2005 [1] and Wisitrassameewong et al. 2012 [45] (Agaricus subrufescens)). Hence, this species is found under different names but is most frequently referred to as Agaricus blazei Murill (sensu Heinemann). Taxonomically this mushroom is classified to the kingdom of Fungi, division Basidiomycetes, order Agaricales, family Agaricaceae, genus Agaricus. AbM is also known as Royal Sun Agaricus, Himematsutake (jp.), Kawariharatake, Agaricus rufotgulis, Songrong, Cogmelo de Dos, and almond mushroom. Agaricus blazei Murill was introduced to the health food market in Japan in the 1960s and effects of AbM and other Basidiomycetes mushrooms such as He [64] and Gf [65] have received an increasing research effort [38].

Inflammatory bowel diseases like ulcerative colitis (UC) and Crohn’s disease (CD) are bothersome conditions of unknown etiology. We have shown in a previous pilot study anti-inflammatory effects by ingestion of AndoSan™ in IBD patients as measured by reduction in pro-inflammatory cytokines and also of fecal calprotectin [66]. On this background, the main task of this study was to determine whether the effect of AndoSan™ could be reproduced clinically and by measurement of cytokines in a prospective and randomized study.

**Composition of AbM and the mushroom extract AndoSan™**

The mushroom extract AndoSan™ was provided by the company Immunopharma AS (organization no. 994924273), Oslo, Norway, and produced by the company ACE CO. LTD., Gifu-ken, Japan. The commercial manufacturing processes of this mushroom extract are GMP (Good Manufacturing Practices) certified, with established and identified optimal growth conditions (e.g., substrate, temperature, fermentation, and timing) and extraction processes. Its complex and time-consuming production process guarantees a formula that is safe and consistent. 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.

The AbM mixed powder contains per 100 g the following constituents (specified by the manufacturer): 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. AndoSan™ is a mushroom extract from the mycelia of three different Basidiomycetes and contains 82.4% from AbM 14.7% from He and 2.9% from Gf, and its final concentration was 340 g/l. The amount per liter 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 amebocyte lysate test (COAMATIC Chromo-LAL; Chromogenix, Falmouth, MA, USA) with detection limit 0.005 EU/ml (1 EU = 0.1 ng/ml), to be a minuscule concentration of <0.5 pg/ml. The concentrations of heavy metals were conformable with strict Japanese regulations for health foods. AndoSan™ had been heat-sterilized (124 °C for 1 h) by the producer and quality controlled by an independent company, Meiji Co, Japan.

The fruiting body of AbM (fig. 2) is particularly rich in proteoglucans and different forms of the β-glucans [37, 38]. The main structure of β-glucans in AbM is a β- (1→3)-backbone with β- (1→6)-side branches. Differences in biological activity of β-D-glucans could be correlated to solubility in water, the size of the molecules, branching rate and form, and the β- (1→6) bounding system in the β- (1→3) major chain [67]. Kept together by hydrogen bounding, the three β- (1→3)-D-polymers with β- (1→6)-D-branches form a triple helical structure that links covalently to chitin in the cell wall, forming an insoluble complex in an alkaline milieu (fig. 3) [68]. A reduction in pH alters this triple confirmation into a single helical and random coil structure [69]. The host’s immune responses to biological response modifiers (BRM), such as β-glucans, are related to their structural composition.
Figure 1. The medicinal mushroom, *Agaricus blazei* Murill

Figure 2. The basic structure of β-glucans of AbM

β-Glucans comprise a major component of many fungal cell walls and occur mainly in linear (β-(1→3)) or branched (β-(1→6)) forms. This figure is a modified version of two figures (used with permission) from Dag T Førlands thesis “Studies on a medicinal Agaricus blazei Murill based mushroom extract” (2011).
Figure 3. Structure of the outer wall of fungi including β-glucans and receptors

The figure shows the principal layers from the cell wall of mushrooms with key receptor-ligand interactions, also including receptors with unknown ligands. CR3, complement receptor 3; DC-SIGN, DC-specific ICAM-3-grabbing nonintegrin; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MR, mannose receptor; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; TNF, tumor necrosis factor. Reprinted from the International Journal of Medicinal Mushrooms with permission from Begell House, Inc. [70].

An antiallergic effect of AndoSan™ has been demonstrated in a previous study as measured by reduction of IgE levels in mice sensitized with the allergen ovalbumin (OVA). When AndoSan™ prior to oral ingestion was dialyzed against a membrane with a cut-off of 12.5 kDa, the observed reduction in specific IgE antibodies in serum was rendered not statistically significant [71]. Hence, small molecular substance(s) in AndoSan™ contribute(s) to its anti-allergy effect(s) [71]. Such substances are most probably not β-glucans, both because they are usually bigger molecules and because it has previously been shown that β-glucan from yeast rather had a positive adjuvant effect on OVA sensitization in the very same allergy model in mice [71]. Therefore, these low-molecular-weight substances (yet not identified) may also contribute to the immunomodulatory effects of AndoSan™, e.g. by lowering the anti-
inflammatory response [71]. After more specific analyses the carbohydrate content was found to be 2% of the 4.5 mg/ml dry material [72] after lyophilization. Further, the glucan content in AndoSan™ was less than stated by the manufacturer, with β-glucan 0.1% vs. 2.8% [72]. This is probably so because the mycelial extract of the three Basidiomycetes contains less carbohydrate than their respective fruiting bodies. In addition, the carbohydrate profile of the extract was analyzed with the findings of 26% xylose, 23% glucose, 11% arabinose, and 10% mannose [72]. Together with researchers at Norwegian University for Life Sciences at Aas, protein profiles and identification of peptides were done on the digested fractions of AndoSan™, with findings of protein concentration of 13 mg/ml [73]. All these proteins, mainly containing actin, histone H4 and endo-xylanase with molecular weights (MW) of 97, 30 and 14 kDa, respectively, were degraded into peptides and amino acids when exposed to human gastrointestinal enzymes in vitro [73].

Although several sophisticated methods are available for isolation of AbM, the laboratory procedures have certain established isolation steps in common [6, 69]. Initially, the dried mushroom is denaturated using different solvents (e.g. NaOH, EtOH, MeOH, Hexane, Chloroform) before boiling. Then there is a new round with the use of solvents and freeze-drying to obtain a precipitate containing polysaccharides that are isolated (e.g. by chromatography) and tested for biological effect. It is crucial to be aware of that differently available AbM extracts exhibit different effects, as demonstrated in a sepsis study [41] where most were ineffective because they are prepared by using different mushroom strains and subspecies, grown on different substrates and by different protocols. Previous reports show that there are various compositions of β-glucans in AbM extracts [74] and the concentration of active ingredients in each component depends on the methods of extraction [49, 75] and on the substrate (rotting woods) they are grown on.

**Toxicology and safety of AbM and AndoSan™**

The Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway, has previously investigated AndoSan™ for *in vitro* inhibitory potential on P-gp-mediated transport of digoxin in the Caco-2 intestinal cell line [76]. They found inhibition of P-gp *in vitro* by AndoSan™ in a similar concentration as for green tea without affecting the viability of the cells [76]. Thus, AndoSan™ may interact with P-gp substrates such as vinblastine anticancer agent, digoxin cardiac agent and cyclosporine immunosuppressive agent [77] and loperamide antidiarrhea agent – hence, it
should not be given to individuals using such drugs. AndoSantm and AbM should also not be
given together with other P-gp inhibitors such as verapamil and quinidine. AndoSantm was
also tested for in vitro inhibition of cytochrome P-450 (CYP3A4 isoform) metabolism and
found to have an inhibitory effect, but 20 times less than did green tea [78]. The P-450
enzyme is involved in the metabolism of 50% of drugs [79] and is therefore also of interest
for drug-herb interactions. Researchers Engdal and Nilsen concluded, “although Agaricus
inhibited CYP3A4 metabolism in vitro, clinical relevant systemic or intestinal interactions
with CYP3A4 were considered unlikely” [78].

Depending on the manufacturer, accumulation of unwanted harmful chemicals in the dried
mass from fruiting body and mycelium vary substantially. Cultivated mushrooms may
generate toxic compounds from non-toxic substrates, like the hydrazine-derived substance
agaritine, which makes up approximately 1% of the dried mass of the fruiting bodies [80, 81].
AbM has been studied and assessed for possible side effects of agaritine and its derivates [82,
83] as these are suspected to be genotoxic and possibly carcinogenic or tumorigenic agents.
This molecule is thought to be capable of binding to the DNA of organs after administration
to mice models [84]. The genotoxicity of agaritine is, however, very limited. To date, such
effects of AbM have not been demonstrated [84].

Several tests from Japan Food Research Laboratories (authorized by the Japanese
Government) were done in March 2012, December 2013, October 2014, April 2015 and
February 2016. The tests were for pH, arsenic, lead, cadmium, tin, aerobic plate count,
coliform bacteria (MPN), viable molds count, viable yeasts count, mesophilic aerobic spores,
refractometric brix degree and specific gravity (15°C) – and all of the results were within the
recommended quantitation limits. AndoSantm also passed the water quality test (acceptable
levels of bacteria, as well as ions, pH, taste, colour and odor). An accelerated aging test
(within four months) was performed with an almost unchanged character of the mushroom
drink. Since the Fukushima accident in 2011 in Japan, AndoSantm has also been tested for
radioactivity, with no detection of Cesium-137, Cesium-134 and Iodine-131 (Meiji Co,
Japan). In addition, the Norwegian Food Safety Authorities found no radioactivity in
AndoSantm (2013, data not shown).

Several human studies [55, 66, 85-90] have demonstrated the safety of AndoSantm and
AbM when taken orally. In these studies, there were no subjective side effects or adverse
effects on hematological parameters, electrolyte balance, liver, pancreatic and renal function
[78]. Regarding safety, AndoSantm has also been tested at HLF Sports Science, an Olympic
committee-approved antidoping facility in Oxford, UK, and found by Liquid Chromatography
and Mass Spectrometry to be free of any of the 130 illegal substances on the international antidoping drug list (WADA). It was also found by gas chromatography and mass spectrometry to be free of steroids, and thus it was cleared for the use by competing athletes [91].

**Immunological and clinical effects of AbM and AndoSan™**

AbM *per se* and the AbM-based extract, AndoSan™, have been shown to exhibit multiple biological effects including antitumor, antiallergic and both pro-inflammatory and anti-inflammatory effects as reviewed [38, 92]. At the Norwegian Inst. of Public Health, Hetland et al. [41] compared, in 2003, five AbM-based extracts from main Japanese health food producers in a lethal Gram-positive pneumococcal infection model in mice. The only AbM extract with statistically significant decrease in bacteremia and resulting in significant increase of survival rate was Agaricus Gold Label, later named AndoSan™, produced by ACE Co. Ltd., Gifu-ken. [93]. Accordingly, this particular mushroom extract was chosen for use in further animal and clinical studies.

Several studies have previously demonstrated immunomodulatory effects of glucans, especially β-glucans, on monocytes, macrophages, and natural killer (NK) cells [39-41]. All of these cells originate from a common precursor cell found in bone marrow. The influx of new cells from bone marrow is steady but limited. β-glucans are shown *in vivo* to stimulate the production of precursor cells in bone marrow, resulting in a more rapid flow of new cells into the bloodstream and all lymphoid organs [94].

*In vitro*, AndoSan™ stimulates human monocytes and human vein endothelial cells (HUVEC) to secrete the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα [93], and in addition, also the chemokine MIP-1β [95] in monocyte-derived dendritic cells. One mechanism behind these effects is probably mediated by binding of glucans in the extract to Toll-like receptor 2 [96, 97] as well as to the dectin-1 receptor [98], the lectin-binding site of CR3 CD11b/18 [99] and possibly CR4 CD11c/18 [100]. Lactosylceramide (LacCer), a glycosphingolipid receptor in the plasma membrane of many cells, is found to be stimulated by β-glucans [101]. These receptors also stimulate the cells to release nitric oxide and hydrogen peroxide in order to kill intruding microbes [102, 103]. The results on cytokine synthesis in HUVEC were supported by the fact that AndoSan™-stimulated promonocytic THP-1 tumor cells [104] demonstrated upregulation of genes for IL-1β, IL-8, TLR-2 and the co-operative molecule MyD88, but not for TLR-4. However, in another *in vivo* study, daily
consumption of 60 ml of AndoSan™ for a week in patients with chronic HCV infection had no effect on expression of these genes in blood cells [55]. Rather, foremost genes associated with antitumor properties were upregulated. Further, AndoSan™ was found to activate innate immune cells by inducing NF-κB activation via stimulation of their TLR-2, and also to inhibit TLR-4-induced NF-κB activation [97]. In this experiment, also the pure mushroom ingredients contained in this mixed extract were tested in the NF-κB activation assay to examine which mushroom was most responsible for the all-over stimulatory effect of AndoSan™ on TLR-2. The observed contributions of He and Gf to NF-κB activation were equally negligible, although a potential synergetic effect could not be ruled out [97]. Another commercial mycelial AbM-based extract, obtained from Chang Gung Biotechnology, Taipei, Taiwan, demonstrated activation of the NLRP3 inflammasome in vitro in a monocytic leukemia THP-1 cell line, causing caspase-1-dependent IL-1β secretion [105]. The increased levels of this pro-inflammatory cytokine is important in stimulation of the innate immune response with the recruitment of phagocytic cells in the defense against tumors, infections and inflammation [105].

Since AndoSan™, which is an extract of the mushrooms’ mycelium and not their fruiting bodies, has recently been found to contain less β-glucan than anticipated [72], action of other yet not identified immunomodulating substances in the extract is believed to part-take to render the observed effects [106]. An example is an isolated polar high-molecular-weight fraction of AndoSan™ that was found to inhibit the activity of macrophages in vitro of the tumor-associated and pro-inflammatory protease, legumain (asparaginyl endopeptidase) [106]. Legumain probably activates pro matrix metalloproteinases and processing of cathepsins, leading to pro-inflammatory activity [106].

Antitumor effects of AbM have also been reported in mice (e.g. fibrosarcoma, myeloma, ovarian-, lung-, and prostate cancer), in humans (gynecological cancer and leukemia) and in vitro in cancer cell cultures [44, 86, 90, 92, 107, 108]. Interestingly, AndoSan™ has recently been found to inhibit intestinal tumorigenesis in mice, in which also IL-1β and IL-12 were elevated [109]. In addition to β-glucan in AbM and AndoSan™, ergosterol and agaritine also exhibit antitumor activity, respectively, by oral administration in sarcoma 180 bearing mice [44] and by induction of apoptosis in leukemic cells [48]. Moreover, isoflavonoids, another isolated subcomponent of AbM, demonstrated a reduction in blood glucose levels in diabetic rats [49]. There was also an antiallergic effect in mice sensitized to ovalbumin (OVA), as demonstrated by the reduction of specific anti-OVA IgE antibodies, both when AndoSan™ was given before or after the OVA immunization [110]. Additionally, in this allergy model,
there was an increase in T\textsubscript{H1} relative to T\textsubscript{H2} cytokines in spleen cell cultures \textit{ex vivo} obtained from the animals treated with AndoSan\textsuperscript{TM}. This finding is in line with the reduced specific IgE levels in these animals, supporting an antiallergic effect of AndoSan\textsuperscript{TM}, through engagement of the adaptive immune response. This observation is compatible with the improvement of the Th1/Th2 imbalance in tumor-bearing and asthma-induced mice, after ingestion of \textit{Agaricus blazei} extracts [111]. Extracts of AbM have also been used successfully as adjuvants in DNA vaccines to improve their efficacy against hepatitis B virus infection and foot-and-mouth disease [59, 60]. Antiviral activities have also been reported about the AbM’s mycelia \textit{in vitro}, but not their fruiting bodies, with inhibition of the toxic effect of Western equine encephalitis virus on VERO cells in culture [54].

In a human pilot study, with oral intake of AndoSan\textsuperscript{TM} (60 ml/day) over 12 days in eight healthy volunteers, there was a reduction in intracellular levels of ROS (mainly superoxide ion) in granulocytes and monocytes \textit{in vivo}, also supporting an anti-inflammatory effect [112]. In a randomized placebo-controlled clinical study in 57 elderly females, there were no difference in levels of the chosen cytokines IL-6, TNF\textalpha, and IFN\gamma after daily consumption of the AbM dry extract (900 mg) or placebo (600 mg) for 60 days [113]. Thus, AbM had no modulating effect on the levels of these classical pro-inflammatory cytokines. However, in a placebo-controlled study in 100 patients with gynecological cancer, treatment with AbM (AbM Kyowa) in addition to chemotherapy was reported to increase NK cell activity in blood and improved quality of life [86]. In another study with this AbM-based mushroom extract, \textit{ex vivo} stimulation of whole blood resulted in a pronounced release of many cytokines being pro-inflammatory (IL-1\beta, IL-6, TNF\alpha), anti-inflammatory (IL-10), chemokines (IL-8, MIP-1\beta, MCP-1, leukocyte growth factors (G-CSF, GM-CSF), pleiotropic (IL-7, IL-17) as well as of the T\textsubscript{H1}-type (IFN\gamma, IL-2, IL-12) and T\textsubscript{H2}-type (IL-4, IL-5, IL-13) cytokines [85]. In addition, when eighth healthy volunteers were given AndoSan (60 ml per day) for a 12 day periode, there was a significant reduction in cytokine levels in plasma of IL-1\beta, TNF\alpha, IL-6, IL-2 and IL-17, whilst levels of the remaining 12 cytokines in the analysis were unaltered, pointing to an anti-inflammatory effect \textit{in vivo}, when given by the oral route [85]. Similarly, as for healthy individuals, 11 patients with CD and 10 patients with UC who likewise consumed the mushroom extract, cytokine levels were reduced in both untreated and in LPS-stimulated blood \textit{ex vivo} [66]. For CD respective reductions in cytokine levels were for IL-2, IL-8, IL-17 and IL-1\beta, MIP-1\beta, MCP-1, IL-8, IL-17, G-CSF and GM-CSF, while MCP-1 and MIP-1\beta, IL-6, IL-1\beta, IL-8, G-CSF, MCP-1, GM-CSF were reduced in UC. For the UC patients, the level of fecal calprotectin was also reduced [66].
The divergent results of levels of cytokines \textit{ex vivo} and \textit{in vivo} in human pilot studies, also pointed to a potential anti-inflammatory effect of AndoSan\textsuperscript{TM} when given the oral route, that deserved further studies.

**The immune response**

The immune system is divided into the innate and adaptive immune system that protects the host against a wide range of pathogens. The innate immune response acts in a rapid, non-specific and conserved manner, and is the dominant system of host defense in most organisms. The host cells express pattern recognition receptors (PRRs), particularly represented by macrophages, DCs and NK cells, that sense pathogen-associated molecular patterns (PAMPs), which makes it possible to discriminate “self” from “non-self” cells. Importantly, the immune system also senses endogenous alarm or danger signals from infected or damaged host tissue, many of which signal through the same receptors as do PAMPs. Different PRRs initiate downstream intracellular events that promote the activation of the immune system, with the specific immune response generated depending on the cell type involved. The innate immune system is usually sufficient to fight off most pathogens on its own but also has the ability to alarm and activate the adaptive immune system when needed.

The adaptive immune system allows for a stronger immune response as well as immunological memory, where each pathogen is “remembered” by a signature antigen (Ag). The leukocytes of the adaptive immune system are the lymphocytes that are divided into B- and T-cells. The adaptive immune response is Ag-specific and requires the recognition of specific “non-self” Ag when DCs, mononuclear phagocytes and B cells present them to T cells. For the activation of the adaptive immune system, Ags must be internalized and processed by Ag-presenting cells (APCs; dendritic cells (DCs), macrophages and B cells). In lymph nodes, the Ag is presented to T cells together with a peptide of the major histocompatibility complex (MHC) class I or II. Then, clonal expansion of activated lymphocytes occurs, with the generation of antibody producing plasma cells and helper- and cytotoxic T cells. In contrast to the innate immune system, the Ag-specific adaptive immune system needs time to get operational. B cells are involved in the humoral immune response, whereas T cells are involved in the cell-mediated immune response. There are two major subtypes of T cells: the cytotoxic T cell and the helper T cell (T\textsubscript{H}). In addition, there are regulatory T cells that have a role in modulating the immune response. Killer T cells only
recognize Ag coupled to class I MHC molecules, while T_H and regulatory T cells (T_{reg}) only recognize antigens coupled to class II MHC molecules. These two mechanisms of Ag presentation reflect the different roles of the two subtypes of T cells.

The reference for this chapter, meant as a brief presentation of the immune system, is the textbook “Janeway’s Immunobiology. 9th ed.” [160].

**Immune stimulation by mushrooms**

The complex fungal cell wall is the main source of PAMPs that are recognized by PRRs on mammalian cells [114]. From inner- to outmost, the three layers of the fungal wall are i) chitin, which is a polymer of N-acetylglucosamine, ii) β-glucans and iii) mannans, which are chains of mannose molecules coupled to fungal proteins by N- or O-linkages (Fig. 3) [70].

The edible and harmless medicinal mushrooms like AbM, He, and Gf, share PAMPs with other highly poisonous species, resulting in an effective and rapid engagement of the innate immune system by pathogen recognition receptor (PRRs) [70]. Such mushrooms and fungi are usually a health threat due to their action of toxins. β-glucan, which is a major cell wall component of fungi and has no host-cell analogs, acts as a major PAMP and serves as a key foreign molecule with immunostimulating and antitumor activities [70]. Representative PRRs of the innate immune system include Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs) that detect invading bacteria, fungi, and viruses, and initiate downstream intracellular events that promote generation of a specific immune response, depending on the cell type involved.

Mushroom β-glucans activate PRRs expressed on the innate immune cells to protect the host from invading pathogens [70]. Mammalian non-immune and immune cells, including NK cells, DCs, monocytes, and macrophages, as well as B- and T-cells, all express TLRs, and thereby play critical roles in the early innate immune response and also induce adaptive immunity [115,116]. In addition, fungal β-glucans bind to the dectin-1 receptor in combination with the dimers of TLR2/TLR4, TLR2/TLR6, or TLR4/TLR6 of macrophages or DCs, thereby activating adaptive immunity, such as targeted cell lysis and humoral- and cell-mediated responses [115, 116].

In addition to PAMPs, mammalian PRRs also recognize damage-associated molecular patterns (DAMPs), mainly being damaged host cell components exemplified by nucleic acids and alarmins [117]. The PAMP- and DAMP-induced immune responses are coordinated by the alarmin S100B via the signals from TLRs and the receptor for advanced glycation end-products (RAGE) [118]. The cross-talk between RAGE and TLRs represents a regulatory
circuit in infection, whereby an endogenous danger signal protects the host against pathogen-induced inflammation and a nucleic acid-sensing mechanism terminates the inflammation induced by the endogenous danger signal.

In summary, TLRs and CLRs activate multiple intracellular pathways upon binding to specific fungal PAMPs, including β-glucans, chitin, mannans, β-(1→2)-linked oligomannosides and fungal nucleic acids. These signals activate NF-κB and the NLRP3 inflammasome, and this culminates in the production of defensins, chemokines, cytokines, and reactive oxygen species (ROS) [119]. The major signaling pathways, which further will be described, are elegantly detailed in figure 4.
Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) that are present during fungal infections are recognized by pattern recognition receptors (PRRs). The major PRRs are Toll-like receptors (TLRs); C-type lectin receptors (CLRs; such as dectin-1 and -2, DC-SIGN, mincle and the mannose receptor; galectin family proteins (such as galectin 3) and receptor for advanced glycation end-products (RAGE). TLRs and CLRs activate multiple intracellular pathways upon binding to specific fungal PAMPs, including β-glucans, chitin, mannans linked to proteins through N- or O-linkages, β-(1,2)-linked oligomannosides and fungal nucleic acids. These signals activate canonical or non-canonical NF-κB and the NLRP3 inflammasome, and this culminate in the production of defensins, chemokines, cytokines, ROS and IDO. Complement receptor 3 and members of the scavenger receptor family (such as CD36) mediate recognition of β-glucans and the fungal adhesin BAD1 (Blastomyces adhesion 1). After TLR activation, protease-activated receptors (PARs) sense proteolytic virulence factors and tissue injury and contribute to fungal recognition. In addition, the alarmin S100B, through the spatio-temporal integration of signals from TLRs and RAGE, allows the immune system to discriminate between pathogen-derived and endogenous danger signals. By forming
complexes with various TLR2 ligands, S100B, and this accounts for its anti-inflammatory activity. However, the ability of S100B to bind nucleic acids results in the activation of intracellular TLRs that signal through TIR domain-containing adaptor protein inducing IFNβ (TRIF) and this eventually resolves damage-associated inflammation through transcriptional downregulation of S100B gene expression. ASC; apoptosis-associated speck-like protein containing a CARD; BCL-10, B cell lymphoma 10; CARD9, caspase recruitment domain-containing protein 9; ERK, extracellular signal-regulated kinase; FcRγ, Fc receptor γ-chain; IL, interleukin; IRF3, IFN-regulatory factor 3; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MYD88, myeloid differentiation primary response protein 88; SYK, spleen tyrosine kinase.

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**CLRss**

C-type lectin receptors (CLRss) are a large family of proteins that have one or more carbohydrate-recognition domains. CLRss exist as transmembrane and soluble proteins, and include type 1; decalektin and mannose receptor (MR), and type 2; dectin-1, dectin-2, macrophage-inducible C-type lectin (mincle), DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), and DC, NK lectin group receptor-1 (DNGR-1) [120]. To protect the host from fungal infection, various receptors are involved in cytokine secretion and phagocytosis through N-linked mannann recognition. This includes the MR on macrophages and DCs, DC-SIGN of DCs [121], the β-galactoside receptor galectin-3 of macrophages [122], the lectin receptor mincle of macrophages [123], and FcRγ-coupled dectin-2 of macrophages and DCs [124]. Mincle is not only an essential component of the innate response that protects against infection by pathogenic fungi but also is a receptor for endogenous DAMPs of damaged necrotic cells [125]. DC-SIGN is a CLR expressed on the surface of DCs and plays important roles in orchestrating the adaptive immunity of DCs with T lymphocytes against the bacterial, fungal, and viral pathogens [126].

The immune system detects infection and tissue damage through PRRs. DNGR-1 is one of the immune receptors expressed by a small subset of DCs that interacts with damaged cell components. DNGR-1 is responsible for the recognition of intracellular DAMPs derived from damaged body components, engulfs necrotic cells and digests them in endosomes [127]. It is not known whether fungal β-glucans regulate the expression of DNGR-1 on DCs.

Dendritic-cell-associated C-type lectin-1 (dectin-1) exists on many cell types including DCs, macrophages, monocytes, polymorphonuclear granulocytes and a subset of T cells [128, 129]. Dectin-1 is found abundantly at the portals of pathogen entry (lung and intestine) [130], and its expression is influenced by various cytokines, chemokines and microbial stimuli [131]. The expression of dectin-1 is markedly increased by Th2 response cytokines, especially IL-4 and IL-13, whereas IL-10 and LPS down-regulate this expression [131]. Dectin-1 is the main PRR that recognizes β-glucans and, following ligand binding, it induces the production
of pro- and anti-inflammatory cytokines and chemokines [132], and also plays a dual role in the internalization and cellular immune responses to β-glucan in macrophages and DCs. The immune response is achieved through the activation of two distinct signaling pathways downstream of dectin-1, the SYK/CARD9 (spleen tyrosine kinase - caspase recruitment domain-containing protein 9) pathway and the MAPK (mitogen-activated protein kinase) pathway. These pathways mutually interact to activate nuclear factor-κB (NF-κB) for modulation of cytokine gene expression [133]. The SYK-CARD9 pathway also activates the NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome, which causes proteolytic activation of the cytokines IL-1β and IL-18 by caspase 1 [119]. Furthermore, recent studies indicate that dectin-1 and TLR2/TLR6 cooperate to induce NF-κB and inflammatory cytokines such as IL-12 and TNFα in the presence of β-glucan [134, 135]. When TLRs are activated, dectin-1 activation stimulates macrophages and DCs to produce ROS [136].

**TLRs**

Toll-like receptors (TLRs) are a family of membrane-spanning proteins that recognize PAMPs as well as DAMPs at the surface of cells. There are 11 different TLRs found in humans, TLR1-11. They are the most important sensors of the innate immune system and signal to the host that a microbial pathogen is present or that tissue damage has occurred. The TLRs are characterized by an ectodomain with varying numbers of leucine-rich repeat motifs and a cytoplasmic Toll/IL-1 receptor (TIR) domain that recruits adaptors, such as the myeloid differentiation primary response protein 88 (MyD88) and TIR-domain-containing adaptor protein including IFNβ (TRIF) [119]. TLRs -2, -4 and -9 are involved in detecting fungal components like zymosan, phospholipomannan, O-linked mannans and fungal DNA [137]. A study in mice has shown that the lack of MyD88 is highly susceptible to infections with various fungi [138]. This finding support that TLRs are involved in the defense against fungal infections, although their relative contribution in this battle is still unclear. Presentation of fungal Ags by DCs are promoted by TLRs, which is a prerequisite for adequate T cell responses and further processing of both self and non-self components [139].

In addition, during inflammation, host- and fungal-derived proteases induce the presence of protease-activated receptors (PARs), which belongs to the family of G protein-coupled receptors (GPCRs) [140]. The stimulation of TLRs by fungi unmasks the divergent roles of PAR1 and PAR2 in downstream signaling and inflammation. After fungal recognition by TLRs, PARs become activated to sense proteolytic virulence factors and tissue injury, to
mediate pro-inflammatory (PAR1) or anti-inflammatory (PAR2) responses and to modulate the activity of TLRs. Thus, TLRs regulate PAR signaling and vice versa [140].

**NLRs**

NLRs are a family of cytosolic proteins that recognize PAMPs and endogenous ligands. The recognitions of ligands induce a signaling cascade leading to activation of NF-κB, or the inflammasome, to produce pro-inflammatory cytokines. NLRs are also involved in signaling for cell death. Although cytoplasmic receptors for fungi have yet to be described, the NLRs are implicated in sensing fungi and, once activated, these receptors induce production of IL-1β and IL-18 through the formation of inflammasomes [137, 141, 142].

**Non-dectin-1 β-glucan receptors (other than TLR2/TLR6)**

Immune cells like NK cells and non-immune cells like EC, alveolar epithelial cells and fibroblasts, do not express dectin-1 but have an important role in antifungal immunity [143] and in mediating the protective effects of β-glucans. These cells express complement receptor 3 (CR3, CD11b/18), lactosylceramide (LacCer) and scavenger receptors (ScR) (fig. 3 and 4) such as CD36, which can recognize certain carbohydrates. Except for the scavenger receptors, these receptors signal through NF-κB to activate innate immunity and mainly recognize β-glucan as ligands [144]. The NK cells of the innate immune response are classified as non-phagocytic large granular lymphocytes, which without activation can damage various target cells.

**T cell responses to fungi**

Innate sensing mechanisms are aimed to activate distinct T cells with protective and non-protective functions against fungi in cooperation with highly adaptive DC subsets [145, 146] through their PRRs, which lead to different T cell immune responses in infection [119]. Inflammatory DCs initiate antifungal T H17 and T H2 cell responses, whereas tolerogenic DCs activate T H1 and T reg cells. The different signaling pathways in the DC subsets regulate the balance between CD4^+ effector T cells and T reg cells [119].

A dominant T H1 cell response correlates with protective immunity against fungi [147-150] and effective fungal vaccines [151]. T H1 cell activation is determined by the DC response to the combination of TLR- and CLR-specific signals provided by the fungi. The combination of the synthesis of IFNγ and opsonizing antibodies contribute to the T H1 cell-induced activation of phagocytes at inflammatory sites [119].
The initial differentiation of naïve T cells to T\(_H\)2 cells is mainly dependent on IL-4 and IL-13. The T\(_H\)17 cells have an important function in the host response against extracellular pathogens, but they are also associated with the pathogenesis of many autoimmune and allergic disorders. T\(_H\)17 cell activation occurs in fungal infections, mainly through the SYK-CARD9, MyD88 and MR signaling pathways in DCs and macrophages. Inhibition of T\(_H\)17 cells is mediated by the RAF and TRIF-type I IFN signaling pathways. The activation and inhibition of T\(_H\)17 cells are believed to be presented downstream of both CLRs and TLRs [119].

IDO is a metabolic enzyme that affects the T\(_{reg}\)/T\(_H\)17 cell balance during fungal infections, by diminishing inflammatory responses through induction of T\(_{reg}\) cells and inhibition of T\(_H\)17 cell development [152, 153].

**Intestinal absorption of β-glucans**

Mammals enzymatically digest α- but not β-glucans. Therefore, orally administered β-glucans can reach the small intestine without being degraded. The general notion is that carbohydrates larger than monosaccharides are not absorbed from the human gut. However, orally administered β-glucans in rodents have been shown to interact with innate immune cells in the gastrointestinal tract via two mechanisms [70]. The first mechanism involves microfold cells (M cells) between the mucosal enterocytes that are responsible for the transportation of the β-glucans to lymphoid tissue known as Peyers patches, where innate immune cells reside [115, 154]. M cells (see fig. 6) pinocytose particles and transport luminal, soluble macromolecules, particles, and whole microorganisms to the Payers patch. In situ, macrophages or DCs recognize transported β-glucans that are internalized and fragmented in the endosome. The β-glucan fragments are released from the macrophages and taken up by other immune cells, leading to a cascade of immune responses. The second mechanism involves the intraluminal pseudopods of DCs [155], where they capture luminal particles like β-glucans, which then are presented and processed in Peyers patches as already described.

Many medicinal mushroom β-D-glucans have been shown to induce the biological responses through the lectin-binding site of complement receptor type three (CR3 (CD11b/CD18)) on immune effector cells [156]. β-glucans also interact following binding to an additional β-glucan receptor dectin-1 in neutrophils, macrophages, DCs and some T-cells, but not in NK cells [157, 158], in which CR3 probably is the key glucan-receptor. Binding of
β-glucans to dectin-1 activates phagocytosis, ROS production, and release of inflammatory cytokines [158].

Following a single oral dose in rodents of three structurally distinct soluble β-(1→3)-glucans of molecular weight less than 10^3 kDa, the glucans were probably internalized by M cells and DCs prior to rapid detection in the circulation [159]. Based on this study the possibility for a similar uptake of β-glucans in humans is reasonable to assume. The internalization of soluble β-glucans is mediated by TLR2 and dectin-1 in granulocytes, macrophages and DCs, of which the two latter cells also are integrated in gut-associated lymphoid tissue. This uptake is dependent on TLR2 on IECs that lack dectin-1 [115].

Despite challenges associated with low bioavailability, due to limited intestinal barrier penetration and possible gastrointestinal degradation, oral administration is still the obvious and safe delivery route of β-glucans.

**Cytokines**

Cytokines are a category of small proteins (about 25 kDa) secreted by immune cells, usually in response to an activating stimulus, and that induce responses through binding to specific receptors. It is more than 60 different cytokines which can act in an autocrine, paracrine or endocrine manner [160], important in cell signaling and cross-talk. Cytokines include chemokines, interferons, interleukins, (growth factors), and tumor necrosis factors. A broad range of cells produces cytokines, including immune cells like macrophages, B-lymphocytes, T-lymphocytes and mast cells, as well as endothelial cells, fibroblasts and various stromal cells. In the following, the 17 different cytokines from studies in this thesis are presented.

Unless otherwise referred to in the text, the following description of different cytokines is based on the textbook “Janeway’s Immunobiology. 9th ed.” [160].

**Interleukin 1β**

IL-1β is a member of the interleukin 1 family of cytokines. IL-1β is a pro-inflammatory cytokine intensely produced by tissue macrophages, monocytes, fibroblasts and DCs, but also expressed by B lymphocytes, NK cells and epithelial cells. The exposure of the innate immune cells to alarmins, which are endogenous molecules, that signal tissue and cell damage, together with NF-κB, also induces the expression of IL-1β. They form an important part of the inflammatory response, often in synergy with TNFα [161]. Immunologically activated T-cells, immune complexes, complement fragment 5a (C5a) and IFNγ can stimulate IL-1β production. Importantly, IL-1β induce the release of IL-2 that stimulate proliferation of
CD4+ T cell, promote growth and differentiation of B-cells, stimulate synthesis of IL-6 and enhance adhesion between leukocytes and endothelial cells. IL-1β increases the expression of adhesion factors on endothelial cells to enable transmigration of immunocompetent cells, including phagocytes and lymphocytes, to inflammatory sites. It also affects the hypothalamus, raising the body temperature, and thereby acts as an endocrine pyrogen. IL-1β also causes hyperalgesia, myalgia, arthralgia, vasodilation and hypotension [162]. IL-1β induces, after binding to the IL-1 receptor (IL-1RI), several transcription factors, especially NF-κB. IL-1β also stimulates synthesis of acute-phase proteins by the liver [160].

**Tumor necrosis factor α**

The pro-inflammatory cytokine TNFα is produced mainly by activated macrophages, monocytes, NK cells and T cells, but also by a broad variety of other cell types, such as CD4+ lymphocytes, mast cells, endothelial cells, and neurons. The primary role of TNFα is in the regulation of immune cells, where it acts synergistically with IL-1, and exhibits mainly overlapping effects. TNFα can induce fever, apoptosis, cachexia, inflammation as well as inhibit carcinogenesis and viral replication. A local increase of TNFα will cause the cardinal signs of inflammation to occur; calor, rubor, tumor, dolor and functio laesa [160].

Macrophages and T lymphocytes produce large amounts of TNFα that plays a pivotal role in the pathogenesis of IBD [163]. When TNFα binds to its corresponding receptors (TNFR1 and TNFR2), three different signaling pathways can be initiated; activation of NF-κB, activation of the MAPK pathways, and induction of programmed cell death. Furthermore, TNF signaling in colitis can drive pleiotropic pro-inflammatory effects, including augmented angiogenesis, the induction of Paneth cell death via necroptosis, the production of matrix metalloproteinases by myofibroblasts, the activation of macrophages and effector T cells, and the direct damage of intestinal epithelial cells (IECs) via myosin light chain kinase activation [164-168]. TNFα-inhibitors, such as infliximab and adalimumab, are crucial in the treatment of IBD.

**Interleukin-6**

IL-6 is a pleiotropic cytokine with the possibility to act in both a pro- and anti-inflammatory way. IL-6 is produced by activated monocytes, macrophages, endothelial cells, activated T cells and liver cells in response to IL-1 and TNFα. This cytokine is important for i) synthesis of acute-phase proteins in the liver, ii) mucosal IgA production, iii) pathogen-induced clearance of neutrophils, and iv) end-stage differentiation of B cells. IL-6 can exert pro-
inflammatory functions by activating multiple target cells, including APCs and T cells. In particular, the IL-6-sIL-6R complex prevents programmed cell death (apoptosis) of mucosal T cells and activates pro-inflammatory cytokine production by these cells [169]. However, IL-6 may also have important homeostatic functions by stimulating the proliferation and expansion of IECs [170]. The anti-inflammatory effect of IL-6 can be explained by inhibition of the pro-inflammatory cytokines TNFα and IL-1, as well as stimulation of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist.

**Chemokines**

Chemokines (IL-8, MIP-1β, MCP-1) are inflammatory products with chemotactic and other leukocyte-activating properties, including trafficking of different leukocyte subsets between blood, tissues and lymphatics.

IL-8, a member of the CXC (cysteine-x-cysteine) chemokine family, is produced by macrophages and other cell types such as epithelial and endothelial cells and is an important mediator in the innate immune response. There are many receptors capable of binding IL-8, in which TLRs are of importance in the innate immunity. IL-8 induces chemotaxins in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. In addition, IL-8 also induces phagocytosis once they have arrived. It is also known to be a potent promoter of angiogenesis, together with stimulating the activation and mobility of T cells, eosinophils, basophils and monocytes. In B cells, IL-8 inhibits IL-4-induced IgE production.

The CC (cysteine-cysteine) chemokines, such as MIP-1β (macrophage inflammatory protein-1β, CCL4) and MCP-1 (monocyte chemotactic protein-1, CCL2), induce the migration of monocytes and other cell types such as NK cells and DCs and are primarily produced by activated T cells and DC. MIP-1β also selectively attracts CD4+ cells but not CD8+ cells. In addition, MIP-1β also binds to the chemokine receptor CCR5 on T_{H1} cells and macrophages. MCP-1, by binding to CCR2, induces monocytes to leave the bloodstream and enter the surrounding tissue to become tissue macrophages, together with T_{H2} cells acting on T cells, NK cells, basophils and immature DCs. In general, neutrophils are unresponsive to the group of CC chemokines.

**Interleukin 2**

The existence of IL-2 has been recognized for over 35 years, and it remains one of the most extensively studied cytokines. IL-2 exerts a wide spectrum of effects on the immune system.
and plays crucial roles in regulating both immune activation and homeostasis [171]. IL-2 is primarily produced by T cells upon activation of the immune system. IL-2 binds to IL-2 receptors and promotes the differentiation of T cells into effector T cells and memory T cells when the initial T cell is also stimulated by an Ag and stimulated with IL-1, thus helping the body to fight off infections. The primary function of IL-2 is to stimulate the proliferation of activated T\(_{H1}\) cells (CD4\(^+\)/CD8\(^-\)). This cytokine is also chemotactic on T cells and stimulates NK- and B-cell proliferation, including antibody production. Unlike other cytokines, IL-2 is crucial in discriminating between foreign (“non-self”) and “self”, i.e. induction of self-tolerance.

**Interleukin 17**

IL-17 is a pro-inflammatory cytokine produced by T-helper cells, T\(_{H17}\) cells, and NK cells, and is induced by IL-23. The T\(_{H17}\) cells also produce IL-21 and IL-22, which together with IL-17, acts on epithelial cells and endothelial cells in order to stimulate secretion of IL-6, IL-8 and G-CSF. IL-17 serves as a potent mediator in delayed-type reactions by increasing chemokine production in various tissues, thereby recruiting monocytes and neutrophils to the site of inflammation, similar to IFN\(\gamma\). IL-17 has been demonstrated to act synergistically with TNF and IL-1 [172]. This activity can also be redirected towards the host and result in autoimmune disorders that involve chronic inflammation. High levels of IL-17 has for example been found in the synovial tissue of patients with rheumatoid arthritis. On the other hand, inadequate levels of T\(_{H17}\) cytokines (IL-17 included) can give excessive inflammation as seen in autoimmune diseases like rheumatoid arthritis and IBD [173]. Studies in mouse models of experimental colitis have shown that the absence or neutralization of IL-17A or IL-17F alone had no effect, or even aggravated disease activity, in a T cell transfer model of colitis [174]. To date, clinical targeting of T\(_{H17}\) cells in patients with Crohn’s disease (CD) has been restricted to the use of secukinumab, an IL17A-specific neutralizing antibody. However, secukinumab treatment has been reported to be ineffective in treating CD and is associated with higher rates of adverse events than placebo therapy [175].

**Interferon \(\gamma\)**

IFN\(\gamma\) is mainly produced by NK cells and natural killer T cells of the innate immunity, as well as by CD4\(^+\) T\(_{H1}\) and CD8\(^+\) cytotoxic T cells of antigen-specific immunity. Moreover, IFN\(\gamma\) activates macrophages and induce expression of class II molecules of the major histocompatibility complex (MHC) on APCs. IFN\(\gamma\) has antiviral, immunoregulatory and
antitumor properties [176]. It alters transcription in up to 30 genes producing a variety of physiological and cellular responses. Regardless the types of interferon, the two major functions are antiviral activity and antiproliferative activity. It activates macrophages to synthesize inflammatory cytokines such as TNF, IL-1β, IL-12 and is also responsible for the intracellular generation of antimicrobial nitric oxide and ROS. Increased expression of MHC class I and II are mediated by INF-γ on several cell types. The IFN-γR is expressed in all cell types except erythrocytes. IFNγ also has a key role in granuloma formation in different infectious or inflammatory diseases, such as CD, where it activates macrophages so that they can be more powerful in killing intracellular organisms. Chemokine IL-8 participates in recruiting monocytic cells to the site of infection. A granuloma is the host’s way of dealing with substances it cannot remove or sterilize. This is a process, including Th1 cells, IL-1, and IL-12 that ultimately results in aggregation of macrophages that transform into fibroblast-like cells walling off the infection or substance. Patients with steroid-refractory ulcerative colitis (UC) have been treated with recombinant IFNβ1a without therapeutic benefit [177]. In contrast, several similar patients were successfully treated with a CpG (bacterial motif)-containing oligonucleotide, which indicates that immunostimulatory approaches to induce IFNγ production might be useful in IBD therapy [178].

**Interleukin 10**

IL-10 is an anti-inflammatory cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation. The cells that produce most of the IL-10 are activated monocytes, macrophages and T_h2 cells. In addition, other cells like DCs, B cells, eosinophils, mast cells and hepatocytes also synthesize IL-10. The major role of IL-10 is to reduce inflammatory responses by affecting mainly monocytes, macrophages, neutrophils, eosinophils and mast cells. The anti-inflammatory effect of IL-10 is for a large part effectuated by inhibiting pro-inflammatory cytokines by inhibiting NF-κB-activated transcription of genes encoding particularly for TNF, IL-1β, IL-6, IL-8 and IL-12. It also enhances B cell survival, proliferation, and antibody production. Accordingly, IL-10 has a crucial role in the reduction of the cytokine release during sepsis as well as inhibition leukocyte-mediated ROS-dependent killing of microbes. Regarding the immunostimulatory effect of IL-10, a T_h2 response is promoted through inhibition of IFNγ and IL-2 secretion by T_h1 cells. There have been several clinical trials with recombinant human IL-10 treatment in patients suffering from autoimmune diseases but with no significant effects in patients with CD [179] or rheumatoid arthritis [180]. This treatment has also shown pro-inflammatory
results in another study with CD patients [181]. The two receptors for IL-10, IL-10R1 and -R2, are expressed mainly on hematopoietic cells.

**Interleukin 4**

IL-4 induces the differentiation of T\(_{H0}\) into T\(_{H2}\) cells, and T\(_{H2}\) cells subsequently produce additional IL-4 in a positive feedback loop. Moreover, IL-4 produced by activated T\(_{H2}\) cells may amplify T\(_{H2}\) development from naive T-cell precursors. IL-4 is a strong pleiotropic T\(_{H2}\) cytokine, synthesized by CD4\(^+\) T cells and to a lesser degree by basophils and mast cells. This cytokine influences all types of cells through interaction with IL-4R. IL-4 stimulate the differentiation of B cells into plasma cells and the proliferation T-cells. IL-4 induces B-cell synthesis of IgE and IgG1 and up-regulates MHC class II production. IL-4 also stimulates IL-12 synthesis in macrophages and DCs, which functions as a negative feedback mechanism for the T\(_{H2}\) response. IL-4 decreases the production of T\(_{H1}\) cells, inhibiting production of IFN\(\gamma\) and IL-1\(\beta\). Macrophages play a major role in chronic inflammation and wound repair. Increased tissue IL-4 shifts the differentiation of macrophages from M1 into M2 cells, also called repair macrophages. Combined with increased synthesis of IL-10 and TGF\(\beta\), the M2 cells reduce pathological inflammation and promote wound repair and fibrosis. IL-4 upon stimulation exerts degranulation and proliferative effects on mast cells. Generally, the effects of IL-4 are antagonistic to those of IFN\(\gamma\).

**Interleukin 12**

It is primarily synthesized by macrophages, but to some extent also by neutrophils, DCs, monocytes and B cells. IL-12 plays a key role in the immune response by linking macrophages and DCs and thereby activating them for microbial ingestion. The innate response is activated through an NK cell activation, which further differentiates naive T\(_{H0}\) cells into T\(_{H1}\) cells. The increased levels of these cytokines are important to exhibit an adequate host defense against intracellular pathogens (e.g. mycobacteria). IL-12 also reduces IL-4-mediated suppression of IFN\(\gamma\), which contributes to an antiangiogenic effect. The result of the IL-12-mediated T\(_{H1}\)-cell response is increased levels of antibodies IgG2a, IgG2b and IgG3, but not the T\(_{H2}\) associated antibodies IgG1 and IgE. Since CD has been considered a T\(_{H1}\)-cell disorder, IL-12 may contribute to the pathogenesis of this disease. The IL-12 receptor (IL-12R) is more densely expressed on activated T- and NK-cells, compared with DCs and B cells.
Other cytokines (IL-5, IL-7, IL-13)

IL-5 is especially pertinent for the differentiation, activation and chemotaxis of eosinophils. Other crucial functions of IL-5 is i) histamine release from mast cells, ii) IgA synthesis in B cells, and iii) development of cytotoxic T lymphocytes. This cytokine is primarily synthesized by T_{H}2 cells, but in a smaller scale by activated mast cells, eosinophils, NK cells and B cells.

IL-7 stimulates the development of B- and T-cells and also contributes to the formation of memory T cells. The main source of IL-7 production is from stromal cells in the bone marrow and thymus, but also by keratinocytes, DCs, epithelial cells and neurons, but not by normal lymphocytes.

Activated T cells produce IL-13, in particular, T_{H}2 cells, but also basophils and mast cells. Except for the differentiation of T_{H}0 into T_{H}2 cells, IL-13 exhibits similar activities as IL-4, but the response is smaller in magnitude. It inhibits synthesis of pro-inflammatory cytokines (IL-1ß, IL-6, TNFα and IL-8) in macrophages, but phagocytosis is not blocked. B cell proliferation and change of isotype to increased synthesis of IgE are stimulated by this cytokine.

Colony stimulating factors

Granulocyte colony-stimulating factor (G-CSF) and granulocyte-monocyte colony-stimulating factor (GM-CSF) are mainly produced by stromal cells, mononuclear phagocytes, endothelial cells, activated T cells and fibroblasts. They are glycoproteins that bind to receptor proteins on the surfaces of hematopoietic stem cells, thereby activating intracellular signaling pathways to proliferate and differentiate into a specific kind of blood cell. G-CSF promotes the development and recruitment of leukocytes and their migration from bone marrow to blood. GM-CSF promotes development from pluripotent hematopoietic stem cells, into subsets of leukocytes as well as megakaryocytes and erythrocytes.
**Inflammatory bowel disease**

Inflammatory bowel disease (IBD) is characterized by a chronic and relapsing inflammation of the gastrointestinal tract that leads to structural damage of the bowel wall [163, 182, 183]. The main forms of IBD are Crohn’s disease (CD) and ulcerative colitis (UC). About 10% of the IBD cases are called indeterminate colitis, with an overlapping disease presentation that makes the CD and UC diagnosis difficult to distinguish. The target in UC is the colonic mucosa while CD affects all layers of the intestinal wall. In UC, the continuous inflammation usually originates in the distal colon or rectum, with disease progression in the proximal direction. In CD, healthy and diseased patches of bowel are often interspersed. The small bowel can give stenosis with malabsorption and subileus, and the transmural inflammation can also cause fistulation to other epithelial lined organs. Clinically, IBD patients can suffer from chronic diarrhea, malabsorption, weight loss, rectal bleeding, abdominal pain, stenosis, abscesses, and fistula formation, and many patients require surgery over time [184]. In addition, both CD and UC can be complicated with various extraintestinal manifestations such as primary sclerosing cholangitis, ankylosing spondylitis, iridocyclitis, pyoderma gangrenosum, erythema nodosum [184]. Finally, there is an increased risk of colitis-associated neoplasias in IBD, particularly in patients with ulcerative pancolitis and colonic CD [185, 186].

The highest occurrence of IBD is reported in Northern Europe and North America, and in Norway, the adult incidence has been found to be $5.8 \times 10^5$ and $13.6 \times 10^5$ for CD and UC, respectively [187, 188]. IBD affects children, adolescents, and adults, with a peak incidence between 15 and 34 years [187, 188]. The diagnoses are confirmed by a combination of specific clinical, endoscopic, histological, and radiological criteria [187, 188]. The disease course is highly individual and variable, often with unpredictable periods of symptom flares and remission. A high proportion of patients are on lifelong medication and in need of frequent contact with the healthcare system. Many patients do not respond to medical treatment, where the key treatment goal is mucosal healing on endoscopy [189, 190], and may also be associated with adverse drug reactions, highlighting the relevance of finding new targets for IBD therapy. Several studies have also shown a lower health-related quality of life (HRQoL) [191, 192] and more fatigue [193] in IBD patients when related to the background population.
The pathogenesis behind both CD and UC are multifactorial comprising environmental changes, more than 200 gene variants, abnormal gut microbiota together with a dysregulated immune response. Despite a considerable research effort and improved treatment of these patients, the pathogenesis of IBD is still far from resolved [194].

**Immunopathogenesis in IBD**

The adaptive immune response has traditionally been considered to play a significant role in the pathogenesis of IBD. However, advances in genome-wide association studies (GWAS) and immunological studies have recently moved the focus of IBD pathogenesis on to mucosal innate immune responses, such as epithelial barrier integrity, innate microbial sensing, autophagy and unfolded protein response, as central pathogenic pathways in IBD [195]. With the new knowledge from GWAS, the overall pathogenesis of IBD must be interpreted in a broader context in which genetics, the microbiota and environmental factors contribute to the pathological immune response [194]. The “hygiene hypothesis” indicates a correlation between the decrease in infectious diseases, lack of parasites, use of antibiotics, vaccination and general improvement in food, water and sanitary conditions with an increase in the incidence of autoimmune and chronic inflammatory disorders [196]. This finding supports the fact that the microbiota is fundamental to the “education” of the immune system after birth. A balanced gut microbiota promotes the differentiation of naive gut DCs into tolerogenic DCs followed by generation of regulatory (T_{reg}) T cells and the establishment of immune homeostasis. This understanding is supported by epidemiological studies of IBD, demonstrating a link between IBD immunopathogenesis and dysbiosis of the gut microbiota [197].

**The gut microbiota and epithelial barrier**

The gastrointestinal tract harbours a microbial community estimated to contain more than 1000 bacterial species and a microbial load of about $10^{13}$–$10^{14}$ microorganisms, and metagenomic studies have revealed an impressive number of microbial genes that powerfully influence host gene expression [198]. Early environmental exposures, including delivery mode, milk, food, hygiene and several other factors exert a fundamental effect on shaping the intestinal microbiota in childhood [199], while in adulthood, the gut microbiota is more stable. Microbe-associated molecular patterns (MAMPs; including pathogen-associated molecular patterns, PAMPs) are sensed by innate immune receptors, such as TLRs and cytosolic NLRs, a process essential to intestinal homeostasis [200, 201]. Abnormalities of the
gut microbiota (dysbiosis) are present in both forms of IBD, either quantitatively or qualitatively [202], and evidence of abnormal gut microbiota in patients with UC has also been documented, but to a somewhat lesser degree than for CD [203]. The microbial dysbiosis is a result of complex interactions with environmental factors such as diet, smoking, infections, and geographical regions as well as genetic modifications associated with IBD susceptibility gene pathways that include microbiota recognition (CARD15/NOD2 and TLR4), microbial clearance (autophagy genes - ATG16L1, IRGM), immune response (IL-23R, JAK2, TNFSF15), and mucosal barrier function (IBD5) [204]. The impaired bacterial recognition and clearance due to defects in the innate immune response, including neutrophil dysfunction, allows the entry of microbial species into the epithelial cells and a breach in the mucosal integrity forming an important hypothesis for IBD pathogenesis [205].

Inadequate acute inflammatory response to intestinal MAMPs could impair innate removal of antigens and trigger a compensatory adaptive immune response, eventually leading to a damaging chronic inflammation [206]. Patients with IBD have a compromised mucus layer and an epithelial surface that is densely coated with bacteria, which may contribute to the barrier defect observed in IBD [207].

To date, human microbiome studies have mostly focused on bacterial components of the microbiome, though emerging data suggest that the viral components of the microbiome (virome) can have a profound impact on the host. The functionality of the virome is not well defined although recent studies on mice suggest the virome does, in fact, play an important role [208]. New evidence demonstrates that disease-specific alterations exist in the gut virome in IBD [209]. This study showed that the IBD gut virome is abnormal. Importantly, the expansion and diversification of the gut virome were found to be independent of alterations in bacterial populations.

The research on the human fungal microbial populations (mycobiome) in IBD is of novel date. Fungal DNA accounts for approximately 0.2% of the mucosa-associated microbiota and 0.3% of the fecal microbiota [210]. Only a few studies have investigated the mycobiome for IBD patients were data revealed a skewed mycobiome but of unknown clinical relevance [211, 212].

**Genetics**

At present (2017), genome-wide association studies (GWAS) have identified 215 IBD-associated loci that have substantially expanded understanding of the biology underlying these diseases [213]. In 2012, 163 genetic IBD-loci had been found, and 110 were associated with
both forms of IBD, 30 and 23 specific to CD and UC, respectively [214]. In addition, multiple interactions among gene variants are probably involved in defective microbial processing in IBD [215]. The concordance rate in monozygotic twins of 10-15% in UC compared with 30-35% in CD suggests that non-genetic factors may have an even more important role in UC than in CD [216]. Studies on dysregulated genes in IBD have demonstrated aberrations concerning intestinal barrier function, epithelial restitution, microbial defense, innate and adaptive immune regulation, autophagy and endoplasmatic reticulum (ER) stress [217].

A few epigenome-wide association studies (EWAS) on IBD have been performed since 2011, and are believed to play a role in IBD pathogenesis, as in the regulation of the TNFα and IL-1β gene expression with different sensitivity to DNA methylation [218]. Epigenetics refers to the mechanisms that modulate gene expression without altering the DNA sequence. Epigenetic processes involve DNA methylation, histone modifications that modulate chromatin structure or noncoding RNAs (ncRNAs) such as microRNAs or PIWI-interacting (pi) RNAs that are involved in the regulation of the posttranscriptional steps. All these mechanisms can be affected by exposure to environmental factors and are transmissible from cell generation to cell generation, but can also be reversed [219].

**Innate immunity**

The innermost intestinal barrier (fig. 5) is formed primarily of mucus glycoproteins, trefoil peptides, IgA and antimicrobial peptides (AMPs). Goblet cells generate the mucus layer, which is essentially devoid of microbes. In IBD patients the mucus layer is compromised and contain an increased load of mucolytic bacteria [220]. Antimicrobial peptides (AMPs), that prevents microbial invasion and control the composition of the gut microflora, are secreted by Paneth cells in the crypts of the small intestine. Also the mucosal epithelial cells produce AMPs, cytokines and chemokines for mucosal protection against invading microbes [221]. The AMPs are small and mainly cationic proteins [222] with antimicrobial activity against bacteria, fungi, viruses and protozoa [223]. In mammals, defensins and cathelicidins are the two major classes of AMPs [224, 225], produced by granulocytes, Paneth cells of the small intestine (α-defensins) and enterocytes (β-defensins). Paneth cells produce large amounts of α-defensins and other antimicrobial peptides, such as lysozymes and secretory phospholipase A2 (sPLA2) [221, 222]. The intestinal barrier integrity in IBD is genetically affected, including the structure of tight and adherens junctions and desmosomes [226-229], thereby suggesting that epithelial barrier defects represent a primary pathogenetic mechanism [207].
Defective epithelial barrier and increased intestinal permeability have long been observed in patients with both CD and UC [230].

Behind the pre-epithelial physical defense are key players of the cellular innate immune system, such as macrophages releasing cytokines for recruitment of neutrophils to generate an inflammation for the elimination of the pathogen.

Figure 5. Innate immune responses in the gut

Mucous layer and epithelial barrier represent the first line of defense against bacterial invasion. Epithelial cells, stromal cells and innate immune cells, such as macrophages and dendritic cells (DCs) can sense invading bacteria through extracellular and intracellular pattern recognition. Innate lymphoid cells (ILCs) are also found in the human lamina propria where they may contribute to cytokine production and inflammatory cell recruitment. Reprinted with permission. Copyright © 2013 Elsevier B.V.

The contribution of neutrophils in IBD pathogenesis includes oxidative and proteolytic damage of epithelial barrier function, tissues and the continuous release of mediators maintaining inflammation [231]. The first line of cellular defense for killing bacteria and fungi are neutrophils [232]. Interestingly, neutrophils may also reduce inflammation, for example by synthesizing lipoxin A₄. Impaired secretion of the anti-inflammatory lipoxin A₄ has been demonstrated in gut mucosa from patients with [207].

Pivotal functions of dendritic cells are monitoring of the microenvironment, sampling of antigens to create immune tolerance or develop a host defense pro-inflammatory response.
This dual function puts DCs in the unique position of controlling the interaction between innate and adaptive immunity [233]. Mucosal DCs through interactions with T- and B cells, intestinal epithelium and stromal cells, are enabled to maintain mucosal homeostasis or induce inflammation [234]. In IBD, the DCs express increased levels of TLR2 and TLR4 compared with normal control mucosa [235].

Intestinal epithelial cells (IECs) perform both barrier and signal-transduction functions by sensing luminal contents through surface receptors and consequently secrete regulatory products that can create an appropriate immune response in the underlying lamina propria [236]. IBD-associated IECs are impaired in their capacity of inducing CD8⁺ T suppressor cells, suggesting a possible defect in mucosal immunoregulation predisposing to IBD [236].

Autophagy contributes to the degradation and recycling of cytosolic contents and organelles, as well as to resistance against infection and removal of intracellular microbes [237]. Defects in autophagy in IBD patients can reduce clearance of bacteria and result in an increased immune response [238]. Closely related to autophagy and innate immunity, dysregulation of the unfolded protein response (UPR) may also contribute to IBD pathogenesis. The unfolded protein response is induced by endoplasmic reticulum (ER) stress due to an accumulation of misfolded or unfolded proteins within the ER [239].

When there is a mucosal injury or inflammation of the gut wall, surrounding IECs migrate to the lesion to initiate wound healing, a process is called “epithelial restitution” [240]. This process is followed by additional steps in wound healing such as cell proliferation, expansion, migration, and differentiation, and ultimately leads to closure of erosions and ulcerations. This process is supported by antimicrobial peptides and products released by Paneth cells such as defensins and REG (regenerating protein family) proteins [183, 241] as well as by mucins that prevent bacterial translocation and immune cell activation. Several transcription factors are involved in epithelial regeneration, which controls crypt cell proliferation and IEC differentiation [242-244].
Figure 6. The Intestinal Immune System

Mucus from goblet cells, antimicrobial peptides from Paneth cells, and production of IgA provide additional protection from luminal microbiota. Innate microbial sensing by epithelial cells, dendritic cells, and macrophages is mediated through PRRs such as TLRs and NOD proteins. Dendritic cells present antigens to naive CD4+ T cells in secondary lymphoid organs (Peyers patches and mesenteric lymph nodes), where factors such as the phenotype of the antigen-presenting cells and the cytokine milieu (TGF-β, and IL-10) modulate differentiation of CD4+ T-cell subgroups with characteristic cytokine profiles (regulatory T cells, e.g., Treg, and helper T cells, e.g., Th1, Th2, and Th17), and enterotropic molecules (e.g., α4β7) are induced that provide for gut homing of lymphocytes from the systemic circulation. These activated CD4+ T cells then circulate to the intestinal lamina propria, where they carry out effector functions.

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Adaptive immunity
In the cellular adaptive immune response, T\textsubscript{H}0 cells can become activated and either differentiate into T\textsubscript{H}1, T\textsubscript{H}2 or T\textsubscript{H}17 cells. This is an essential process, which contributes to the clearance of specific pathogens. In particular T\textsubscript{H}1 cells are essential for the elimination of intracellular pathogens, T\textsubscript{H}2 cells are protective against parasites and mediate allergic reactions, and T\textsubscript{H}17 may contribute to the clearance of extracellular bacteria and fungi \cite{246, 247}. However, a dysregulated T cell response with abnormal development of activated T cell subsets may lead to the onset of inflammation by an excessive release of cytokines and chemokines, which have multiple pathogenic effects on the components of both the adaptive and the innate branches of the immune system.

On activation, CD4\textsuperscript{+} T cells secrete a particular set of cytokines that determine the type of ensuing immune response and the phenotype of the mature effector T cells. CD4\textsuperscript{+} T cells activated in the presence of IL-12 and the absence of IL-4 acquire a T helper 1 (T\textsubscript{H}1) phenotype, resulting in IFN\textgamma\textsuperscript{-}-producing cells effective in the control of intracellular pathogens. CD4\textsuperscript{+} T cells activated in the presence of IL-4 acquire a T\textsubscript{H}2 phenotype, generating IL-4-, IL-5- and IL-13-producing cells effective in the clearance of parasitic infections and allergic responses. CD4\textsuperscript{+} T cell activation in the presence of IL-6, TGF-\beta, and IL-23, results in T\textsubscript{H}17 phenotype (i.e., IL-17 and IL-6 producing T cells responsible for acute inflammation and recruitment of granulocytes).

Regulatory T cells (T\textsubscript{reg}), characterized by the expression of Foxp3, may be defined as T cells able to suppress T\textsubscript{H}0 cell proliferation both \textit{in vitro} and \textit{in vivo} \cite{248}. T\textsubscript{reg} cells are crucially involved in the maintenance of gut mucosal homeostasis by suppressing abnormal immune responses against the commensal flora or dietary antigens. In particular, T\textsubscript{reg} cells exert their function by producing the anti-inflammatory cytokines IL-10 and TGF-\beta and by preventing both the activation and the effector function of T cells that have escaped other mechanisms of tolerance \cite{249}. IBD immunopathogenesis is due, at least in part, to insufficient suppressor function \cite{250}. Boosting T\textsubscript{reg} cell function may have beneficial effects as a target for IBD treatment, a possibility supported by finding that TNF-blockade restores the suppressive function of T\textsubscript{reg} cells \cite{249}.

CD4\textsuperscript{+} T cell on activation by bacterial antigen recognition interacts with B cells (CD40) through their cell-surface molecule CD40 ligand. This interaction results in the activation of B cells leading to their proliferation and initiation of antibody secretion of IgA and IgM that protect the epithelial barrier from commensal and pathogenic bacteria \cite{251}. These IgA can bind and coat the pathogens to neutralize them, thus protecting the intestine against bacterial
penetration and infection [252]. Homing pathways coordinate the movement of lymphocytes into the site of intestinal inflammation. Chemokine receptor and ligand interactions are essential for the process of migration of lymphocytes from the vasculature to the tissue microenvironment in inflammation [253].

**Cytokines in IBD**

The cytokine responses characterizing IBD are key pathophysiologic elements that govern the initiation, evolution and, ultimately, the resolution of these forms of inflammation [254]. Cytokines not only drive intestinal inflammation and associated symptoms, such as diarrhea, but many also regulate extraintestinal disease manifestations (for example, arthralgia or arthritis) and systemic effects in IBD [255, 256]. Furthermore, cytokines seem to have a crucial role in the pathogenesis of progressive and destructive forms of IBD that are associated with complications such as intestinal stenosis, rectal bleeding, abscess- and fistula formation [183, 184].

Until less than a decade ago, CD was designated as a T\(\text{H}1\) condition based on an elevated production of IL-12 and IFN\(\gamma\), whereas UC was characterized as an atypical T\(\text{H}2\) condition based on an enhanced production of IL-5 and IL-13, but low levels of IL-4. This T\(\text{H}1\)/T\(\text{H}2\) paradigm was later revised with the discovery of IL-17-producing T\(\text{H}17\) cells and the interplay among T\(\text{H}1\), T\(\text{H}2\), T\(\text{H}17\) and T\(\text{reg}\) cells [257]. The mucosal lymphocytes in CD produce both IL-17 and IFN\(\gamma\), redefining this form of IBD as a mix T\(\text{H}1\) and T\(\text{H}1\)/T\(\text{H}17\) condition [258], although the T\(\text{H}1\) response is believed to be quantitatively greater and thus more likely to be the driving force of inflammation [170]. Increased IL-17 expression is found in the mucosa and serum of most patients with IBD, but is consistently higher in those with CD than those with UC [259].

Cytokine expression profiles from both forms of IBD mucosa have found increased levels of IL-1\(\beta\), IL-6, and TNF\(\alpha\), together with decreased levels of IL-4. Furthermore, CD and UC show different cytokine profiles from the mucosa, with elevated levels of IL-2, IFN\(\gamma\), IL-12 and IL-18 in CD, and IL-5 and IL-13 for UC.

Most data on cytokine profiles in IBD are from mucosal samples. However, in a recent study by Korolkova et al. (2015), 23 out of 38 cytokines were measurable in serum samples from 25 and 28 patients with UC and CD, respectively, and with a control group of 30 persons. They found significantly increased levels in serum for the cytokines eotaxin, GRO and TNF\(\alpha\) for UC, and IFN\(\gamma\), IL-6 and IL-7 for CD, compared with the control group. IL-8
was increased for both conditions [260]. Other studies have detected increased serum levels of IL-6 [87] and TNFα [261] in IBD, while increased MIP-1β was only found in UC [262].

A general notion is that the extent and degree of disease (acute or chronic) affects the cytokine levels in the course of IBD [170]. Moreover, the pathological mechanisms driving the mucosal inflammation are likely to differ between patients, and, in addition, a blockade of a single cytokine in patients with IBD may lead to the development of alternative compensatory pro-inflammatory cytokine pathways. This may explain why the attempts of targeting of a single pro-inflammatory cytokine are not effective in many cases [170].

**Other factors contributing to the pathogenesis of IBD**

*Intestinal fibrosis.* The excessive deposition of extracellular matrix (ECM), constitutes a common complication of IBD leading to stricture formation and obstruction [263]. Fibrosis in CD is more pronounced and usually transmural [264, 265], but fibrosis also occurs in UC, in which ECM deposits in the superficial layers of the intestinal wall [266]. Multiple metalloproteinases (MMPs) are highly expressed in IBD tissues [267], the breakdown of collagen fibers being mediated by interstitial collagenase (MMP-1) and MMP-2, whereas fistula formation in CD has been associated with increased expression of MMP-3 and MMP-9 [268].

*Angiogenesis* is also thought to contribute to the IBD pathogenesis [269] because mucosal and plasma levels of vascular endothelial growth factor (VEGF)-A are upregulated in IBD patients. The gut microbiota is also believed to have potential stimulating and inhibiting contributions in the angiogenesis [270-272], and thereby a therapeutic potential in IBD.

*Lymphangiogenesis.* The lymphatic vasculature is essential to immune regulation as it is responsible for draining and removal of antigens and leukocytes from sites of inflammation [273]. Chronic inflammation may lead to leukocyte retention and reduced antigen processing, which then promotes lymphangiogenesis and inflammation [274]. In mice, it has been shown that administration of the pro-lymphangiogenic factor VEGF-C protected against acute and chronic colitis. This effect was explained by increased recruitment and bacterial clearance by leukocytes from inflamed tissue to draining lymph nodes [275].

*The redox state.* The reduction and oxidation (redox) state of the gut depends on the equilibrium between oxidants, such as free radicals, ROS or reactive nitrogen species, and antioxidant mechanisms [276]. This redox state affects many signal-transduction pathways, such as NF-kB signaling and AMP activity [276]. ROS have important antimicrobial activity, and contribute to intracellular signaling, promoting the production of pro-inflammatory
cytokines. Genes within several IBD-associated loci may either regulate ROS production or protect against oxidative stress. In addition to pro-inflammatory pathways, ROS are also involved in T$_{reg}$ cell polarization and function [277, 278].
Methodological considerations

Ethics
The actual study was approved on April 8, 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 (December 15, 2011). The authors confirm that all ongoing and related trials for AndoSan™ are registered.

Competing Interests
Two of the authors (GH and EJ) have patent/patent applications and financial interests relating to the material (AndoSan™) pertinent to studies in this thesis: i) WO2005065063 A2, Appl. No.: 10/ 585600, NO- and PCT-filed Jan 2004 and Jan 2005, respectively, by Inventor Geir Hetland, and ii) NO20090003383, Appl. No.: NO20090003383 20091119, by Inventors Geir Hetland and Egil Johnson and filed by Applicant Immunopharma AS in Nov 2009 and financial interest of Geir Hetland as a shareholder in Immunopharma AS of Norway, commercializing AndoSan™.

Criteria for inclusion and exclusion of patients
173 patients with CD and 210 patients with UC were phone-interviewed, and those with simple Crohn’s disease activity index (SCDAI-) and clinical activity index (CAI) score of at least 3 were given the opportunity to join the study. At the first attendance, SCDAI and CAI were re-recorded, and criteria for exclusion were pregnancy, biological treatment with antibodies to TNFα (Adalimumab, Infliximab), daily use of more than 5 mg of prednisolone, change of medication and/or consumption of mushroom products from two weeks before till end of the study. (Additional reasons for exclusions of patients are explained in flow charts in both paper I and II).

Experimental Design and Randomization
This is a single-center randomized two-armed patient-blinded study designed to determine in
this setting whether daily oral intake of a mushroom extract, AndoSan™, in addition to measuring clinical symptoms, fatigue and quality of life in patients with CD or UC during the 21-day study period, influenced the level of 17 different cytokines, with emphasis on the pro-inflammatory cytokines. Patient evaluation, fecal tests 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 assays from visit 1 and 3 were analyzed. The placebo group was evaluated likewise but received an equal volume of color-like drink with ionized water containing 0.5 ml per liter of caramel color (E150c) with salt.

Block-randomization was done after the phone interview, with uneven and even numbers given for AndoSan™ or placebo, respectively. The patients, one by one, were placed in one pile, and the group affiliations were placed in another pile. The randomization was performed by combining one selection from each pile, both anonymized. The first author performed the randomization, enrolled the participants, and assigned participants to interventions. A few patients were excluded throughout the study by not attending or because of intercurrent incidents (disease, unexpected life events). Accordingly, a slight imbalance of the study groups occurred that was corrected for in the latter rounds of randomization. Patients in the AndoSan™ group and the placebo group self-reported, in writing, at visit 1, 2 and 3 regarding symptoms, fatigue and health-related quality of life. Patient-derived blood samples and fecal calprotectin from these visits were also analyzed. All data were stored in a secure database (Access–Microsoft Office) at a server at Oslo University Hospital, Ullevål, Norway. A study number anonymized the patients. 50 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.

**Symptom score**

The recruitment of IBD patients was in collaboration with the Department of Medicine, Oslo University Hospital, Ullevål, and the gastroenterologists recommended the symptom score questionnaires we chose to use in our studies.

The patient-reported symptom score was for CD the SCDAI, a.k.a. the Harvey-Bradshaw index [279]. This index was devised in 1980 as a simpler version of the Crohn’s disease activity index (CDAI) for data collection purposes. Multiple studies have demonstrated that the SCDAI show a poor correlation with mucosal inflammation [280, 281], but is well correlated with both fatigue and HRQoL [193, 282]. 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 extraintestinal manifestations (arthritis, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula, abscess (score 1 per item)). The symptom score ranges from 0–21. Scores 3–5 meant mild, 6–9 moderate and over 9 severe disease activity. A criterion for inclusion was a score beyond 2. In a review from 2011, the simple Crohn’s Disease Activity Index was found to be a valid, reliable and responsive tool for the measurement of CD activity, when compared to the original CDAI [283].

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) [284]. 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 0–15. There are very limited studies on the modified CAI, making it somewhat difficult to compare our results with previous studies done on this parameter. Other symptom scorings on UC, like Simple Clinical Colitis Activity Index (SCCAI), have shown a good correlation with mucosal inflammation, fatigue and HRQoL [193, 282]. In a review article by D’Haens et al. [285], investigating six symptom-based activity scores for UC, including the CAI, they found that virtually none of the instruments have been validated. All of the activity scores included bowel frequency and blood, four out of six included abdominal pain and general well-being, and three included stool consistency. The five items in the modified CAI represent the most frequently used items in symptom-based activity scores for UC [285].

The symptom score questionnaires, constituting a total of 1500 questions for both CD and UC in this study, were answered correctly without any missing values.

**Health-related quality of life, HRQoL**

The short form 36 (SF-36) is a generic HRQoL questionnaire designed to assess functional status, well-being and general perception of health. The questionnaire has been shown to have high validity and reliability [286, 287] and is one of the most frequently used generic HRQoL instruments.

Self-reported health-related quality of life (HRQoL) was assessed with the SF-36 (IQOLA SF-36 Norwegian version 1.2), which consists of 36 items, of which 35 are grouped into the following eight health domains: (1) physical functioning (PF), (2) social functioning (SF), (3) role limitations due to physical problems (RP), (4) role limitation due to emotional problems...
(RE), (5) mental health (MH), (6) vitality (VT), (7) bodily pain (BP) and (8) general health perception (GH). Each domain is graded on a scale of 0–100, and the higher the score, the better the HRQoL. The validity and reliability of the SF-36 form have been demonstrated for a number of countries including Norway (version 1) [288]. The data were compared with published norms from 2323 individuals in the general population [288].

In the CD patients, only 30 out of 5400 HRQoL questions were unanswered, and accordingly, 17 out of 1200 dimensions were lacking. Using a scoring algorithm for missing data outlined in the SF 36 survey manual, still, 5 out of 17 dimensions involving 3 patients in the placebo group, could not be included in the results.

In the UC patients, only 11 out of 5400 HRQoL questions were unanswered, and accordingly, 10 out of 1200 dimensions were lacking. Using a scoring algorithm for missing data outlined in the SF 36 survey manual, these 10 dimensions could also be included in the results.

Fatigue
Fatigue, which is expressed as a “persistent feeling of tiredness, reduced energy levels, reduced muscle strength and cognitive impairment” [289], is recognized as a major concern for IBD patients [290]. The fatigue questionnaire (FQ) [291] is translated to Norwegian and validated in a Norwegian reference population [292], and consists of total fatigue (11 items of graded questions with score 0–3 per question), which is the sum of physical fatigue (7 items) and mental fatigue (4 items). The respective scores for total, mental and physical fatigue are 0–33, 0–21 and 0–12, and the higher score, the more fatigue. The items of physical (1–7) and mental (8–11) fatigue were: 1) Do you have problems with tiredness? 2) Do you need to rest more? 3) Do you feel sleepy or drowsy? 4) Do you have problems with starting things? 5) Are you lacking in energy? 6) Do you have less strength in your muscles? 7) Do you feel weak? 8) Do you have difficulty concentrating? 9) Do you have problems thinking clearly? 10) Do you make slips of the tongue when speaking? 11) How is your memory? Criteria for chronic fatigue syndrome was a dichotomized score >4 and duration>6 months [291, 292].

The fatigue questionnaires, constituting a total of 3300 questions for both CD and UC in this study, were answered correctly without any missing values. The data were compared with published norms from 2323 individuals in the general population [292].
**Multiplex cytokine assay**

We used the multiplex bead-based sandwich immunoassay performed using Bio-Plex xMAP technology (Bio-Rad, Austin Texas, USA) with a Luminex IS 100 instrument (Bio-Rad, Hercules, California, USA), powered using Bio-Plex Manager (version 6.0.1) software for analysis of 17 different cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, IFNγ, MCP-1, MIP-1β and TNFα) following manufacturers’ instructions and detailed elsewhere [293]. These particular cytokines were chosen because they cover typical pro- (TNFα, IL-1β, -6, -12) and anti-inflammatory (IL-6, -10, T₃H1- (IFNγ, IL-2, -12), T₃H2 (IL-4, -5, -13) and T₃H17 (IL-17) type of cytokines and growth factors (IL-7, G- and GM-SCF) and chemokines (IL-5, -8, MCP-1, MIP-1β) for leukocytes. Briefly, samples were thawed on ice, vortexed, and then spun down at 10,000xg for 10 min at 4 °C before dilution and further processing. The StatLIA software package (ver. 3.2, Brendan Scientific Carlsbad, California, USA), incorporating a weighted, five-parameter logistic curve-fitting method, was used to calculate sample cytokine concentrations. Controls were used in order to validate interassay variation. One test of each sample (visit 1 and 3) was run on the same plate, thereby avoiding intra-assay variation. In order to optimize the assay for low-level detection, the cytokine analysis process included i) a standard point in addition to the vendor’s recommendation and ii) use of a magnetic plate washer to yield more reliable results. There was an interassay coefficient of variation, usually from 10-30%, 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, in order 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. The baseline for upper out of range levels was given as the highest measurable cytokine concentration, while undetectable levels were set to 0 pg/ml that excluded the possibility of comparing the results by using relative indices.

The number of cytokines in upper out of range levels at baseline for both CD (n=50) and UC (n=50) were 30% for MIP-1β, but less than 2% for the remaining 16 cytokines. The lower detection limits (pg/ml) for the 17 cytokines were; IL-1β (0.39), IL-2 (1.79), IL-4 (0.17), IL-5 (0.16), IL-6 (8.25), IL-7 (0.23), IL-8 (33.1), IL-10 (1.79) IL-12 (0.87), IL-13 (0.33), IL-17 (7.61), G-CSF (6.12), GM-CSF (2.32), IFNγ (2.36), MCP-1 (31.24), MIP-1β (95.1) and TNFα (5.24).
Statistical analysis

In paper I and II, the data are presented as the mean and standard deviation or as median and range values. Paired sample t-test and Wilcoxon test are used for within-group analysis. The judgment of whether the distributions of the main efficacy variables were so close to the normal distribution that normality-based significance tests might be used, is based on the finding in a paper by Fagerland and Sandvik [294]. Mixed models corrected for baseline values were used for measuring P values between the AndoSan™ and placebo groups, using visit 1, 2 and 3 with time as a continuous variable. P values below 0.05 were considered statistically significant. The SPSS statistical program for the social sciences, version 22 (IBM), was used in the analyses.

In Paper III, data are presented as median and range values. Wilcoxon test and Mann-Whitney test were used for within-group- or inter-group 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.

This study on clinical outcome is a follow-up of a previous pilot study [66] in which there was a reduction of pro-inflammatory cytokines and chemokines in patients receiving the same daily dose of AndoSan™, but for 12 days. Prospective differences of 20% between the experimental and placebo group and assumed standard deviation of 20% for the different parameters with a significance level of 5% and a power of 90% (β = 0.10), demanded about 25 patients per randomized arm (calculated in cooperation Oslo Center for Biostatistics and Epidemiology, Oslo University Hospital).

Aims of the study

Inflammatory bowel diseases like ulcerative colitis (UC) and Crohn´s disease (CD) are bothersome conditions of unknown etiology. We have shown in a previous pilot study anti-inflammatory effects by ingestion of AndoSan™ in IBD patients as measured by reduction in pro-inflammatory cytokines and also fecal calprotectin [66]. On this background, we wanted to examine whether AndoSan™, in a placebo-controlled randomized study, improved clinical parameters and level of cytokines in these patients.
General summary

Paper I:
Ingestion of AndoSantm has previously been shown to exhibit anti-inflammatory effects as shown by of reduction of pro-inflammatory cytokines in healthy individuals and patients with ulcerative colitis. In this randomized single-blinded placebo-controlled study we examined whether intake of AndoSantm also resulted in clinical effects.

50 patients with symptomatic ulcerative colitis were block-randomized and blinded for oral daily intake of AndoSantm or placebo for the 21 days’ experimental period. The patients reported scores for symptoms, fatigue and health-related quality of life (HRQoL) at days 0, 14 and 21. Fecal calprotectin and general blood parameters were also analyzed. In the AndoSantm group (n=24) symptoms improved from baseline (day 0) to days 14 and 21, with respective mean scores (95% CI) of 5.88 (4.92-6.83), 4.71 (3.90-5.52) (p=0.002) and 4.50 (3.70-5.30) (p=0.001). Corresponding improved mean scores (±SD) for total fatigue were 16.6 (5.59), 14.1 (4.50) (p=0.001) and 15.1 (4.09) (p=0.023). These scores in the placebo group (n=26) were not improved. When comparing the two study groups using mixed model statistics, we found significantly better scores for the AndoSantm-patients. HRQoL for dimensions bodily pain, vitality, social functioning and mental health improved in the AndoSantm group. There were no alterations in general blood samples and fecal calprotectin.

Beneficiary effects on symptoms, fatigue and HRQoL from AndoSantm consumption were demonstrated in this per-protocol study, supporting its use as a supplement to conventional medication for patients with mild to moderate symptoms from ulcerative colitis. The patients did not report any harms or unintended effects of AndoSantm in this study.

Paper II:
50 patients with symptomatic CD were randomized for oral daily consumption of AndoSantm or placebo for a 21-day experimental period, in this per-protocol study. Patients reported validated scores for symptoms, fatigue and health-related quality of life (HRQoL) at days 0, 14 and 21. Fecal calprotectin and general blood parameters were also analyzed. In the AndoSantm group (n=25) symptoms improved from baseline (day 0) to days 14 and 21, with respective mean scores (95% CI) of 5.52 (4.64-6.40), 4.48 (3.69-5.27) and 4.08 (3.22-4.94) (p<0.001). We found significant improvements in symptom score for both genders in the AndoSantm group and no significant changes in the placebo (n=25) group. There were,
however, no significant differences between the groups (p=0.106), although a marginal effect in symptom score for men (p=0.054). There were comparable improvements in physical, mental and total fatigue for both groups. HRQoL versus baseline were at day 21 improved for bodily pain and vitality in the AndoSan™ group and for vitality and social functioning in the placebo group. No crucial changes in general blood samples and fecal calprotectin were detected. The results from this single-blinded randomized clinical trial show significant improvement in symptoms, for both genders, in the AndoSan™ group, but no significant differences between the study groups. The results on fatigue, HRQoL, fecal calprotectin and blood samples were quite similar compared with placebo. The patients did not report any harms or unintended effects of AndoSan™. CD patients with mild to moderate symptoms may have beneficiary effects of AndoSan™ as a safe supplement in addition to conventional medication.

**Paper III:**
Ingestion of the mushroom extract AndoSan™ has been shown in randomized placebo-controlled studies to improve symptoms of 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 analyzed for cytokines IL-1ß, IL-2, IL-4-8, IL-10, IL-12-13, IL-17, G-CSF, GM-CSF, IFNγ, MCP-1, MIP-1ß and TNFα. 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-1ß, IL-6 and G-CSF in the patients with CD, the cytokine levels at visit 3 were significantly lower in the Andosan™ group – versus the placebo group. In UC levels of IL-2, IL-5 and MIP-1ß 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 antiallergic and antioxidant activities.
General discussion

Inflammatory bowel diseases like ulcerative colitis (UC) and Crohn’s disease (CD) are bothersome conditions of unknown etiology. We have shown in a previous pilot study anti-inflammatory effects by ingestion of AndoSan™ in IBD patients as measured by reduction in pro-inflammatory cytokines and also fecal calprotectin [66]. On this background, the aim of this thesis was to determine whether the effect of AndoSan™ could be reproduced clinically and by measurement of cytokines in a prospective, placebo-controlled randomized study. This thesis is based on three single-blinded randomized studies on 50 symptomatic UC and CD patients who ingested 60 ml/day of the AbM-based mushroom extract AndoSan™ or placebo for a three-week study period. The major findings were improvements in clinical parameters, especially for the UC patients, both within the AndoSan™ group and when compared with placebo. However, AndoSan™ did largely not influence the plasma levels of the 17 different cytokines analyzed throughout the study period. At best, a marginal anti-inflammatory effect for the CD patients consuming AndoSan™ may be interpreted from the cytokine analysis.

All of the patients, 210 with UC and 173 with CD, interviewed to join this study had been diagnosed with IBD by a gastroenterologist, at the Department of Medicine at Oslo University Hospital, Ullevål. The included patients in this per-protocol study were randomized, resulting in comparable patient characteristics at baseline between the placebo and AndoSan™ groups, for both CD and UC patients. The randomization was not blinded for the authors, leaving a possible bias of the result. This is especially true for the first author who was responsible for the inclusion and randomization of participants, the implementation of the practical aspects of and in meeting with the patients, and also in the analysis of the results. 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 three weeks.

The moderate reductions in the patient-reported symptom scores were significant for patients in the AndoSan™ group for both CD and UC, but only significant in UC patients when compared with placebo. However, there was a close to significant p-value (0.054) when comparing the symptom score for men among CD patients. The reductions in symptom score in the AndoSan™ group were 23% and 26% in UC and CD patients, respectively. Interestingly, in the subgroup analysis, there was a significant reduction in stool frequency for both CD and UC, together with a patient-reported improved stool consistency in UC patients,
all in the AndoSan™ groups. No such effects were found in the placebo groups. Deterioration in stool consistency is probably one of the most frequent and bothersome concerns of symptomatic IBD.

The second major finding was significant improvements in total, physical and mental fatigue for the UC patients when compared with placebo. Such effects were not present in CD patients, where the fatigue scores actually improved more in the placebo group than in the AndoSan™ group. Obviously, the clinical improvement due to the intake of this mushroom extract in patients with CD was more limited vs. those with UC. This may partly be explained by the fact that CD is pan-intestinal and characterized by transmural inflammation complicated by stenosis and/or development of fistulas, together with a higher rate of extraintestinal manifestations than in UC. The suggestion that environmental rather than genetical factors are more important in UC than in CD [216] may partly also explain why AndoSan™ had a more clinical impact in the former disease.

We compared the fatigue scores with a Norwegian background population [292] with significant lower fatigue scores for both CD and UC in our study. We also found chronic fatigue syndrome (CFS) at 40% and 30%, in CD and UC, respectively, compared with about 11% in the background population [292]. Previous studies are inconsistent on this matter [295-298], with different findings regarding fatigue scores, CFS, and fatigue scores compared with a background population. Fatigue is particularly problematic during active disease with prevalence rates reported as high as 86% [299]. Some studies report that between 40% to 83% of patients continue to experience high rates of fatigue, even during remission [295, 296, 300], that are comparable with levels experienced by oncology patients [301]. Huppertz-Hauss et al. [193] published in 2017 fatigue scores in a Norwegian population-based cohort of patients with IBD 20 years after diagnosis (the IBSEN study). The main findings from this study were significantly higher fatigue scores for both CD and UC patients with active disease than those with quiescent disease, and CFS was more frequent among IBD patients than in the reference population [292]. Factors associated with fatigue included self-perceived disease activity, poor sleep quality, anxiety and depression. Women had higher fatigue scores and experienced more frequent CFS than men, and they found no statistically significant difference in mean fatigue scores between IBD patients with subjectively perceived inactive disease and the reference population. This study was a 20-year follow-up of IBD patients, and one may speculate if the impact of IBD on fatigue might decrease over time, e.g. due to improved coping abilities [292].
The third major finding was significant improvements in four out of eight dimensions in the health-related quality of life questionnaire (SF-36) in the AndoSan™ group in UC patients. The dimensions were bodily pain, vitality, social functioning and mental health. In addition, four out of eight dimensions were significantly improved when AndoSan™ was compared with placebo in UC patients, and these dimensions were physical functioning, role limitation physical, bodily pain and social functioning. The results were unaltered or worsened in the placebo group in UC patients. The HRQoL in CD patients showed significant improvements in bodily pain and vitality in the AndoSan™ group, together with significant improvements in vitality and social functioning in the placeo group. When comparing the intervention groups, we found no significant p-values, which consequently led to our conclusion that the effect of AndoSan™ on HRQoL in CD patients was not different from placebo. It was though interesting that the dimension bodily pain was significantly improved in the AndoSan™ group in CD patients, as this is a major concern in active CD.

We compared the HRQoL scores in our study with a Norwegian background population [292] and found significantly lower scores in seven out of eight health dimensions, for both CD and UC. The dimensions vitality and general health had high Z-scores (data not shown) for both CD and UC, indicating a large difference compared with the background population, which is in line with previous studies [191, 192].

In paper III, we analyzed the cytokine levels for the included patients. We chose to study cytokine levels in blood instead of gut mucosa for several reasons. In our study protocol, we planned to do sigmoidoscopy for biopsies and endoscopic evaluation of treatment for the UC patients, but this was not feasible mainly for practical reasons. Secondly, granted that findings in blood reflect disease activity, the representativity of a blood sample would surpass the challenge of hitting the hot spots of disease within the gut mucosa.

The fourth, and final, major finding in this thesis is that oral intake of AndoSan™ influenced cytokine levels marginally for UC and moderately for CD, supporting a weak systemic anti-inflammatory effect (paper III). Therefore, the reported beneficial clinical effects mediated by AndoSan™ for these patients probably also involve other anti-inflammatory compounds, not examined. In more detail, there was an all-over trend in CD of lower pro-inflammatory cytokines, chemokines and growth factor analytes after AndoSan™ compared with consumption of placebo. Since CD is more generalized disease than UC, one may speculate that the AndoSan™ impact in CD, although clinically less than in UC, also was more systemic as shown by greater changes in serum cytokines. With the exception of IL-2 in CD and IL-2, IL-5 and MIP-1β in UC, all other within or intergroup comparisons were
insignificant, also when comparing between the diseases. Despite the fact that a moderate anti-inflammatory effect was present in CD, the more pronounced clinical effect, comprising symptoms, fatigue and HRQoL, was found in patients with UC. Thus, we were not able to demonstrate the same degree of anti-inflammatory effect as previously indicated by the decline in plasma cytokines in the two similar pilot studies [66, 85], without control groups.

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 CD and UC, translated into the reported clinical effects in these IBD patients [88, 89], also must be explained by other mechanisms, which may not affect the cytokine levels in blood. Some of these relevant mechanisms are previously described in the literature, and will be discussed more precisely in the following, and include among others: antioxidant effects including reduction in ROS, antiallergic effects, local immunological effects in the gut by the mushroom extract, and beneficiary changes in the gut microbiota.

In a study on healthy volunteers ingesting AndoSan™ [112], there was a reduction in vivo of ROS mainly reflecting superoxide ions, and again pointing to an anti-inflammatory effect. However, this result was not demonstrated in the CD patients in the pilot study mentioned above (data not shown). One explanation for the reduced superoxide anions may be related to reduction of IL-1β, because ROS inhibitors have been shown to reduce the synthesis of this cytokine in macrophages [302].

In addition, a water-soluble polysaccharide isolated from AbM that was given orally for eight weeks to ovariectionized and osteopenic rats markedly decreased ICAM-1, cyclooxygenase-2, inducible nitric oxide synthetase and total antioxidant status [303]. Moreover, AbM contains absorbable low molecular weight antioxidant substances [50], which downregulate the levels of ROS in vitro [304].

Substances such as β-glucan may further be transported by dendritic cells to lymphocytes in GALT and induce local immune responses there, or systemic if circulated in blood [305, 306]. There also was an antiallergic effect in mice sensitized to ovalbumin (OVA), as demonstrated by a reduction of specific anti-OVA IgE antibodies, both when AndoSan™ was given before or after the OVA immunization [110]. Additionally, in this allergy model, there was an increase in T_H1 relative to T_H2 cytokines in spleen cell cultures ex vivo obtained from the animals treated with AndoSan™. Although it has been speculated that CD is a T_H1 (IL-2, IFNγ, IL-12) – and that UC is a T_H2 ((IL-4), IL-5, IL-13)-related disease based on the T_H1/T_H2 dichotomy [307], the cytokine data presented in paper III actually show comparable baseline cytokine levels and therefore do not clearly support this paradigm. This is also in line
with previous [66] cytokine measurements where the same cytokines were elevated (TNFα, IFNγ, IL-2, IL-6, IL-8, IL-12, IL-17, MCP-1, GM-CSF, MIP-1β) 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.

The inhibitory effect of an isolated carbohydrate fraction of AndoSan™ [106] on the tissue degrading pro-inflammatory and tumor-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. In fact, in an intestinal tumor model in Min/+ mice, AndoSan™ did both reduce tumor load and legumain expression in the intestines [109]. Alkaline and aqueous substances isolated from AbM [51] 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 edema 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 down-regulating the immune system by interactions with β-glucans of the extract [51].

In line with several human studies [55, 66, 85-87], we also demonstrated the safety of AndoSan™ when taken orally. In these studies, there were no subjective side effects or adverse effects on hematological parameters, electrolyte balance, liver-, pancreatic- and renal function. Regarding possible drug interactions, AndoSan™ did less than green tea inhibit the transmembrane efflux P-glycoprotein (P-gp) pump present in intestines and liver and hence important for drug absorption and excretion [76]. However, possible interactions may occur with P-gp substrates, e.g. some anticancer, diarrhea (loperamide) and cardiac (digoxin) agents, and P-gp inhibitors, e.g. verapamil. When testing AndoSan™ on cytochrome P-450 metabolism, the extract inhibited it, but far less than green tea and clinically relevant systemic interactions were therefore considered unlikely [78]. In our clinical study, none of the IBD patients were concomitantly treated with the above-mentioned anticancer-, heart-, or diarrhea drugs.

Moreover, contrary to a previous pilot study in which 10 UC patients received the same amount of AndoSan™ for 12 days with a significant reduction of fecal calprotectin, we did not find such effect. One reason for lack of reduction of fecal calprotectin in our studies could be the large variability of baseline calprotectin levels (range 10–6000) that was not seen in the previous small pilot study (128–1683) [66].
In paper I with UC patients consuming AndoSan™, the improvements in addition to symptoms irrefutably were also evident for fatigue and HRQoL. Obviously, the clinical improvement due to the intake of this mushroom extract in patients with CD (paper II) was more limited compared with UC. This may partly be explained by the fact that CD is a more severe disease than UC. Higher dosage of AndoSan™ and longer treatment may have given more results.

There are several studies of AbM, He and Gf, isolated or in the mixture together as AndoSan™, showing beneficial immunomodulatory, antioxidant, antihyperglycemic, and antitumor effects [36, 38, 92, 112, 308]. However, most data, to date, were produced using rodents or cell cultures, there are a very limited number of studies measuring their effects in humans. Even though AbM is the dominating mushroom (82%) of the mushroom extract compared with He (15%) and Gf (3%), biologic activities exerted by the latter two mushrooms isolated or in synergy must be kept in mind. All of these mushrooms are described in the literature as being rich in polysaccharides, where especially β-glucans have been credited the biological effects observed. However, since AndoSan™, which is an extract of the mushrooms’ mycelium and not their fruit bodies, recently was shown to contain less β-glucan than anticipated from the published data of β-glucan content in the fruiting bodies, action also of other yet not identified immunomodulating substances in the extract must part-take to render the observed effects [106]. Although, despite these findings, we still believe β-glucan is a major contributor to the observed biological effects, as it is found to be a potent immunomodulator in several studies [70, 91, 92].

**Conclusion**

In conclusion, this thesis demonstrates clinically significant effects by consumption of AndoSan™ for symptoms in both UC and CD, as well as improvement in quality of life and fatigue for the patients with UC. A relevant interpretation of the results supports the use of this mushroom extract as an adjuvant to routine medical treatment of IBD patients with moderate disease. However, since there only was a trend in reduction of pro-inflammatory cytokines and chemokines in CD and basically no effect in UC, this study could not unmask the main mechanisms behind the complex AndoSan™-mediated improved clinical effects.
Future perspectives

When AndoSan™ is used as an adjuvant in the treatment of IBD patients in the near future, we recommend the use of validated patient-reported outcomes (PROs) combined with classical inflammatory parameters (leukocytes, C-reactive protein, fecal calprotectin).

References


72. Karppinen PK. Search for biologically active compounds in AndoSanTM, a medicinal mushroom extract. Norwegian University of Life Sciences, Ås; 2013.
73. Ingvaldsen IA. Proteiner i det medisinske soppekstraktet AndoSanTM. Norwegian University of Life Sciences, Ås; 2014.


76. Engdal S, Nilsen OG (2008) Inhibition of P-glycoprotein in Caco-2 cells: effects of herbal remedies frequently used by cancer patients. Xenobiotica 38: 559-73. doi: 10.1080/00498250801986969 PMID: 18570158


variants protecting against inflammatory bowel disease. Nat Genet 43: 43-7. doi: 10.1038/ng.733 PMID: 21151126


293. Dahl J, Ormstad H, Aass HC, Malt UF, Bendz LT, Sandvik L, et al. (2014) The plasma levels of various cytokines are increased during ongoing depression and are reduced to normal levels after recovery. Psychoneuroendocrinology 45: 77-86. doi: 10.1016/j.psyneuen.2014.03.019 PMID: 24845179


Effect of a Medicinal Agaricus blazei Murill-Based Mushroom Extract, AndoSan™, on Symptoms, Fatigue and Quality of Life in Patients with Ulcerative Colitis in a Randomized Single-Blinded Placebo Controlled Study

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Abstract

Background

Ingestion of AndoSan™, based on the mushroom Agaricus blazei Murill, has previously been shown to exhibit anti-inflammatory effects because of reduction of pro-inflammatory cytokines in healthy individuals and patients with ulcerative colitis. In this randomized single-blinded placebo controlled study we examined whether intake of AndoSan™ also resulted in clinical effects.

Methods and Findings

50 patients with symptomatic ulcerative colitis were block-randomized and blinded for oral daily intake of AndoSan™ or placebo for the 21 days’ experimental period. The patients reported scores for symptoms, fatigue and health related quality of life (HRQoL) at days 0, 14 and 21. Fecal calprotectin and general blood parameters were also analyzed. In the AndoSan™ group (n = 24) symptoms improved from baseline (day 0) to days 14 and 21, with respective mean scores (95% CI) of 5.88 (4.92–6.83), 4.71 (3.90–5.52) (p = 0.002) and 4.50 (3.70–5.30) (p = 0.001). Corresponding improved mean scores (±SD) for total fatigue were 16.6 (5.59), 14.1 (4.50) (p = 0.001) and 15.1 (4.09) (p = 0.023). These scores in the placebo group (n = 26) were not improved. When comparing the two study groups using mixed model statistics, we found significant better scores for the AndoSan™-patients. HRQoL for dimensions bodily pain, vitality, social functioning and mental health improved in the AndoSan™ group. There were no alterations in general blood samples and fecal calprotectin.
Conclusions
Beneficiary effects on symptoms, fatigue and HRQoL from AndoSan™ consumption were demonstrated in this per-protocol study, supporting its use as a supplement to conventional medication for patients with mild to moderate symptoms from ulcerative colitis. The patients did not report any harms or unintended effects of AndoSan™ in this study.

Trial Registration
ClinicalTrials.gov NCT01496053

1. Introduction
Agaricus blazei Murill, a mushroom of the Basidiomycetes family, grows in the wild in the Piauí area outside of São Paulo, Brazil, and the local population has for centuries utilized it as a health food ingredient. Serious diseases such as atherosclerosis, hyperlipidemia, diabetes and cancer were less prevalent in the Piauí population compared with counterparts in neighboring regions [1], presumably owing to consumption of AbM. The mushroom was brought to Japan in 1966 and introduced to the health food market 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 (82.4%), He (14.7%) and Gf (2.9%), 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], anti-oxidant substances [10], and anti-inflammatory substances such as isolated alkaline and aqueous extracts [11] and the steroid 4-hydroxy-17-methylincisterol (4-HM) [12].

AbM per se and the AbM based extract, AndoSan™, have been shown to exhibit multiple biological effects including anti-tumor, anti-allergic and both pro-inflammatory and anti-inflammatory effects as reviewed [13, 14]. AbM stimulation in vitro of mononuclear phagocytes induced secretion of nitric oxide [15] and pro-inflammatory cytokines IL-1β, IL-6 and TNFα and IL-8 using AndoSan™ [16], which in monocyte-derived dendritic cells also stimulated such cytokine production as well as that of chemokine MIP-1β [17]. One mechanism behind these effects is probably mediated by binding of glucans in the extract to Toll-like receptor 2 [18] as well as to the dectin-1 receptor [19], the lectin-binding site of CD11b/18 [20] and possibly CR4 CD11c/18 [21]. However, since AndoSan™, which is an extract of the mushrooms’ mycelium and not their fruit bodies, was recently shown to contain less β-glucan than anticipated from the published data of β-glucan content in the fruit bodies [22], action also of other yet unidentified immunomodulating substances in the extract must take part to render the observed effects. The in vitro results above were supported by microarray expression analysis in AbM stimulated promonocytic THP-1 tumor cells [23], demonstrating markedly upregulated genes for IL-1β, IL-8, moderately for TLR-2 and co-operative molecule MyD88, but not for TLR-4. However, in another in vivo study, daily consumption of 60 ml of AndoSan™ for a week in chronic hepatitis C patients [24] had no effect on expression of these genes in blood cells. Ex vivo stimulation of whole blood with this AbM-based mushroom extract resulted in a pronounced release, mainly from monocytes, of many cytokines being pro-inflammatory (IL-1β, IL-6, TNFα), anti-inflammatory (IL-10), chemokines (IL-8, MIP-1β, MCP-1, leukocyte...
growth factors (G-CSF, GM-CSF), pleiotropic (IL-7, IL-17) as well as of the Th1- (IFN-\(\gamma\), IL-2, IL-12) and Th-2 types (IL-4, IL-5, IL-13) [25]. However, after daily consumption of 60 ml of AndoSan™ for 12 days in 8 healthy volunteers, there was a significant reduction in cytokine levels in plasma of IL-1ß, TNF-\(\alpha\), IL-6, IL-2 and IL-17, whilst levels of the remaining 12 cytokines in the kit were unaltered, thereby pointing to an anti-inflammatory effect \(in vivo\), when given by the oral route.

In patients with ulcerative colitis (UC), increased mucosal levels have been demonstrated for MIP-1ß, MCP-1 and IL-8 [26], IL-1ß [27], IL-6 and TNF-\(\alpha\) [28]. Cytokine levels in serum are less well studied but increased levels have been reported for IL-6 [29], TNF-\(\alpha\) [30, 31] and MIP-1ß [32]. Moreover, in a recent extensive review [33] the cytokines eotaxin, GRO (chemokine), TNF-\(\alpha\) and IL-8 were considered to be persistently elevated in blood of UC patients compared with findings in healthy individuals.

In 10 patients with UC who likewise consumed the mushroom extract, there was an anti-inflammatory cytokine effect [34] as demonstrated by reduction at day 12 from baseline values \(in vivo\) levels of one cytokine (MCP-1-ß) in untreated blood and of 7 other cytokines (MIP-1ß, IL-6, IL-1ß, IL-8, G-CSF, MCP-1, GM-CSF) in LPS-stimulated blood \(ex vivo\). The level of fecal calprotectin was also reduced as a consequence of consumption of the mushroom extract. Accordingly, the next step was to examine whether a decline in pathological levels of cytokines mediated by the mushroom extract \(in vivo\), does result in a putative beneficial clinical effect in patients with UC.

The aim of the present study was to examine whether consumption of AndoSan™ for 21 days had a positive impact in patients with UC on clinical symptoms, fatigue and quality of life in a randomized single-blinded placebo controlled study. It should be noted that this trial also includes Crohn's disease patients and that those results are being reported separately.

2. Materials and Methods

2.1. 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, and its extract contained a business secret, part of which has not been revealed until recently. 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 \(\beta\)-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 Hr (Yamabushitake) [2] and 2.9% from Gf (Maitake) [3], and its final concentration was 340 g / l. The amount per liter 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 amebocyte lysate test (COAMATIC Chromo-LAL; Chromogenix, Falmouth, MA, USA) with 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 by examination of the Norwegian Food Safety Authorities.

2.2. 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, 14 and 21) analyzed for
haemoglobin, haematocrite, mean cellular volume, mean cellular haemoglobin, reticulocytes, immature reticulocytes, leukocytes 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. Fecal calprotectin concentrations (mg/kg) (normal value <50 mg/kg) at days 0, 14, 21 were determined in duplicates as reported [25, 35].

The mainly patient-reported symptom score was a modified version of the Clinical Activity Index (CAI), including only the 4 clinical items and adding one item defining stool consistency (normal = 0, soft = 1, watery = 2) [36]. 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 0–15. Scores 0–2 meant patients in remission and 3–15 gradually increasing disease activity. The modified CAI, rather than the simple clinical colitis activity index (SCCAI) as described in the study-protocol, was used because of recommendation from the participating gastroenterologist in this study.

Self reported health-related quality of life (HRQoL) was assessed with the short form 36 (IQOLA SF-36 Norwegian Version 1.2), which is a generic HRQoL questionnaire consisting of 36 items, of which 35 are grouped into the following eight health domains: (1) physical functioning (PF), (2) social functioning (SF), (3) role limitations due to physical problems (RP), (4) role limitation due to emotional problems (RE), (5) mental health (MH), (6) vitality (VT), (7) bodily pain (BP) and (8) general health perception (GH). Each domain is graded on a scale of 0–100, and the higher the score the better the HRQoL. The validity and reliability of the SF-36 form have been demonstrated for a number of countries including Norway (version 1) [37]. The data from our study were compared with published norms from 2323 individuals in the general population. Only 11 out of 5400 HRQoL questions were unanswered and, accordingly, 10 out of 1200 dimensions were lacking. Using a scoring algorithm for missing data outlined in the SF 36 survey manual, these 10 dimensions could also be included in the results.

Total fatigue score consists of 11 items of graded questions with score 0–3 per question, which is the sum of physical fatigue (7 items) and mental fatigue (4 items). This score has been validated in a Norwegian general population [38]. The respective scores for total, mental and physical fatigue are 0–33, 0–21 and 0–12, and the higher score the more fatigue. The items of physical (1–7) and mental (8–11) fatigue were: 1) Do you have problems with tiredness? 2) Do you need to rest more? 3) Do you feel sleepy or drowsy? 4) Do you have problems with starting things? 5) Are you lacking in energy? 6) Do you have less strength in your muscles? 7) Do you feel weak? 8) Do you have difficulty concentrating? 9) Do you have problems thinking clearly? 10) Do you make slips of the tongue when speaking? 11) How is your memory?

Rectosigmoidoscopy with biopsies, as described in the study-protocol, were not performed because of lack of resources.

2.3. Inclusion and Exclusion of Patients

210 patients with UC were phone interviewed and those with CAI score of at least 3 were given the opportunity to join the study. At the first attendance CAI was re-recorded and criteria for exclusion were pregnancy, biological treatment with antibodies to TNFα (Adalimumab, Infliximab), daily use of more than 5 mg of prednisolone, change of medication and/or consumption of mushroom products from two weeks before till end of the study. A flow chart reveals additional reasons for exclusions (Fig 1).
Fig 1. An algorithm showing the scheme for inclusion of the patients in the study.

doi:10.1371/journal.pone.0150191.g001
2.4. Experimental Design and Randomization

This is a single-center randomized two-armed patient-blinded study designed to determine whether daily oral intake of a mushroom extract, AndoSan™, improved clinical symptoms, fatigue and quality of life in patients with UC during the 21 days’ study period. The patients were evaluated before (visit 1, day 0), during (visit 2, day 14) and after (visit 3, day 21) AndoSan™ or placebo consumption (30 ml twice daily). This dose (60 ml/day) reduced levels of pro-inflammatory cytokines and chemokines in healthy volunteers [25] and in patients with UC and CD [34], whilst half dosage (30 ml/day) had no detectable effects (unpublished data). The placebo group was evaluated likewise but received an equal volume of color-like drink with ionized water containing 0.5 ml per liter of caramel color (E150c) with salt.

The 50 patients were divided into 13 groups and manually randomized with the overall allocation ratio of one to one. Block-randomization was done after the phone interview, with uneven and even numbers given for AndoSan™ or placebo, respectively. The patients, one by one, were placed in one pile, and the group affiliations were placed in another pile. The randomization was performed by combining one selection from each pile, both anonymized. The first author performed the randomization, enrolled the participants, and assigned participants to interventions. A few patients were excluded throughout the study by not attending or because of intercurrent incidents (disease, unexpected life events). Accordingly, a slight imbalance of the study groups occurred that was corrected in the latter rounds of randomization. More specifically, the 50 UC patients were divided into 13 study groups (range 2–9 per group), each with a study period of 3 weeks. The included 50 symptomatic patients had no missing data and were randomized and blinded for oral daily consumption (30 ml twice daily) of AndoSan™ or placebo for the 21 days’ experimental period. Patients in the AndoSan™ group and the placebo group self-reported, in written, at visit 1 (day 0), visit 2 (day 14) and visit 3 (day 21) regarding symptoms, fatigue and health-related quality of life. Patient derived blood samples and fecal calprotectin from these visits were also analyzed. All data were stored in a secure database (Access—Microsoft Office) at a server at Oslo University Hospital, Ullevål, Norway. A study number anonymized the patients.

This study is a follow up of a previous pilot study [34] in which there was a reduction of pro-inflammatory cytokines and chemokines in patients receiving the same daily dose of AndoSanTM, but for 12 days. We calculated for prospective differences of 20% between the experimental and placebo group and assumed standard deviation of 20% for the different parameters with a significant level of 5% and a power of 90% (ß = 0.10), demands about 25 patients per randomized arm (calculated in cooperation Oslo Center for Biostatistics and Epidemiology, Oslo University Hospital).

2.5. Patient Characteristics

Clinical disease data concerning duration, anatomic extent and activity were registered. The study groups were comparable and with no significant differences with respect to details of demographic data and patient characteristics (Table 1). Eight patients in the AndoSan™ group used several medications, Azathioprine and 5-ASA in 4 and Azathioprine and Prednisolone in 1 patient(s), respectively. Three patients used oral 5-ASA combined with rectal enema with Budesonide, Prednisolone and 5-ASA, respectively. In the placebo group 2 patients used Azathioprine and 5-ASA and 5 patients used 5-ASA by oral and rectal route. The medication was unaltered from baseline and throughout the study period, in both groups.

2.6. Statistical Analysis

Data are presented as mean and standard deviation or as median and range values. Paired sample t-test and Wilcoxon test are used for within-group analysis. The judgement of whether the
distributions of the main efficacy variables were so close to the normal distribution that normality-based significance tests may be used, is based on the finding in a paper by Fagerland and Sandvik [39]. Mixed models corrected for baseline values were used for measuring P values between the study groups. P values below 0.05 were considered statistically significant. The SPSS statistical program for the social sciences, version 22 (IBM), was used in the analyses.

2.7. Ethical Considerations
The study was approved on April 8, 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 (December 15, 2011). The authors confirm that all ongoing and related trials for this drug/intervention are registered.

3. Results
3.1. Exclusion of randomized patients
A total of 62 patients, 31 in both study groups, were randomized for inclusion in this study. 12 of these patients were excluded according to the criteria of the study protocol, because three

Table 1. Demographic and patient data.

<table>
<thead>
<tr>
<th></th>
<th>AndoSan™</th>
<th>Placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>24</td>
<td>26</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Age</strong> (years)</td>
<td>40.5 (23–56)</td>
<td>40.0 (23–67)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Gender</strong> (male, female)</td>
<td>13, 11</td>
<td>12, 14</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Duration of diagnosis</strong> (months)</td>
<td>96 (4–324)</td>
<td>66 (4–260)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Anatomic extent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rectosigmoidal</td>
<td>5</td>
<td>7</td>
<td>ns</td>
</tr>
<tr>
<td>left colon</td>
<td>6</td>
<td>10</td>
<td>ns</td>
</tr>
<tr>
<td>entire colon</td>
<td>13</td>
<td>9</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Disease pattern</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>continuous</td>
<td>4</td>
<td>2</td>
<td>ns</td>
</tr>
<tr>
<td>episodic</td>
<td>20</td>
<td>24</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-ASA</td>
<td>13</td>
<td>17</td>
<td>ns</td>
</tr>
<tr>
<td>Azathioprin</td>
<td>2</td>
<td>0</td>
<td>ns</td>
</tr>
<tr>
<td>Several medications</td>
<td>8</td>
<td>7</td>
<td>ns</td>
</tr>
<tr>
<td>No medication</td>
<td>1</td>
<td>2</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Comorbidity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthralgia</td>
<td>8</td>
<td>6</td>
<td>ns</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>0</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes mellitus type 2</td>
<td>1</td>
<td>0</td>
<td>ns</td>
</tr>
<tr>
<td>PSC, livercirrhosis and anemia</td>
<td>0</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>Myalgic encephalomyelitis</td>
<td>1</td>
<td>0</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values for age and duration of diagnosis are given as median (range).

doi:10.1371/journal.pone.0150191.t001
and two changed their medical treatment just prior to or during the study period, two in each group had missing laboratory data, and two and one had missing attendance, in the AndoSan™ and placebo group, respectively. Thereby we ended up with 24 patients in the AndoSan™ group and 26 in the placebo group.

3.2. Age and Gender
Median age for the 50 included patients with UC was 40.5 years (range 23–67). There were 13 men and 11 women in the AndoSan™ group and 12 men and 14 women in the placebo group. Respective ages in the two groups were median 35 (range 23–64) and 41.5 (27–50) for men (p = 0.611) and 44 (24–67) and 35.5 (23–56) for women (p = 0.075).

3.3. Symptom Score
The symptom scores using the CAI were similar at inclusion in the AndoSan™ and placebo groups, with respective mean scores of 5.88 and 5.81. Compared with baseline only the patients in the AndoSan™ group reported a significant reduction of symptoms that was also further reduced from visit 2 (day 14) to visit 3 (day 21) after the mushroom extract intake (Table 2). There were no significant differences in baseline symptom scores between male and females within the two groups. When comparing the two groups using mixed models corrected for baseline values, there also was a significant difference (p = 0.023) in favor of the AndoSan™ group.

Regardless of disease activity at inclusion the patients had a reduction of symptom score (data not shown). Within the AndoSan™ group from visit 1 to 3, there was a significant reduction regarding stool frequency (p = 0.005), consistency (p = 0.02), and the doctor’s evaluation of disease activity (p = 0.02). For blood in stool (p = 0.08) and abdominal pain (p = 0.07) there was a trend favoring improvement. In the placebo group there were no significant improvements in these parameters, but a trend in favor of reduction of stool frequency (p = 0.07). The doctor’s evaluation of disease activity could be a source of bias in the CAI score because of lack of blinding. Therefore we also examined the symptoms without this item, and the p values for difference of scores were the same (data not shown). The patients did not report any harms or unintended effects of AndoSan™ in this study.

3.4. Fatigue Score
Firstly, age-adjusted normative fatigue scores in the Norwegian population were compared with such scores in the UC patients at inclusion in this study (Table 3). There were for both

Table 2. Symptom score for the UC patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>P V1V2</th>
<th>P V1V3</th>
<th>P between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>AndoSan™</td>
<td>5.88 (4.92–6.83)</td>
<td>4.71 (3.90–5.52)</td>
<td>4.50 (3.70–5.30)</td>
<td>0.002</td>
<td>0.001</td>
<td>0.023</td>
</tr>
<tr>
<td>M (n = 13)</td>
<td>6.15 (4.70–7.61)</td>
<td>5.08 (3.78–6.37)</td>
<td>5.08 (3.93–6.22)</td>
<td>0.037</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>F (n = 11)</td>
<td>5.55 (4.09–7.00)</td>
<td>4.27 (3.19–5.36)</td>
<td>3.82 (2.66–4.97)</td>
<td>0.031</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>5.81 (4.81–6.80)</td>
<td>5.58 (4.42–6.73)</td>
<td>5.27 (4.28–6.26)</td>
<td>0.471</td>
<td>0.114</td>
<td></td>
</tr>
<tr>
<td>M (n = 12)</td>
<td>6.33 (4.82–7.85)</td>
<td>6.42 (4.76–8.07)</td>
<td>5.83 (4.48–7.18)</td>
<td>0.878</td>
<td>0.309</td>
<td></td>
</tr>
<tr>
<td>F (n = 14)</td>
<td>5.36 (3.90–6.82)</td>
<td>4.86 (3.15–6.56)</td>
<td>4.79 (3.25–6.26)</td>
<td>0.205</td>
<td>0.252</td>
<td></td>
</tr>
</tbody>
</table>

V1: visit 1 (day 0), V2: visit 2 (day 14), V3: visit 3 (day 21). M: male, F: female.
Values are given as means and 95% confidence intervals. Paired sampled t-test for the p-values.
P between groups is measured with mixed models corrected for baseline values.

doi:10.1371/journal.pone.0150191.t002
genders a significant increase of physical, mental and total fatigue scores in the UC patients compared with the general population. This effect was more pronounced for physical fatigue than for mental fatigue.

The scores for genders on inclusion were quite similar within and between the groups. In the AndoSan™ group (Table 4) for both genders the UC patients reported a significant decline in mental and total fatigue, that was more pronounced at visit 2 (day 14) vs. visit 3 (day 21).

When broken down into gender the reduction in mental and total fatigue was not significant at visit 3. There was, however, a significant decline in physical fatigue at visit 2 (p = 0.007), but not at visit 3 (p = 0.128). The improvement in physical fatigue at visit 2 was significant for men (0.042) but not for women (0.055). In the placebo group the fatigue scores were unaltered throughout the experimental period as a whole and by division into gender.

However, when comparing the AndoSan™ and placebo groups using mixed models corrected for baseline values, there were significant improvements in the AndoSan™ group for the three scores of total (p = 0.018), mental (p = 0.022) and physical (p = 0.037) fatigue (Table 4).

### 3.5. Quality of Life

HRQoL score (SF-36) for UC patients were compared with age-adjusted normative data for the Norwegian population (Table 5). We found significantly much lower scores for HRQoL in

<table>
<thead>
<tr>
<th>Table 3. Mean fatigue scale scores. Normative data in the Norwegian population compared to patients with UC on inclusion.</th>
</tr>
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<tbody>
<tr>
<td><strong>Normative data</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Physical</td>
</tr>
<tr>
<td>Mental</td>
</tr>
</tbody>
</table>

Normative data from the general Norwegian population. Values are given as mean and standard deviation (SD). Paired sample t-test for the p-values.

doi:10.1371/journal.pone.0150191.t003

<table>
<thead>
<tr>
<th>Table 4. Fatigue scores for the patients with (n = 24 AndoSan™ and n = 26 placebo) UC.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AndoSan™ group</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>TF</td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>PhF</td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>MF</td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td>F</td>
</tr>
</tbody>
</table>

V1; visit 1 (day 0), V2; visit 2 (day 14), V3; visit 3 (day 21). TF; total fatigue, PhF; physical fatigue, MF; mental fatigue. Values are given as mean and standard deviation (SD). Paired sampled t-test for p-values. P between groups is measured with mixed models corrected for baseline values.

doi:10.1371/journal.pone.0150191.t004
the UC patients for both genders regarding 7 out of 8 dimensions. Only for physical functioning (PF) there were no differences in both genders relative to the general population. The reduction of quality life scores in the UC patients were for both genders most pronounced for the dimensions; general health (GH), vitality (VT) and social functioning (SF) (p values < 0.0001) as compared with the general population.

In the AndoSan™ group as a whole the results were significantly improved (Table 6) for bodily pain (BP), VT, SF and mental health (MH), of which BP and MH also scored significantly but less pronounced at visit 2. Although not significant, there was an increase of scores for the remaining four dimensions GH, PF, role limitation, physical (RP) and role limitation, emotional (RE). In the placebo group, except from an improvement of BP (p = 0.036) at visit 2, there were no significant alterations in the 8 quality of life dimensions throughout the study. When comparing the two groups, using mixed models corrected for baseline values, we found significant improvement of SF-36 scores for PF, RP, BP and SF in the AndoSan™ group.

Except for unaltered score in GH for females in the AndoSan™ group, there were improvements of scores in all dimensions for both genders. In the placebo group, however, the corresponding general pattern was a gender-like unchanged score during the three weeks study period (Table 6).

### 3.6. Calprotectin in Feces

The patients delivered fecal tests at visits 1, 2 and 3 (Table 7). In the AndoSan™ group the median (range) values for fecal calprotectin (mg/kg) were 439 (10–6000), 366 (10–6000) and 489 (18–6000). In the placebo group the values were 328 (16–5361), 521 (18–6000) and 563 (10–6000). There were no significant differences in levels of calprotectin within or between the groups. However, for men there was rather a significant increase of calprotectin in the placebo group from visit 1 to visit 3 (p = 0.019).

### 3.7. Effect on General Blood Parameters

The following blood samples were analyzed at visit 1 and 3: CRP, leukocytes, eosinophils, basophils, neutrophils, lymphocytes, monocytes, hemoglobin, haematocrite, mean cellular volume, mean cellular haemoglobin, immature reticulocytes, reticulocytes, thrombocytes, urea,
creatinine, and GFR (glomerular filtration rate), bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, \(\gamma\)-glutamine transferase, alkaline phosphatase and pancreatic amylase. Significant changes in the blood samples were neither found in the Ando-San™—nor the placebo group.

Table 6. Mean SF-36 scale scores (n = 24 AndoSan™ and n = 26 placebo) UC.

<table>
<thead>
<tr>
<th>Group</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>(P_{V1V2})</th>
<th>(P_{V1V3})</th>
<th>(P_{V2V3})</th>
<th>(P_{AvsP})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AndoSan™ group</td>
<td>PF 85.0 (16.9)</td>
<td>87.6 (13.7)</td>
<td>88.8 (13.4)</td>
<td>0.096</td>
<td>0.101</td>
<td>86.4 (15.5)</td>
<td>85.8 (14.6)</td>
</tr>
<tr>
<td></td>
<td>M 86.9</td>
<td>89.9</td>
<td>90.4</td>
<td>0.148</td>
<td>0.145</td>
<td>84.6</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>F 82.7</td>
<td>84.9</td>
<td>86.8</td>
<td>0.399</td>
<td>0.347</td>
<td>87.9</td>
<td>86.4</td>
</tr>
<tr>
<td></td>
<td>RP 55.2 (43.0)</td>
<td>59.4 (39.6)</td>
<td>64.6 (40.3)</td>
<td>0.426</td>
<td>0.059</td>
<td>52.9 (39.6)</td>
<td>47.1 (43.2)</td>
</tr>
<tr>
<td></td>
<td>M 61.5</td>
<td>71.2</td>
<td>69.2</td>
<td>0.096</td>
<td>0.219</td>
<td>56.3</td>
<td>60.4</td>
</tr>
<tr>
<td></td>
<td>F 47.7</td>
<td>45.5</td>
<td>59.1</td>
<td>0.810</td>
<td>0.176</td>
<td>50.0</td>
<td>35.7</td>
</tr>
<tr>
<td>Placebo group</td>
<td>BP 57.1 (26.1)</td>
<td>67.3 (24.0)</td>
<td>73.6 (21.5)</td>
<td>0.015</td>
<td>0.000</td>
<td>59.3 (23.0)</td>
<td>64.7 (25.0)</td>
</tr>
<tr>
<td></td>
<td>M 59.3</td>
<td>70.8</td>
<td>77.8</td>
<td>0.114</td>
<td>0.015</td>
<td>59.3</td>
<td>68.5</td>
</tr>
<tr>
<td></td>
<td>F 54.5</td>
<td>63.2</td>
<td>68.7</td>
<td>0.022</td>
<td>0.010</td>
<td>59.4</td>
<td>61.5</td>
</tr>
<tr>
<td></td>
<td>GH 53.0 (23.2)</td>
<td>56.5 (24.0)</td>
<td>54.8 (22.2)</td>
<td>0.174</td>
<td>0.536</td>
<td>52.9 (23.1)</td>
<td>50.7 (23.4)</td>
</tr>
<tr>
<td></td>
<td>M 49.5</td>
<td>55.8</td>
<td>53.5</td>
<td>0.137</td>
<td>0.374</td>
<td>53.8</td>
<td>55.7</td>
</tr>
<tr>
<td></td>
<td>F 57.3</td>
<td>57.4</td>
<td>56.4</td>
<td>0.972</td>
<td>0.789</td>
<td>52.1</td>
<td>46.5</td>
</tr>
<tr>
<td></td>
<td>RP 38.8 (19.9)</td>
<td>44.6 (18.9)</td>
<td>46.9 (17.9)</td>
<td>0.076</td>
<td>0.018</td>
<td>38.4 (18.5)</td>
<td>40.6 (19.4)</td>
</tr>
<tr>
<td></td>
<td>M 39.2</td>
<td>46.9</td>
<td>48.1</td>
<td>0.139</td>
<td>0.046</td>
<td>38.8</td>
<td>47.1</td>
</tr>
<tr>
<td></td>
<td>F 38.2</td>
<td>41.8</td>
<td>45.5</td>
<td>0.371</td>
<td>0.205</td>
<td>38.1</td>
<td>35.0</td>
</tr>
<tr>
<td></td>
<td>SF 65.1 (26.6)</td>
<td>75.5 (22.9)</td>
<td>75.0 (19.8)</td>
<td>0.013</td>
<td>0.015</td>
<td>66.3 (21.6)</td>
<td>65.9 (21.1)</td>
</tr>
<tr>
<td></td>
<td>M 67.3</td>
<td>81.7</td>
<td>75.0</td>
<td>0.050</td>
<td>0.180</td>
<td>67.7</td>
<td>75.0</td>
</tr>
<tr>
<td></td>
<td>F 62.5</td>
<td>68.2</td>
<td>75.0</td>
<td>0.096</td>
<td>0.041</td>
<td>68.8</td>
<td>58.0</td>
</tr>
<tr>
<td></td>
<td>RE 68.1 (39.9)</td>
<td>69.4 (40.4)</td>
<td>75.0 (39.6)</td>
<td>0.846</td>
<td>0.423</td>
<td>61.5 (44.9)</td>
<td>52.6 (44.4)</td>
</tr>
<tr>
<td></td>
<td>M 64.1</td>
<td>64.1</td>
<td>71.8</td>
<td>1.000</td>
<td>0.461</td>
<td>69.4</td>
<td>72.2</td>
</tr>
<tr>
<td></td>
<td>F 72.7</td>
<td>75.8</td>
<td>78.8</td>
<td>0.588</td>
<td>0.690</td>
<td>54.8</td>
<td>35.7</td>
</tr>
<tr>
<td></td>
<td>MH 65.3 (15.7)</td>
<td>69.5 (14.4)</td>
<td>71.0 (13.7)</td>
<td>0.032</td>
<td>0.005</td>
<td>66.8 (15.7)</td>
<td>69.2 (17.1)</td>
</tr>
<tr>
<td></td>
<td>M 63.1</td>
<td>67.4</td>
<td>68.0</td>
<td>0.141</td>
<td>0.075</td>
<td>64.0</td>
<td>70.7</td>
</tr>
<tr>
<td></td>
<td>F 68.0</td>
<td>72.0</td>
<td>74.5</td>
<td>0.137</td>
<td>0.036</td>
<td>69.1</td>
<td>68.0</td>
</tr>
</tbody>
</table>

Paired sampled t-test for the p-values.

P between groups is measured with mixed models corrected for baseline values.

doi:10.1371/journal.pone.0150191.t006

Table 7. Fecal test in the 50 UC patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>(P_{V1V2})</th>
<th>(P_{V1V3})</th>
<th>(P_{V2V3})</th>
<th>(P_{between\ groups})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AndoSan™ (n = 24)</td>
<td>439 (10–6000)</td>
<td>366 (10–6000)</td>
<td>489 (18–6000)</td>
<td>0.673</td>
<td>0.808</td>
<td>0.705</td>
<td></td>
</tr>
<tr>
<td>M 358</td>
<td>382</td>
<td>527</td>
<td>0.727</td>
<td>0.600</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F 517</td>
<td>317</td>
<td>366</td>
<td>0.314</td>
<td>0.959</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo (n = 26)</td>
<td>328 (16–5361)</td>
<td>521 (18–6000)</td>
<td>563 (10–6000)</td>
<td>0.298</td>
<td>0.551</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M 234</td>
<td>570</td>
<td>1702</td>
<td>0.060</td>
<td>0.019</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F 679</td>
<td>480</td>
<td>445</td>
<td>0.875</td>
<td>0.245</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are given as median (range). Non-parametric Wilcoxon test for the p-values.

P between groups is measured with Mann-Whitney U test.

doi:10.1371/journal.pone.0150191.t007
The median and range haemoglobin (g/l), leukocyte counts (10⁹/l) and CRP levels for visit 1 and 3 the AndoSan™ group were 13.55 (11.5–17.2) versus 13.55 (11.0–17.3), 6.20 (3.1–9.2) versus 5.95 (3.3–10.5) and 2.00 (0.6–30.4) versus 1.80 (0.6–16.9), respectively. Corresponding values in the placebo group were 13.7 (5.7–16.1) versus 13.5 (6.0–15.8) for haemoglobin, 5.85 (4.4–10.3) versus 6.55 (3.1–14.6) for leukocytes and 1.60 (0.6–63) versus 1.95 (0.6–58.0) for CRP. Accordingly, there were no statistical changes of these parameters neither within nor between the groups.

4. Discussion

The present study demonstrates for the first time, in a randomized patient-blinded placebo controlled study, that the immunomodulatory Agaricus blazei Murill based mushroom extract AndoSan™ [14] improved clinical symptoms as well as fatigue and quality of life in patients with UC during a 3 weeks’ study period. Moreover, there was no effect in the placebo group, whatsoever, and when comparing the two groups the difference was significant in favor of AndoSan™. The moderate, but significant reduction (about 20%) in symptom score occurred already after 2 weeks and persisted after 3 weeks.

Compared with the normal population the UC patients had considerably more fatigue (women 32%, men 37%). However, the general effect on fatigue subsided after 3 weeks but still was significant for mental fatigue. This may be due to a reduced effect of AndoSan™ over time, but external factors influencing the patients’ physical status like intercurrent subclinical disease must also be considered.

Regarding limitations of this study we need to adress some issues. The study was not blinded for the authors leaving possible biases of the results. This is especially true for the first author who was responsible for the inclusion and randomization of participants, the implementation of the practical aspects of and in meeting with the patients, and also in the analysis of the results. This is a relatively small study, althought with significant results, with its limitations. The study was conducted with participants from the Oslo (Norway) area, and the results may not be transferable to other geographical areas with other patient characteristics. 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 after the study period of three weeks. The modified CAI is not used much in previous studies, making it somewhat difficult to compare our results with previous studies done on this parameter.

For HRQoL in the AndoSan™ group, which is a broad coverage of the patient’s well-being, there was a persistent an even improved effect on bodily pain (29% reduction), vitality (21%), social functioning (15%) and mental health (9%). Although no within-group significance for physical functioning and role limitation, there was a significant effect in favor of the AndoSan™ compared with the placebo group.

In line with a previous study [34] in which 10 UC patients received the same amount of AndoSan™ for 12 days, there were no alterations in general blood parameters including CRP. However, contrary to this study there was a significant reduction of fecal calprotectin in those patients during the study period. However, in the placebo group for men in the present study there was, on the other hand, a significant increase in level of fecal calprotectin (p = 0.019), implying a prospective stabilizing effect on calprotectin levels in the AndoSan™ group that was not seen for the controls. One reason for lack of reduction of fecal calprotectin in this study could be the large variability of baseline calprotectin levels (range 10–6000) that was not seen in the previous small pilot study (128–1683).

The AndoSan™ and placebo groups had similar baseline values with respect to symptom score, fatigue and quality of life. The groups were also comparable with regard to disease
duration, extent of disease, disease pattern, arthralgia and relevant comorbidity, and thereby well-fitted for comparison. Hence, putatively increased symptoms tolerance in patients with extensive or prolonged disease experience, should not affect the SF-36 results.

The patients did not report any obvious clinical side-effects from consumption of the mushroom extract, which is similar to clinical studies in patients with chronic hepatitis B [40] or hepatitis C infection [24] where liver function was either normalized or unaltered. However, although other causative factors such as cancer chemotherapy and hepatitis virus could not be ruled out, consumption of Agaricus blazei extract for days to months may have induced severe hepatic dysfunction in three patients receiving concomitant chemotherapy for breast and ovarian cancer [41]. In a phase I study different doses (1.8 g, 3.6 g and 5.8 g) of AbM granulated powder was consumed in orally for 6 months in 78 patients with different cancers [42].

Adverse effects were observed in 12%, mainly nausea and diarrhea. Only in one case with ovarian cancer receiving six cycles of chemotherapy (Paclitaxel/Carboplatin) after surgery, generalized urticaria with papulae and moderate liver dysfunction occurred after two months’ AbM consumption, which was the definitive cause as judged by a positive lymphocyte AbM stimulation test. However, in another recent clinical trial done with AndoSan™ or the same placebo as used here, as supplementary treatment 60 ml/day over 7 weeks to high-dose chemotherapy and bone marrow transplantation for 40 patients with multiple myeloma, there were no side effects, neither during nor after the trial [43]. Hence, AndoSan™ is proven to be a safe product per se for very different categories of patients. However, caution is advised for possible interaction with some drugs as referred below.

Herb-drug interactions are associated with cytochrome P-450 metabolism in the liver and the trans-membrane efflux pump P-glycoprotein (P-gp) that is present in normal intestinal lumen where it may limit drug absorption, as well as in the liver, where it may increase excretion of the drug [44]. In this respect, AndoSan™ (called Agaricus from Japan) has previously been investigated by another independent researchers at the Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, for in vitro inhibitory potential on P-gp-mediated transport in an intestinal cell line. It was found that AndoSan™ inhibited P-gp in vitro in a similarly as did green tea. Because the mushroom may interact with some drugs beeing P-gp substrates (i.e. vinblastine, loperamide, digitoxin, cyclosporine, verapamil) concomitant AbM should not be given to patients using these drugs. When tested in vitro for inhibition potential on cytochrome P-450 (CYP3A4 isoform) the AndoSan™ extract was found to inhibit it but far less than green tea [45]. The researchers [45] concluded that “clinical relevant systemic or intestinal interactions with CYP3A4 were considered unlikely”.

In our clinical study, none of the UC patients were concomitantly treated with the above mentioned anticancer-, heart-, immunodepressive- or diarrhea drugs.

The notion of a potential anti-inflammatory effect after intake of AndoSan™ was a result of the surprising finding of reduced serum pro-inflammatory cytokines (IL-1β, IL-6, IL-8) and chemokines (MCP-1, G-CSF, GM-CSF) in a pilot safety study with AndoSan™ in healthy individuals without a placebo control [25]. Recently, a steroid 4-hydroxy-17-methylincisterol (4-HM) [12] isolated from AbM dose-dependently suppressed the synthesis in PHA-stimulated peripheral blood mononuclear cells of cytokines IL-2, IL-4, IFNγ and TNFα by decreasing both NF-AT (nuclear factor of activated T-cells), which belongs to a family of transcription factors required for activation and proliferation of T lymphocytes including production of the first three aforementioned cytokines, and NF-κB—the latter being the “mother” of all inflammation. Substances isolated from AbM had several anti-inflammatory effects in rats, related to IL-1β, TNFα and IL-8 modulations [11,46]. In a study on healthy volunteers ingesting AndoSan™ [47] there was a reduction in vivo of ROS mainly reflecting superoxide ions, and again pointing to an anti-inflammatory effect. However, this result was not demonstrated in the UC
patients. The reason for reduced superoxide anions may be related to reduction of IL-1β because inhibitors of ROS reduce synthesis of this cytokine in macrophages [48].

There also was an anti-allergic effect in mice sensitized to ovalbumin (OVA), regarding reduction of specific anti-OVA IgE antibodies, both when AndoSan™ was given before or after the OVA immunization [49]. 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™ [49]. Moreover, the inhibitory effect of an isolated carbohydrate fraction of AndoSan™ [22] on the tissue degrading enzyme legumain (asparaginyl endopeptidase), which probably activates proMMP and processing of cathepsins may also contribute to less pro-inflammatory activity in the UC patients.

It is commonly believed that carbohydrates larger than monosaccharides are not absorbed from the human gut. However, in murine models [13, 50], uptake of β-1,3 glucans across the gut wall, probably by microfold cells (M cells) but also by dendritic cells (DC) [51], has been demonstrated. The β-glucan may further be transported by DC to lymphocytes in GALT, but also circulated in blood in rodents [52, 53]. Presumably, a similar mechanism is operating in humans for intestinal absorption of small immunomodulatory bioactive β-glucan fragments into the lymphoid system and blood. As mentioned, other yet unidentified small immunomodulatory substances in the mixed mushroom extract are probably also playing a role in this context. This assumption is supported by the fact that when molecules <12.5 kDa, and thus smaller than β-glucans, were dialyzed away from the AndoSan™ extract prior to performing the experiments in the mouse allergy model, the anti-allergic effect of the extract was diminished to no longer statistically significant levels [49]. Hence, since the anti-allergic effect of AbM extract seems to be owing to low-molecular-weight substances in the mouse allergy model, other smaller and simpler substances than β-glucans in AbM could very well be as important for the mushroom’s biological effect in other settings such as UC. In addition, AbM also contains small molecular anti-oxidant and anti-inflammatory substances that may be absorbed actively or by diffusion through the enterocytes in the gut. A key to understanding the function of a changed or down-regulated cytokine response locally in the gut wall, is probably the signals mediated by DC [54] after processing and presenting to CD4 T helper cells of native antigens from the mushrooms or novel bacteria-derived antigens resulting from mushroom-microbiota interactions. As seen from animal models [49, 55], the AbM extracts seem to drive the Th1/Th2 balance towards an increased Th1 response, which also inhibits Treg cells and Th17 cells [14].

In conclusion, the results support that AndoSan™ may be beneficial as a supplement to conventional medication in UC patients with mild to moderate disease activity because of improvements in symptoms, fatigue and quality of life. There is reason to assume that AndoSan™ may stabilize these patients with subsequently less need for increased medical treatment, which may be associated with potentially troublesome and harmful side effects.

Supporting Information

S1 Fig. Flow chart UC kopi.
(TIF)

S1 CONSORT Checklist. CONSORT 2010 Checklist.
(DOC)

(DOCX)

(DOCX)
Acknowledgments

The skillful statistical guidance by professor Leiv Sandvik, Oslo Center for Biostatistics and Epidemiology, Oslo University Hospital, Norway, is gratefully acknowledged.

Author Contributions

Conceived and designed the experiments: SPT EJ. Performed the experiments: SPT EJ. Analyzed the data: SPT EJ. Contributed reagents/materials/analysis tools: SPT GH EJ. Wrote the paper: SPT GH EJ. Inclusion of patients and review of manuscript: IL. Laboratory setup and review of manuscript: TL.

References


Clinical Effects of AndoSan™ on Patients with Ulcerative Colitis


44. Engdal S, Nilsen OG (2008) Inhibition of P-glycoprotein in Caco-2 cells: effects of herbal remedies frequently used by cancer patients. Xenobiota 38: 559–73. doi:10.1080/00498250801986969 PMID: 18570158


Effect of the Medicinal *Agaricus blazei* Murill-Based Mushroom Extract, AndoSan™, on Symptoms, Fatigue and Quality of Life in Patients with Crohn’s Disease in a Randomized Single-Blinded Placebo Controlled Study

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Abstract

**Background**

Ingestion of AndoSan™, based on the mushroom *Agaricus blazei* Murill, has previously shown an anti-inflammatory effect through reduction of pro-inflammatory cytokines in healthy individuals and patients with Crohn’s disease (CD). In this randomized single-blinded placebo-controlled study we examined whether intake of AndoSan™ also resulted in clinical effects.

**Methods and Findings**

50 patients with symptomatic CD were randomized for oral daily consumption of AndoSan™ or placebo for a 21-day experimental period, in this per-protocol study. Patients reported validated scores for symptoms, fatigue and health related quality of life (HRQoL) at days 0, 14 and 21. Fecal calprotectin and general blood parameters were also analyzed. In the AndoSan™ group (n = 25) symptoms improved from baseline (day 0) to days 14 and 21, with respective mean scores (95% CI) of 5.52 (4.64–6.40), 4.48 (3.69–5.27) and 4.08 (3.22–4.94) (p < 0.001). We found significant improvements in symptom score for both genders in the AndoSan™ group, and no significant changes in the placebo (n = 25) group. There were however no significant differences between the groups (p = 0.106), although a marginal effect in symptom score for men (p = 0.054). There were comparable improvements in physical, mental and total fatigue for both groups. HRQoL versus baseline were at day 21 improved for bodily pain and vitality in the AndoSan™ group and for vitality and social functioning in the placebo group. No crucial changes in general blood samples and fecal calprotectin were detected.
Conclusions

The results from this single-blinded randomized clinical trial shows significant improvement on symptoms, for both genders, in the AndoSan™ group, but no significant differences between the study groups. The results on fatigue, HRQoL, fecal calprotectin and blood samples were quite similar compared with placebo. The patients did not report any harms or unintended effects of AndoSan™. CD patients with mild to moderate symptoms may have beneficiary effects of AndoSan™ as a safe supplement in addition to conventional medication.

Trial Registration
ClinicalTrials.gov NCT01496053

1. Introduction

A mushroom Agaricus blazei Murill (AbM) has for centuries been utilized as a health food ingredient by the local population in the Piedade area in Brazil, where prevalence of atherosclerosis, hyperlipidemia, diabetes and cancer was lower than in neighboring regions [1], presumably owing to AbM consumption. In 1966 the mushroom was brought to Japan and introduced to the health food market. Since then, AbM and other Basidiomycetes mushrooms [2, 3] have been subjected to an increasing research effort regarding their effects.

AbM per se and the AbM-based mushroom extract, AndoSan™ (ACE Co. Ltd., Gifu-ken, Japan), composed of AbM (82.4%), Hericium erinaceus (He) (14.7%) [2] and Grifola frondosa (Gf) (2.9%) [3], contain immunomodulatory β-glucans and other biologically active substances like α-glucans [4], proteoglycans [5], lectins [6], ergosterol (provitamin D2) [7], agaritine [8], isoflavonoids [9], anti-oxidant [10], and anti-inflammatory substances [11] including the 4-HM steroid [12].

Depending on the experimental set up, AbM or the AbM-based extract AndoSan™, comprise as reviewed [13, 14] anti-tumor, anti-allergic, and anti-inflammatory effects in vivo.

AndoSan™ is an extract of the mushrooms’ mycelium and not their fruiting bodies and was recently shown to contain less β-glucan [15] than anticipated from the published data on AbM fruiting body. Therefore, action also of other yet not identified immunomodulating substances in this particular extract must part-take to render the observed effects. An example is an isolated fraction of AndoSan™ that was found to inhibit the production in macrophages of the tumor-associated and pro-inflammatory protease, legumain [15].

In patients with Crohn’s disease (CD) increased mucosal levels have been demonstrated for MIP-1ß, MCP-1 and IL-8 [16], IL-1ß [17], IL-6 and TNFα [18]. Cytokine levels in serum are less well studied but increased levels have been reported for IL-6 [18] and TNFα [19, 20]. Moreover, in a recent extensive review [21] the cytokines IFNγ, IL-6, IL-7 and IL-8 were considered to be persistently elevated in blood of CD patients compared with findings in healthy individuals.

In 11 patients with CD who consumed the mushroom extract AndoSan™ for 12 days [22] cytokine levels were reduced in untreated (IL-2, IL-8, IL-17) and in LPS-stimulated blood ex vivo (IL-16, MIP-1ß, MCP-1, IL-8, IL-17, G-CSF and GM-CSF). Then, the next step was to examine whether a decline in pathological levels of cytokines mediated by the mushroom extract in vivo, did result in a putative beneficial clinical effect in patients with CD.

We have in a recent single-blinded randomized placebo controlled study, in which patients with ulcerative colitis received this mushroom extract (AndoSan™) for three weeks [23],
demonstrated improvements in symptoms, fatigue and HRQoL compared with patients in the placebo group.

On this background, it was pertinent to study whether consumption of AndoSan™ had similar effects in patients with CD.

2. Materials and Methods

2.1. 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, 14.7% from He [2] and 2.9% from Gf [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 (lipopolysaccharide) content of AndoSan™ was found, using the Limulus amebocyte lysate test (COAMATIC Chromo-LAL; Chromogenix, Falmouth, MA, USA) with detection limit 0.005 EU / ml (1 EU = 0.1 ng / ml), to be a miniscule concentration of <0.5 pg / ml. AndoSan™ had been heat-sterilized (124°C for 1 h) by the producer. Several tests from Japan Food Research Laboratories (authorized by the Japanese Government) were done in March 2012, December 2013, October 2014, April 2015 and February 2016. The tests were for pH, arsenic, lead, cadmium, tin, aerobic plate count, coliform bacteria (MPN), viable molds count, viable yeasts count, mesophilic aerobic spores, refractometric brix degree and specific gravity (15°C)–and all of the results were within the quantitation limits. AndoSan™ also passed the water quality test (no bacteria, acceptable level of ions, pH, taste, color and odor). An accelerated aging test (up to four months) with almost unchanged character of the mushroom drink. AndoSan™ were also tested for radioactivity, with no detection of Cesium-137, Cesium-134 and Iodine-131 (Meiji Co, Japan). In addition, the Norwegian Food Safety Authorities found no radioactivity.

2.2. 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, 14 and 21) analyzed for haemoglobin, haematocrite, mean cellular volume, mean cellular haemoglobin, reticulocytes, immature reticulocytes, leukocytes 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. Fecal calprotectin concentrations (mg/kg) (normal value <50 mg/kg) at days 0, 14 and 21 were determined in duplicates as reported [24, 25].

The patient-reported symptom score was the simple Crohn’s disease Activity Index (SCDAI) also denounced the Harvey-Bradshaw index [26]. 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 extraintestinal manifestations (arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula, abscess (score 1 per item)).
The symptom score ranges from 0–21. Scores 3–5 meant mild, 6–9 moderate and over 9 severe disease activity. A criterion for inclusion was a score beyond 2.

Self reported health-related quality of life (HRQoL) was assessed with the short form 36 (IQOLA SF-36 Norwegian version 1.2), which is a generic HRQoL questionnaire consisting of 36 items, of which 35 are grouped into the following eight health domains: (1) physical functioning (PF), (2) social functioning (SF), (3) role limitations due to physical problems (RP), (4) role limitation due to emotional problems (RE), (5) mental health (MH), (6) vitality (VT), (7) bodily pain (BP) and (8) general health perception (GH). Each domain is graded on a scale of 0–100, and the higher the score the better the HRQoL. The validity and reliability of the SF-36 form have been demonstrated for a number of countries including Norway (version 1) [27].

The data were compared with published norms from 2323 individuals in the general population. Only 30 out of 5400 HRQoL questions were unanswered, and accordingly, 17 out of 1200 dimensions were lacking. Using a scoring algorithm for missing data outlined in the SF 36 survey manual, still 5 out of 17 dimensions involving 3 patients in the placebo group, could not be included in the results.

Fatigue consists of total fatigue (11 items of graded questions with score 0–3 per question), which is the sum of physical fatigue (7 items) and mental fatigue (4 items), which has been validated in a Norwegian general population [28]. The respective scores for total, mental and physical fatigue are 0–33, 0–21 and 0–12, and the higher the score the more fatigue. The items of physical (1–7) and mental (8–11) fatigue were: 1) Do you have problems with tiredness? 2) Do you need to rest more? 3) Do you feel sleepy or drowsy? 4) Do you have problems with starting things? 5) Are you lacking in energy? 6) Do you have less strength in your muscles? 7) Do you feel weak? 8) Do you have difficulty concentrating? 9) Do you have problems thinking clearly? 10) Do you make slips of the tongue when speaking? 11) How is your memory? Criteria for chronic fatigue syndrome was a dichotomized score >4 and duration >6 months.

2.3. Inclusion of Patients

173 patients with CD were phone interviewed and those with SCDAI score of at least 3 were given the opportunity to join the study. At the first attendance SCDAI was re-recorded 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 of medication and/or consumption of mushroom products from two weeks before till end of the study. A flow chart reveals additional reasons for exclusions (Fig 1). The 23 excluded patients in the initial screening mentioned as “other reasons” were: 10 could not participate because of not able to attend, 5 had moved to a different part of the country, 3 never showed up, and 1 proved to be pregnant, and one each had an ileostomy with high output, drug abuse, severe comorbidity or language difficulties.

2.4. Experimental Design and Randomization

This is a single-center randomized two-armed patient-blinded study designed to determine whether daily oral intake of a mushroom extract, AndoSan™, improved clinical symptoms, fatigue and quality of life in patients with CD during the 21 days’ study period. The patients were evaluated before (visit 1, day 0), during (visit 2, day 14) and after (visit 3, day 21) consumption of AndoSan™ (30 ml twice daily). This dose (60 ml/day) reduced levels of pro-inflammatory cytokines and chemokines in healthy volunteers [24] and in patients with UC and CD [34], whilst half dosage (30 ml/day) had no detectable effects (unpublished data). The placebo group was evaluated likewise but received an equal volume of color-like drink with ionized water containing 0.5 ml per litre of caramel color (E150c) with salt.
Block-randomization was done after the phone interview, with uneven and even numbers given for AndoSan™ or placebo, respectively. The patients, one by one, were placed in one pile, and the group affiliations were placed in another pile. The randomization was performed by combining one selection from each pile, both anonymized. The first author performed the randomization, enrolled the participants, and assigned participants to interventions. A few patients were excluded throughout the study by not attending or because of intercurrent incidents (disease, unexpected life events). Accordingly, a slight imbalance of the study groups...
occurred that was corrected for in the latter rounds of randomization. More specifically, the 50 CD patients were divided into 13 groups (range 1–9 per group), each with a study period of 3 weeks. The included 50 symptomatic patients had almost no missing data (only for 3 patients with missing a total of 5 out of 1200 dimensions for the SF-36 results) and were randomized and blinded for oral daily consumption (30 ml twice daily) of AndoSan™ or placebo for the 21 days’ experimental period. Patients in the AndoSan™ group and the placebo group self-reported, in written, at visit 1 (day 0), visit 2 (day 14) and visit 3 (day 21) regarding symptoms, fatigue and health-related quality of life. Patient derived blood samples and fecal calprotectin from these visits were also analyzed. All data were stored in a secure database (Access–Microsoft Office) at a server at Oslo University Hospital, Ullevål, Norway. A study number anonymized the patients.

This study on clinical outcome is a follow up of a previous pilot study [22] in which there was a reduction of pro-inflammatory cytokines and chemokines in patients receiving the same daily dose of AndoSan™, but for 12 days. Prospective differences of 20% between the experimental and placebo group and assumed standard deviation of 20% for the different parameters with a significant level of 5% and a power of 90% (ß = 0.10), demands about 25 patients per randomized arm (calculated in cooperation Oslo Center for Biostatistics and Epidemiology, Oslo University Hospital).

2.5. Patient Characteristics

Disease duration for the CD patients was 9.7 years (range 0.5–46) and 8.0 years (range 0.5–42) in the AndoSan™ and placebo groups, respectively. Disease location and behavior, number and type of resections as well as proportions of patients subjected to surgery were registered (Table 1). There were 30 and 35 extra-intestinal manifestations in 21 patients in the AndoSan™ group and 22 patients in the placebo group, respectively. Comorbidities, exclusively in the AndoSan™ group, were Mb Bechterew in 3, diabetes mellitus in 1 and chronic obstructive lung disease in 1 patient(s). Combinations of 5-ASA, azathioprine and budesonide or prednisolone were consumed in 3 and 2 patients, respectively. Topical treatment with 5-ASA was used in 3 patients in the AndoSan™ group.

2.6. Statistical Analysis

Data are presented as mean and standard deviation or as median and range values. Paired sample t-test and Wilcoxon test were used for within-group analysis. The judgment of whether the distributions of the main efficacy variables were so close to the normal distribution that normality-based significance tests may be used, also for each individual index at baseline that compose the SCDAI, is based on the finding in a paper by Fagerland and Sandvik [29]. Mixed models corrected for baseline values were used for measuring P values between the AndoSan™ and placebo groups, using V1, V2 and V3 with time as a continuous variable. P values below 0.05 were considered statistically significant. The SPSS statistical program for the social sciences, version 22 (IBM) was used in the analyses.

2.7. Ethical Considerations

The study was approved on April 8, 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 (December 15, 2011). The authors confirm that all ongoing and related trials for this drug/intervention are registered.

3. Results

3.1. Exclusion of randomized patients

A total of 76 patients, 37 in the AndoSan™ group and 39 in the placebo group, were randomized for inclusion in this study. 26 of these patients were excluded according to the criteria of the study protocol, because of missing data on symptom score, laboratory data and not attending in 20, 4 and 1, patient(s), respectively. Thereby we ended up with 25 patients in the AndoSan™ group and 25 in the placebo group (Fig 1).
3.2. Age and Gender
Median age for the 50 included patients with CD was 41 years (range 22–74). There were 11 men and 14 women in the AndoSan™ group and 10 men and 15 women in the placebo group. Respective ages in the two groups were median 42 (range 22–70) and 44.5 (28–74) for men (p = 0.611) and 43 (26–69) and 38 (25–61) for women (p = 0.36).

3.3. Symptom Score
The symptom scores were similar at inclusion in the AndoSan™ and placebo groups, with respective mean scores of 5.52 and 5.04. There were no significant differences in baseline symptom scores between male and females within the two groups. Compared with baseline there were in the AndoSan™ group increasing reductions of symptom score for both genders from visit 2 (day 14) to visit 3 (day 21) (Table 2). In the placebo group, only for women there was a reduction of symptom score from baseline to visit 2 but not visit 3. When comparing the two groups using mixed models corrected for baseline values for both genders there was no difference (p = 0.106). However, for men, there was a close to significant difference (p = 0.054) in favor of the mushroom group.

Within the AndoSan™ group there was for both genders a significant improvement in stool frequency with scores 2.04, 1.56 (p = 0.01) and 1.20 (p < 0.01) at visit 1–3, respectively. There also was a trend for both genders towards reduction of abdominal pain with scores 1.12 and 0.88 (p = 0.06) at visit 1 vs. 3, with significant values for men (p = 0.04). When using mixed models corrected for baseline values there was a significant difference between the study groups for stool frequency (p = 0.011), in favor of the AndoSan™ group, but not for abdominal pain (p = 0.36).

3.4. Fatigue Score
Firstly, the normative fatigue scores in the Norwegian population were compared with scores in the CD patients at inclusion in this study (Table 3). There were for both genders significant decreases of physical, mental and total fatigue scores in the CD patients compared with the general population. This effect was much more pronounced for physical fatigue than for mental fatigue.

Twenty of the 50 patients (40%) had chronic fatigue at visit 1 vs. about 11% in the normative population [28]. The scores for genders on inclusion were quite similar within and between the groups (data not shown). In the AndoSan™ group (Table 4) for both genders the CD patients reported a significant decline in total and physical fatigue, but not mental fatigue, from baseline

---

Table 2. Symptom score (SCDAI) for the CD patients.

<table>
<thead>
<tr>
<th></th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>(P_{V1V2})</th>
<th>(P_{V1V3})</th>
<th>(P_{between\ groups})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AndoSan™</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M (n = 11)</td>
<td>5.52 (4.64–6.40)</td>
<td>4.48 (3.69–5.27)</td>
<td>4.08 (3.22–4.94)</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.106</td>
</tr>
<tr>
<td>F (n = 14)</td>
<td>5.05 (4.07–6.11)</td>
<td>3.64 (2.72–4.55)</td>
<td>3.27 (1.99–4.55)</td>
<td>0.014</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M (n = 10)</td>
<td>5.04 (4.49–5.59)</td>
<td>4.52 (3.72–5.32)</td>
<td>4.68 (3.92–5.44)</td>
<td>0.119</td>
<td>0.327</td>
<td></td>
</tr>
<tr>
<td>F (n = 15)</td>
<td>4.70 (4.11–5.29)</td>
<td>4.50 (2.74–6.26)</td>
<td>4.80 (3.23–6.37)</td>
<td>0.764</td>
<td>0.885</td>
<td></td>
</tr>
</tbody>
</table>

V1; visit 1 (day 0), V2; visit 2 (day 14), V3; visit 3 (day 21).
Values are given as means and 95% confidence intervals. Paired sampled t-test for the p-values.

\(P\) between groups is measured with mixed models corrected for baseline values.

---

V1: visit 1 (day 0), V2: visit 2 (day 14), V3: visit 3 (day 21).
Values are given as means and 95% confidence intervals. Paired sampled t-test for the p-values.

\(P\) between groups is measured with mixed models corrected for baseline values.

---

V1: visit 1 (day 0), V2: visit 2 (day 14), V3: visit 3 (day 21).
Values are given as means and 95% confidence intervals. Paired sampled t-test for the p-values.

\(P\) between groups is measured with mixed models corrected for baseline values.

---

V1: visit 1 (day 0), V2: visit 2 (day 14), V3: visit 3 (day 21).
Values are given as means and 95% confidence intervals. Paired sampled t-test for the p-values.

\(P\) between groups is measured with mixed models corrected for baseline values.
to visit 3 (day 21). Mental fatigue was transiently improved at visit 2 but not at visit 3. In the placebo group, however, the three aspects of fatigue was improved both at visit 2 and 3. Moreover, when comparing the AndoSan™ and placebo groups using mixed models corrected for baseline values there were no differences between the two groups regarding the three fatigue scores.

3.5. Quality of Life

HRQoL scores (SF-36) for CD patients were compared with age-adjusted normative data for the Norwegian population (Table 5). Men had significant reduction in scores of all 8 dimensions whilst women had similar results with the exception of physical functioning (PF), which was within the normal range. The reductions of quality life scores in the CD patients were for both genders most pronounced for the dimensions vitality (VT), general health (GH) and role limitations, physical (RP) (p values < 0.0001) as compared with the general population.

In the AndoSan™ group as a whole the HRQoL score were at visit 3 significantly improved (Table 6) for bodily pain (BP) and VT, whilst VT and SF were improved in the placebo group. When broken into gender the respective improvements at visit 3 were BP for men and SF for women in the AndoSan™ group versus none in the placebo group (data not shown). There were no significant differences when comparing the two groups, using mixed models corrected for baseline values.

Table 3. Mean fatigue scale scores. Normative data in the Norwegian population compared with the included CD patients.

<table>
<thead>
<tr>
<th></th>
<th>Normative data</th>
<th>CD</th>
<th>PNormative data vs UC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M (n = 1112)</td>
<td>F (n = 1175)</td>
<td>M (n = 21)</td>
</tr>
<tr>
<td>Total</td>
<td>11.9 (3.9)</td>
<td>12.6 (4.0)</td>
<td>16.43 (4.48)</td>
</tr>
<tr>
<td>Physical</td>
<td>7.6 (3.0)</td>
<td>8.2 (3.2)</td>
<td>11.38 (3.69)</td>
</tr>
<tr>
<td>Mental</td>
<td>4.3 (1.4)</td>
<td>4.4 (1.4)</td>
<td>5.05 (1.40)</td>
</tr>
</tbody>
</table>

Normative data from the general Norwegian population, age 19–80. Values are given as mean and standard deviation (SD). Independent sample t-test for the p-values.

doi:10.1371/journal.pone.0159288.t003

Table 4. Fatigue scores for the patients with (n = 25 AndoSan™ and n = 25 placebo) CD.

<table>
<thead>
<tr>
<th></th>
<th>AndoSan™ group</th>
<th>Placebo group</th>
<th>Pbetween groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>16.40 (5.07)</td>
<td>18.00 (5.55)</td>
<td>0.813</td>
</tr>
<tr>
<td>V2</td>
<td>15.28 (4.86)</td>
<td>16.28 (4.97)</td>
<td></td>
</tr>
<tr>
<td>V3</td>
<td>14.00 (5.10)</td>
<td>15.36 (4.65)</td>
<td></td>
</tr>
<tr>
<td>PVT2</td>
<td>0.128 (0.55)</td>
<td>0.011 (4.26)</td>
<td></td>
</tr>
<tr>
<td>PVT3</td>
<td>0.032 (0.51)</td>
<td>0.011 (4.01)</td>
<td></td>
</tr>
<tr>
<td>PhF</td>
<td>11.08 (3.51)</td>
<td>12.56 (4.26)</td>
<td>0.824</td>
</tr>
<tr>
<td>(9.04)</td>
<td>(3.74)</td>
<td>(4.00)</td>
<td></td>
</tr>
<tr>
<td>0.289</td>
<td>(3.66)</td>
<td>(4.01)</td>
<td></td>
</tr>
<tr>
<td>0.016</td>
<td>(4.26)</td>
<td>(4.01)</td>
<td></td>
</tr>
<tr>
<td>MF</td>
<td>5.32 (1.97)</td>
<td>5.44 (1.83)</td>
<td>0.824</td>
</tr>
<tr>
<td>(4.80)</td>
<td>(1.56)</td>
<td>(1.43)</td>
<td></td>
</tr>
<tr>
<td>4.96</td>
<td>(1.77)</td>
<td>(1.10)</td>
<td></td>
</tr>
<tr>
<td>0.045</td>
<td>(1.83)</td>
<td>(1.10)</td>
<td></td>
</tr>
<tr>
<td>0.265</td>
<td>(1.43)</td>
<td>(1.10)</td>
<td></td>
</tr>
</tbody>
</table>

V1; visit 1 (day 0), V2; visit 2 (day 14), V3; visit 3 (day 21). TF; total fatigue, PhF; physical fatigue, MF; mental fatigue. Values are given as mean and standard deviation (SD). Paired sampled t-test for p-values. P between groups is measured with mixed models corrected for baseline values.

doi:10.1371/journal.pone.0159288.t004
3.6. Calprotectin in Feces and Effect on General Blood Parameters

The patients delivered fecal tests at visits 1, 2 and 3. In the AndoSanTM group (n = 25) the median (range) values for fecal calprotectin (mg/kg) were 394 (17 – 6000), 398 (20 – 2244) and 472 (36 – 1623), respectively. In the placebo group the corresponding values were 293 (21 – 2783), 515 (12 – 6000) and 342 (10 – 4659). There were no significant differences in levels of calprotectin within or between the groups, also when broken into gender (data not shown).

The following blood samples were analyzed at visit 1 and 3: CRP, leukocytes, eosinophils, basophils, neutrophils, lymphocytes, monocytes, hemoglobin, hematocrit, mean corpuscular volume, and platelets. The levels of these markers were analyzed as part of the routine diagnostic procedures.

Table 5. Mean SF-36 scale scores. Age-adjusted Normative Data from the Norwegian Population compared with patients with CD on inclusion.

<table>
<thead>
<tr>
<th>Normative data</th>
<th>Crohn’s disease</th>
<th>PNormative data vs CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>M (n = 977–1017)</td>
<td>F (n = 1013–67)</td>
<td>M (n = 21)</td>
</tr>
<tr>
<td>PF</td>
<td>91.37 (16.19)</td>
<td>87.72 (17.53)</td>
</tr>
<tr>
<td>RP</td>
<td>83.27 (31.98)</td>
<td>79.18 (34.99)</td>
</tr>
<tr>
<td>BP</td>
<td>78.06 (24.61)</td>
<td>74.46 (26.00)</td>
</tr>
<tr>
<td>GH</td>
<td>78.34 (20.98)</td>
<td>77.46 (22.14)</td>
</tr>
<tr>
<td>VT</td>
<td>63.36 (18.19)</td>
<td>57.57 (21.00)</td>
</tr>
<tr>
<td>SF</td>
<td>88.23 (20.52)</td>
<td>84.78 (22.79)</td>
</tr>
<tr>
<td>RE</td>
<td>85.89 (28.47)</td>
<td>80.94 (33.10)</td>
</tr>
<tr>
<td>MH</td>
<td>79.74 (15.75)</td>
<td>77.64 (16.85)</td>
</tr>
</tbody>
</table>

Age-adjusted normative data from the general Norwegian population, age 19–69.
Values are given as mean and standard deviation (SD). Independent sample t-test for the p-values.


doi:10.1371/journal.pone.0159288.t005

Table 6. Mean SF-36 scale scores (n = 25 AndoSanTM and n = 25 placebo) CD.

<table>
<thead>
<tr>
<th>AndoSanTM group</th>
<th>Placebo group</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>V2</td>
</tr>
<tr>
<td>PF</td>
<td>85.80</td>
</tr>
<tr>
<td>(14.8)</td>
<td>(14.4)</td>
</tr>
<tr>
<td>RP</td>
<td>41.00</td>
</tr>
<tr>
<td>(43.2)</td>
<td>(43.9)</td>
</tr>
<tr>
<td>BP</td>
<td>53.96</td>
</tr>
<tr>
<td>(20.3)</td>
<td>(18.6)</td>
</tr>
<tr>
<td>GH</td>
<td>50.64</td>
</tr>
<tr>
<td>(23.4)</td>
<td>(23.6)</td>
</tr>
<tr>
<td>VT</td>
<td>32.53</td>
</tr>
<tr>
<td>(20.0)</td>
<td>(21.5)</td>
</tr>
<tr>
<td>SF</td>
<td>65.50</td>
</tr>
<tr>
<td>(25.8)</td>
<td>(20.7)</td>
</tr>
<tr>
<td>RE</td>
<td>69.33</td>
</tr>
<tr>
<td>(41.9)</td>
<td>(41.4)</td>
</tr>
<tr>
<td>MH</td>
<td>69.28</td>
</tr>
<tr>
<td>(17.1)</td>
<td>(13.3)</td>
</tr>
</tbody>
</table>

Paired sample t-test for the p-values.
P between groups is measured with mixed models corrected for baseline values.

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volume, mean cellular haemoglobin, immature reticulocytes, reticulocytes, thrombocytes, urea, creatinine, and GFR (glomerular filtration rate), bilirubin, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, γ-glutamine transferase, alkaline phosphatase and pancreatic amylase. Significant changes were for reduction of bilirubin (μmol/L) from 11.4 to 9.2 (p = 0.02) in the AndoSan™ group and for increase of thrombocytes (10⁹/L) from 289 to 302 (p = 0.03) in the placebo group.

The median and range of blood samples of special interest were haemoglobin (g/l), leucocyte counts (10⁹/l) and CRP levels for visit 1 and 3 the AndoSan™ group (n = 25) were 13.4 (12.0–16.2) versus 13.3 (11.4–16.0), 6.1 (3.1–12.2) versus 6.8 (2.7–13.0) and 2.9 (0.6–30.9) versus 2.1 (0.6–25.3), respectively. Corresponding values in the placebo group (n = 25) were 13.7 (10.5–15.5) versus 13.6 (10.7–15.5) for haemoglobin, 6.6 (4.2–13.8) versus 6.7 (3.9–11.0) for leucocytes and 2.2 (0.6–41.6) versus 2.1 (0.6–34.8) for CRP. Accordingly, there were no statistical changes of these parameters neither within nor between the groups.

4. Discussions

The main finding in this placebo-controlled patient blinded prospective study was that the immunomodulatory Agaricus blazei Murill-based mushroom extract AndoSan™ [14] increasingly improved clinical symptoms in patients of both genders during a 3 weeks’ study period. In the placebo group there was an improvement of symptoms exclusively for women, although less than in the AndoSan™ group and not significant. There were no significant differences between the study groups, but a close to significant p-value (0.054) when comparing the symptom score for men.

Of the four items (general well-being, abdominal pain, number of liquid stools per day, complications) of the SCDAI [26] there were improvements of key symptoms such as of number of liquid stools for both genders and pain for men. Interestingly, despite the trend in the AndoSan™ group towards more stricturing disease (p = 0.04), less inflammatory disease (p = 0.04), significantly more ileal (p = 0.01) and ileocolic resections (p = 0.02) (Table 1), which implied increased disease activity vs. the placebo group, the improvement of symptoms as a whole was only evident in the former group.

Compared with the normal population the CD patients had considerably more fatigue, especially physical fatigue probably owing to the somatic manifestations of this chronic granulomatous disease. However, we were not able to demonstrate any advantage using AndoSan™ vs. placebo on outcome of fatigue. At visit 3 there was as a whole an improvement in mental-, physical- and total fatigue in the placebo group vs. only the two latter fatigue scores in the AndoSan™ group (Table 4). Thus, despite external factors that presumably could influence mental fatigue due to intercurrent subclinical and mental disease, we conclude that the mushroom extract vs. placebo had no demonstrable effect on fatigue in these patients.

For HRQoL in the AndoSan™ group as a whole there was an improvement in bodily pain at visit 3, which is interesting because pain was the second item of the SCDAI that was significantly reduced for men at visit 3. Common to the AndoSan™ and placebo groups were the improvements of vitality, whilst social functioning also was improved in the latter group. With the exception of improvement in bodily pain, which is a crucial factor in patients with CD suffering from intestinal and other symptoms, we conclude that the effect of AndoSan™ on HRQoL was not different from placebo. Besides a reduction of bilirubin in the AndoSan™ group and an increase of thrombocytes in the placebo group there was as reported [22] in a one-armed pilot study with 12 days’ AndoSan™ consumption in CD patients, no alterations in general blood parameters including CRP as well as fecal calprotectin.

In a recent similar placebo-controlled study [23] of patients with UC consuming AndoSan™, the improvements in addition to symptoms irrefutably also were evident for fatigue
and HRQoL. Obviously, clinical improvement due to intake of this mushroom extract in patients with CD was more limited vs. those with UC. This may partly be explained by the fact that CD is pan-intestinal and characterized by transmural inflammation complicated by stenosis and/or development of fistulas in addition to more systemic manifestations (e.g. joints and anal fissures).

The AndoSan™ and placebo groups had similar baseline values with respect to symptom score, fatigue and quality of life. The groups were also quite comparable with regard to disease duration, arthralgia and relevant comorbidity. However, there were significantly more resections in the AndoSan™ group (20 vs. 9) and, accordingly, more stricturing and severe disease. Analyses of symptom score and fecal calprotectin were similar when we did calculations comparing the patients with inflammatory and stenotic presentation (data not shown).

The patients did not discontinue consumption of the mushroom extract throughout the study, since there were no side effects and normal blood samples, including liver function. As recently outlined [30], AndoSan™ and AbM per se have been well tolerated by the patients in several clinical studies for hepatitis B [31] and C [30] and different cancers (breast, ovarian, myeloma) [32–34]. AbM induced temporary urticaria and moderate liver dysfunction developed after two months of AbM consumption [33], in one out of 78 patients only, which also received chemotherapy for ovarian cancer. With our knowledge of AndoSan™ and AbM, and review of the literature, we believe it is safe as a supplement to patients with different types of disease.

The patients were not blinded for the authors leaving possible bias with respect to different attitude towards patients in the two groups. This is especially true for the first author who was responsible for the inclusion and randomization of participants, the implementation of the practical aspects of and in meeting with the patients, and also in the analysis of the results. This is a relatively small study, although with some significant results, with its limitations. 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 three weeks.

Regarding possible drug interactions, AndoSan™ did less than green tea inhibit the transmembrane efflux P-gp pump present in intestines and liver and hence important for drug absorption and excretion [35]. However, possible interactions may occur with P-gp substrates, e.g. some anti-cancer, diarrhea (loperamide) and cardiac (digoxin) agents, and P-gp inhibitors, e.g. verapamil. When testing AndoSan™ on cytochrome P-450 metabolism, the extract inhibited it, but far less than green tea and clinically relevant systemic interactions were therefore considered unlikely [36]. In our clinical study, none of the CD patients were concomitantly treated with the above-mentioned anticancer-, heart-, or diarrhea drugs.

There are several studies of Agaricus blazei Murill, Hericium erinaceus and Grifola frondosa, isolated or in the mixture together as AndoSan™, showing beneficial immunomodulatory, antioxidant, antihyperglycemic and anti-tumor effects [13, 14, 37–39]. However, most data, to date, were produced using rodents or cell cultures, and there are a very limited number of studies measuring their effects in humans.

In a placebo-controlled study in patients with gynecological cancer, AbM treatment in addition to chemotherapy was reported to increase NK cell activity in blood and improved the patients quality of life [40].

A randomized double-blind placebo-controlled study on 30 critically ill ICU patients, followed for 7 days, found significant increases in NK cell activities when given immune-enhancing enteral nutrition (IMHP) enriched with β-glucan from the baseline and significantly greater increase than the control group [41]. The authors suggest that β-glucan can be an attractive candidate to add to IMHP for stimulation of protective immunity without enhanced
inflammation in critically ill patients. This finding is in line with previous studies in which β-glucan enhanced NK cell activation in mice [42, 43].

A randomized double-blinded clinical trial in 57 elderly females found no immunomodulatory effect as measured by unaltered plasma levels of IL-6, TNFα and IFNγ after ingesting dried AbM capsules for a 60-day study period [44].

The notion of a potential anti-inflammatory effect of AndoSan™ intake was a result of the surprising finding of reduced serum pro-inflammatory cytokines (IL-1β, IL-6, IL-8) and chemokines (MCP-1, G-CSF, GM-CSF) in a pilot safety study with AndoSan™ in healthy individuals without a placebo control [24].

In a study on healthy volunteers ingesting AndoSan™ [37] there was a reduction in vivo of ROS mainly reflecting superoxide ions, and again pointing to an anti-inflammatory effect. However, this result was not demonstrated in the CD patients in the aforementioned pilot study (data not shown). The reason for reduced superoxide anions may be related to reduction of IL-1β because inhibitors of ROS reduce synthesis of this cytokine in macrophages [45].

Oral administration of AndoSan™ is associated with low bioavailability due to polysaccharides, like β-glucan, that is normally not taken up from the GI-tract as it is a non-degradable cellulose (the human tract normally only takes up monosaccharides). However, Rice et al [46] showed that soluble β-glucans are able to bind directly and undergo internalization by intestinal epithelial cells and gut associated lymphoid tissue (GALT) cells. The internalization of soluble β-glucan by intestinal epithelial cells is not dependent on dectin-1, however in GALT cells dectin-1 and TLR-2 participate in uptake of soluble β-glucan.

Recently, a steroid 4-hydroxy-17-methylincisterol (4-HM) [12] isolated from AbM dose-dependently suppressed the synthesis in PHA-stimulated peripheral blood mononuclear cells of cytokines IL-2, IL-4, IFNγ and TNFα by decreasing both NF-AT (nuclear factor of activated T-cells), which belongs to a family of transcription factors required for activation and proliferation of T lymphocytes including production of the first three aforementioned cytokines, and NF-κB—the latter being the “mother” of all inflammation.

Alkaline and aqueous substances isolated from AbM [11] had, when given orally to rats for 1–2 weeks, several anti-inflammatory effects such as improved healing of stress-induced ulcers and reductions of paw edema in the presence of nystatin or Freund’s adjuvant, as well as reduced neutrophil migration to the peritoneal cavity. It was speculated that these effects in part were related to modulations of cytokine levels for TNFα and IL-8. In another recent study [47] a water soluble polysaccharide isolated from AbM was given orally for 8 weeks to ovariectomized and osteopenic rats, and it markedly decreased serum levels of IL-1β, TNFα, ICAM-1 and total antioxidant status. AbM contains absorbable low-molecular weight anti-oxidant substances [10] that down-regulate the levels of reactive oxygen species (ROS) in vitro. Type 1 diabetes has similarities to CD because it has been regarded both as an autoimmune and as an innate inflammatory disease affecting the pancreas [48]. Anecdotes of possible benefits of AbM for diabetes in folk medicine have been supported by findings in a rodent model for diabetes [9]. Since the hypoglycemic effect of AbM seems to result from its suppression of oxidative stress and pro-inflammatory cytokine production [38], similar therapeutically mechanisms probably play a role in CD patients who responded positively on the AndoSan™ treatment in the current study.

There also was an anti-allergic effect in mice sensitized to ovalbumin (OVA), as demonstrated by reduction of specific anti-OVA IgE antibodies, both when AndoSan™ was given before or after the OVA immunization [49]. In this allergy model also there was an increase in Th1 relative to Th2 cytokines in spleen cell cultures ex vivo obtained from the animals treated with AndoSan™ [49]. In addition, the inhibitory effect of an isolated carbohydrate fraction of AndoSan™ [15] on the tissue degrading pro-inflammatory and tumor-associated enzyme,
legumain (asparaginyl endopeptidase), that probably may also contribute to less inflammatory activity in the CD patients.

Pharmaceutical investigation of AndoSan™ revealed the carbohydrate content to be only 2% of the ~5mg dry material/ml, and that it was concentrated in the polar high molecular weight fraction of AndoSan™ [15]. It was this fraction that was the most potent inhibitor of legumain [15]. The mushroom extract also contains some not yet characterized proteins (personal communication, prof G Vegarud at The Norwegian University of Life Sciences). As to the pharmacokinetics of AndoSan™ that is a mixed water extract of mycelium to the aforementioned three Basidiomycetes mushrooms, it has biological effects when taken orally both in mice and men. Ingredients in AndoSan™ may execute their effects directly after absorption through intestinal mucosa enterocytes to the blood and processing in the liver, where Kupffer cells and endothelial cells may be stimulated similar to our previous findings in monocyte/macrophage and endothelial cell cultures [30, 51]. However, substances in the mushroom extract may have a greater indirect influence on the body by inducing changes in the microbiota and production of analytes by bacteria after their uptake of sugar moieties for energy etc.. Moreover, substances such as β-glucan my further be transported by dendritic cells to lymphocytes in GALT and induce local immune responses there, or systemic if circulated in blood [52, 53].

In conclusion, we found increasingly improved symptom score in the AndoSan™ group, of both genders, foremost regarding stool frequency and abdominal pain. Compared with placebo there was a significant reduction in stool frequency, favoring the AndoSan™ group. For fatigue, quality of life, calprotectin in feces and blood samples there were comparable results between the study groups. We suggest that AndoSan™ may be used as a safe supplement to conventional medication to relieve symptoms in these patients. At present, the effects on cytokine levels from AndoSan™ consumption in these patients are being studied.

Supporting Information

S1 Table. CONSORT 2010 Checklist.
(DOC)

S1 Text. Study Protocol Norwegian version.
(DOCX)

S2 Text. Study Protocol English version.
(DOCX)

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Author Contributions

Conceived and designed the experiments: EJ GH TL IL. Performed the experiments: SPT EJ. Analyzed the data: SPT EJ. Contributed reagents/materials/analysis tools: SPT EJ GH TL. Wrote the paper: SPT EJ GH. Recruitment of patients: IL SPT.

References


