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Selective granulocyte and monocyte apheresis in inflammatory bowel disease: Its past, present and future

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Abstract

The etiology and pathogenesis of inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, are not fully understood so far. Therefore, IBD still remains incurable despite the fact that significant progress has been achieved in recent years in its treatment with innovative medicine. About 20 years ago, selective granulocyte and monocyte apheresis (GMA) was invented in Japan and later approved by the Japanese health authority for IBD treatment. From then on this technique was extensively used for IBD patients in Japan and later in Europe. Clinical trials from Japan and European countries have verified the effectiveness and safety of GMA therapy in patients with IBD. In 2013, GMA therapy was approved by China State Food and Drug Administration for therapeutic use for the Chinese IBD patients. However, GMA therapy has not been extensively used in China, although a few clinical studies also showed that it was effective in clinical and endoscopic induction of remission in Chinese IBD patients with a high safety profile. This article reviews past history, present clinical application as well as the future prospective of GMA therapy for patients with IBD.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Granulocyte and monocyte apheresis; Therapy; Efficacy

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Core tip: Conventional therapies for inflammatory bowel disease (IBD) patients including mesalazine, corticosteroids, and immunosuppressants have been used for decades with unsatisfactory outcomes due to ineffectiveness or side effects. Although the emerging biologic agents have revolutionized IBD treatment, severe opportunistic infections, primary or secondary loss of response, *etc.* are the major clinical concerns of clinicians and patients. In recent years, selective granulocyte and monocyte apheresis has been used in Japan, Europe, China and elsewhere for its advantages of satisfactory

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efficacy and high safety profile. Granulocyte and monocyte apheresis therapy is an important and promising therapeutic option for IBD patients.

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INTRODUCTION

Inflammatory bowel disease (IBD) includes two chronic relapsing and remitting diseases, *i.e.* ulcerative colitis (UC) and Crohn's disease (CD). Millions of individuals worldwide are currently affected by this disease in terms of function and quality of life^[1]. Although it is thought to be an immune disorder of the gastrointestinal tract in genetically susceptible individuals exposed to environmental risk factors^[1-4], IBD is sometimes difficult to define due to either its history of unusual complexity or poor understanding of its etiology and pathogenesis. Therefore, IBD still remains incurable despite significant progress in recent years due to its treatment with innovative medicine. Clinical and experimental studies have indicated that the distressful flare-ups of IBD might be triggered by the impaired function of the intestinal barrier and a dysregulated immune response to the gut microbiota^[5,6].

Treatment of IBD with aminosalicylates and corticosteroids has been used for decades with unsatisfactory outcomes due to either their poor effectiveness or side effects. In recent years, an array of emerging medical therapies including biologic agents, immune cell modulators, mucosal barrier enhancers and stem cells have revolutionized IBD treatment^[7]. Nevertheless, therapies for IBD have been mostly empirical rather than based on understanding of the disease etiology. Furthermore, side effects of long-term medications are inevitable, even though medications are often initially effective in the majority of patients^[8].

It is currently believed that an imbalance between pro-inflammatory and anti-inflammatory cytokines exist in patients with IBD, which is closely related to the onset and development of the disease^[9,10]. Additionally, myeloid leukocytes, especially granulocytes and monocytes, have been shown to play an important role in the occurrence and development of IBD^[11,12]. Based on this theory, selective granulocytes and monocytes apheresis (GMA) therapy targeting these cells and subsequently on inflammatory cytokines was invented and applied to IBD patients in Japan about 20 years ago. It has been generally accepted that GMA therapy is a non-pharmacological therapeutic option for IBD patients due to its good therapeutic effect and incomparable safety, especially when conventional therapies are ineffective^[13,14]. This article reviews the past history, present application, and future prospect of GMA therapy for patients with IBD.

GMA HISTORY

The term "apheresis" is from the Greek "*apairesos*" or Roman "*aphairesis*" meaning to remove or take away something by force or a withdrawal. Before modern medicine, it was a common way to treat diseases, *e.g.*, by bloodletting. At that time, it was thought that the goal of treatment could be achieved by eliminating pathogenic factors from the blood^[15]. The removal of plasma and return of red blood cells was first demonstrated in research animals at Johns Hopkins University in 1914. Since then, apheresis technology was gradually used in clinics, including centrifugation, plasmapheresis, plateletpheresis, photopheresis, *etc.* The technique of selective GMA originated from the separation of blood cells.

As early as the beginning of the 20th century, centrifugation was used to selectively remove blood components that were thought to activate or promote the occurrence of diseases to achieve a therapeutic goal through non-drug therapy. Separation of blood cells was first used for leukemia, tumors, rheumatoid arthritis and other diseases and later gradually applied to treat IBD^[16]. However, centrifugation has certain disadvantages of being expensive to perform and difficult to handle.

In the 1980s, a novel extracorporeal leukocyte removal filter with a commercial

name of Cellsorba was developed by Asahi Medical Co. Japan. The filter consists of non-woven polyester cylindrical fabric. Using this filter, one could partially remove leukocytes from the whole blood during extracorporeal circulation, and it was approved for therapeutic use by the Japanese government in 1989. Cellsorba is simpler to operate and more efficient in the removal of granulocytes and monocytes and hence has greater clinical efficacy than centrifugation^[16]. It has been found that the Cellsorba column is capable of removing about 1.6×10^{10} white blood cells per session including almost 100% of neutrophils and monocytes and 30%-60% of lymphocytes.

Studies have shown that the leukocyte removal filter could reduce the number of activated leukocytes as well as serum levels of pro-inflammatory cytokines^[17-19]. Selective GMA, also known as granulocytapheresis, could selectively remove granulocytes and monocytes using a device commercially named Adacolumn, which was developed by the Japan Immunoresearch Laboratories Co., Ltd. of Takasaki, Japan. The column (G-1 granulocyte removal column) is packed with cellulose acetate beads. About 65% of granulocytes, 55% of monocytes and a significant fraction of lymphocytes are removed from peripheral blood passing through the column^[20]. In the early 2000s, Japan national health reimbursement scheme introduced GMA as an induction of remission therapy for UC patients^[21]. In the same year, Adacolumn was approved by the Ministry of Health of Japan for treating UC patients. Afterwards, Adacolumn became available for clinical application for IBD patients in the European Union countries after it was CE marked^[22]. From then on, there have been accumulating reports on clinical efficacy and safety of Adacolumn in patients with IBD from Japan as well as from European countries^[23-28]. In 2013, GMA therapy was approved for Chinese IBD patients by the Government Health Authority, and since then it has been applied in clinics to treat IBD patients in China mainland^[29].

GMA CURRENTLY

GMA equipment

The Adacolumn (G-1 column), 206 mm in length, 60 mm in diameter and 335 mL in capacity, is made of polycarbonate and filled with 220 g of cellulose acetate beads of 2 mm in diameter (adsorptive carriers) bathed in 130 mL sterile saline^[30] (Figure 1)^[31]. The Adacolumn apheresis system consists of four components: The column, the blood circuit lines, Adamonitor and the pump. The column and its blood circuit lines are allowed for single use. The Adamonitor is the center of the system and is formed by a blood pump and four other functional units^[20,22]. The pump of the Adacolumn system has special functional units, including a flow rate and time setting panel, a pressure monitor as well as a fault detecting alarm system. With the help of these functional units, if the actual pressure of the apheresis blood does not match the preset values, then the system will alarm and automatically stop working. Likewise, in the event of other abnormal conditions, the system will recognize it and alarm. Sometimes it will automatically switch off to ensure the safety of apheresis procedures^[22].

Mechanisms of GMA

GMA reduces inflammatory leukocytes and inhibits their infiltration: Elevation in number and activity of neutrophils and monocytes in peripheral blood contributes to the basic pathophysiology of IBD. In patients with IBD, peripheral circulating activated granulocytes, monocytes and macrophages are increased in number and subsequently lead to an increased level of circulating pro-inflammatory cytokines and infiltration of intestinal mucosa by these inflammatory cells, which are significantly correlated with intestinal inflammation level^[11]. Therefore, removal of these inflammatory cells should be theoretically beneficial to patients with IBD^[9].

Cellulose acetate beads inside the Adacolumn are capable of selectively adsorbing circulating neutrophils and monocytes by binding to IgG fragments (Fcγ) and immune complement complexes^[32], which serve as a “connecting bridge” between leukocytes and the beads (Figure 2). A significant reduction of CD14(+) CD16(+) monocytes in peripheral blood of GMA-treated IBD patients can be observed^[33]. One study showed that soluble cell adhesion molecule-1 and soluble vascular cell adhesion molecule-1 were significantly increased in the peripheral blood of patients with IBD and closely related to the degree of tissue inflammation^[34]. An *in vitro* study observed that the concentration of soluble cell adhesion molecule-1 and soluble vascular cell adhesion molecule-1 in blood samples was significantly decreased after incubation with acetate beads as adsorption carrier at different temperatures when compared with that in the control group without acetate beads incubation^[35]. Therefore, GMA is also capable of inhibiting leukocyte migration by downregulating expression of leukocyte related adhesion molecules and thereby affecting the adhesion between

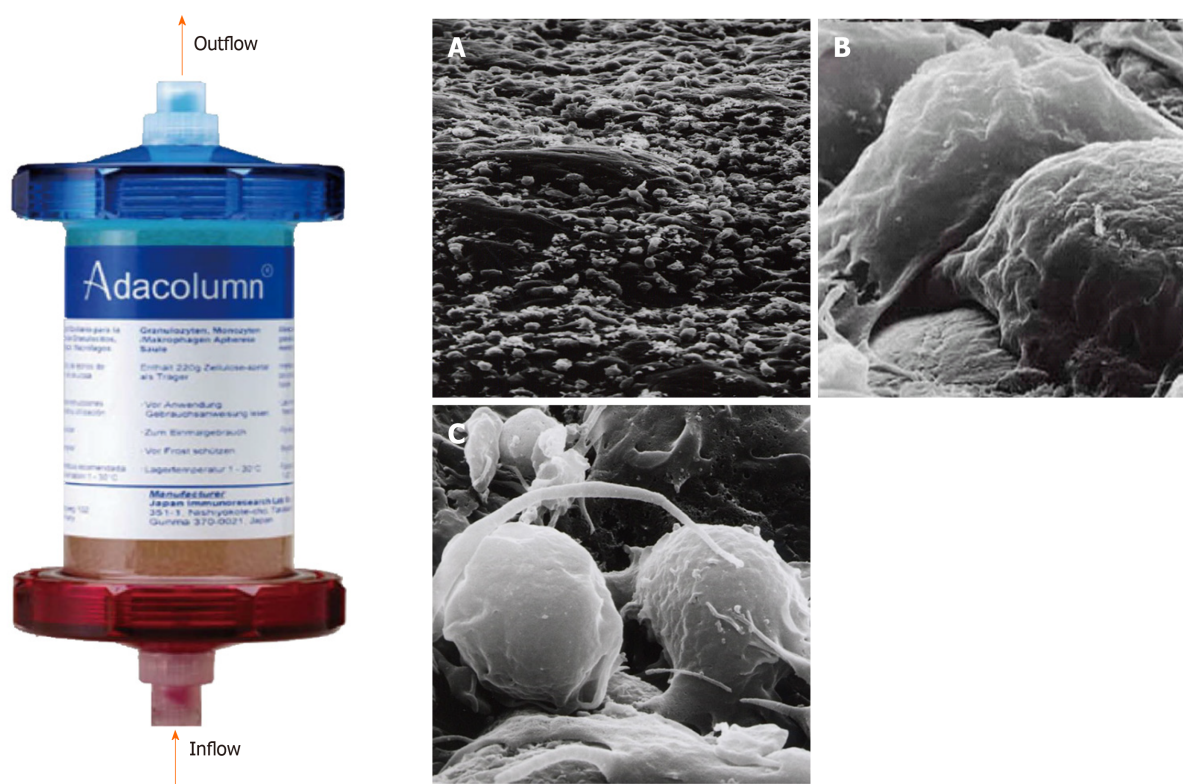


Figure 1 Photograph of Adacolumn and scanning electron photomicrograph of the acetate beads after treatment. Adacolumn is filled with cellulose acetate beads of 2 mm in diameter (adsorptive carriers) bathed in sterile saline. The blood from the antecubital vein of one arm flows into the column and returns to the antecubital vein in the contralateral arm. A: A low power view (400 ×) of the acetate beads in a column after treatment with cells covering the surface of the carrier; B: Viewed at 10000 ×. Neutrophils were adsorbed onto the beads; C: Viewed at 12000 ×. Activated monocyte/macrophages are seen (taken by Dr. A. Saniabadi of Japan Immunoresearch Laboratories). Modified from reference^[31].

cells and vascular endothelium at the initial stage. One study indicated that CX3CR1 played an important role in regulating the dynamic balance of intestinal macrophages, bacterial translocation and inflammatory effector Th17 response in patients with IBD^[36]. Pro-inflammatory monocytes are able to highly express $\alpha 4$ integrin and CX3CR1 in patients with active UC, while GMA is able to selectively remove CD14⁺CD16⁺CX3CR1⁺ monocytes and increase CD14^{hi}CD16⁻CCR2^{low} “immature” monocytes and consequently inhibiting the adhesion and chemotaxis of pro-inflammatory monocytes to a certain extent^[37].

GMA affects functions of other immune cells: One study observed that granulocytes removable by the Adacolumn from peripheral blood were mostly CD10-positive granulocytes, but the number of myeloid CD10-negative premature granulocytes with low pro-inflammatory function increased significantly after GMA therapy. This indicates that GMA therapy may play its therapeutic role indirectly by promoting the migration of “less-inflamed” premature granulocytes from bone marrow to peripheral blood making the granulocyte level in the peripheral blood unchanged^[38]. Another study showed that the Adacolumn adsorption column was involved in the increased induction of myeloid-derived suppressor cells, which are strong anti-inflammatory cells, thus regulating the inflammatory response through immune cells to relieve the disease^[39]. CD4⁺CD25⁺Foxp3⁺T_{reg} cells are necessary for the maintenance of autoimmune tolerance. Kamikozuru *et al*^[40] showed that after five sessions of GMA in active UC patients who achieved remission, the number of CD4⁺CD25⁺Foxp3⁺T_{reg} in the peripheral blood increased to the level of the normal control group at the 10th wk. A clinical study by Muratov *et al*^[41] showed that CD4⁺ T cells producing IFN- γ in peripheral blood of active IBD patients were significantly reduced after GMA therapy, and Waitz *et al*^[42] found that the number of myeloid dendritic cells in the peripheral blood of patients with active UC was significantly higher than that of the normal control group. However, the number of myeloid dendritic cells in peripheral blood after GMA therapy was significantly lower. The mechanism may be that dendritic cells can express a variety of receptors, including Fc gamma, which can be absorbed by the cellulose acetate beads of the Adacolumn, resulting in a transient decline of dendritic cells in the peripheral blood and increase intestinal tolerance to different antigens.

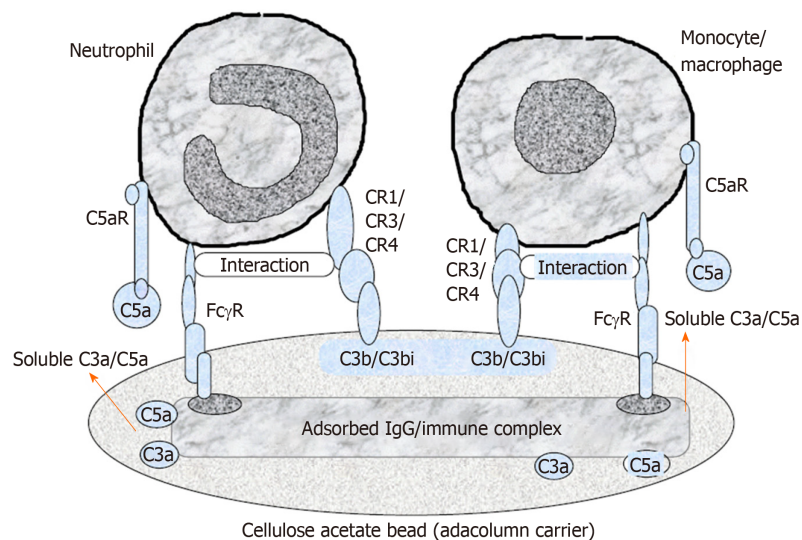


Figure 2 A schematic diagram of the selective adhesion of myeloid granulocyte and monocyte to cellulose acetate carriers. Cellulose acetate beads inside the Adacolumn are capable of selectively adsorbing circulating neutrophils and monocytes by binding to IgG fragments (Fcγ) and immune complements complexes. Lymphocytes are not absorbed as they rarely express complement receptor. Modified from reference^[31].

GMA regulates pro-inflammatory and anti-inflammatory cytokines: As a pro-inflammatory cytokine, TNF- α plays a very important role in IBD. Its level in active IBD patients is significantly higher than that in normal subjects. One study showed that GMA therapy could significantly decrease peripheral blood levels of TNF- α , IL-1 β , IL-6 and IL-8 in active IBD patients to alleviate the disease^[38], and the intestinal tissue levels of these cytokines were significantly lowered in IBD patients who had achieved clinical or endoscopic remission but remained unchanged in patients without remission after GMA therapy^[43]. It is known that TGF- β 1 is a kind of pleiotropic cytokine as well as the most powerful intestinal immunosuppressant. Cellulose acetate in the Adacolumn can absorb soluble human leukocyte antigen I in peripheral blood, and soluble human leukocyte antigen I induces the production and release of soluble Fas ligand, which further leads to the production of TGF- β 1^[44,45], hence inducing the immune suppression effect. On one hand, IL-1, including IL-1 α and IL-1 β , is the major pro-inflammatory cytokine in the early inflammatory response and is significantly enhanced in its expression in the inflamed intestinal tissues, which is correlated well with the disease activity. On the other hand, IL-1Ra, a strong anti-inflammatory inhibitor of IL-1, is increased in active UC patients who responded well to GMA therapy, but no significant change is seen in IL-1Ra levels in patients who did not respond to GMA therapy^[46]. This means that balance of IL-1Ra/IL-1 is very important in regulating inflammatory response in IBD patients.

GMA for IBD

GMA for UC: The first clinical trial of GMA for UC was reported in 2001. It was a multicenter controlled study with a total of 53 patients with active UC receiving five sessions in consecutive weeks of GMA therapy in combination with prednisolone at 14 hospitals in Japan^[21]. By the 7th wk, 58.5% of the patients had achieved remission or improved, and prednisolone dosage was gradually reduced. Only eight non-severe adverse events in 5 patients were reported. Therefore, the first clinical trial indicated that GMA was a potentially effective and safe way to induce remission as well as tapering steroid dosage^[21]. Since then, a large number of clinical applications and studies have been conducted in Japan, most of which showed satisfactory clinical and endoscopic outcomes and proved that GMA is an effective and safe way for UC patients who experienced tapering of corticosteroids as well as lowering the colon resection rate^[24,47-54]. A significant study was reported in 2009 by Hibi *et al*^[55] in which 656 severe or refractory UC patients in 53 medical institutions were observed over 7 years. The results showed the clinical response rates of severe, moderate and mild patients were 63.2%, 65.7% and 80.4%, respectively. Patients treated with GMA had a lower clinical recurrence rate and longer sustained remission.

In recent years, biologic agents and immunosuppressants have been increasingly used in IBD. However, some IBD patients responded poorly to these agents. Many clinical trials were conducted to observe the effect of GMA for IBD patients who failed or were refractory to pharmacological therapy. D'Ovidio *et al*^[24] treated 12 mild to

moderate steroid-dependent/refractory UC patients by GMA. After 6 wk, mucosal healing was accomplished in 3 patients, partial mucosal healing in 8 patients, and 1 patient had no response. In 2016, in a single-arm, open-label, multicenter trial^[56] conducted in 18 centers in the United Kingdom, France and Germany, 84 moderate-to-severe active UC patients having poor response or intolerance to immunosuppressant and/or biologics were enrolled. Each patient received GMA therapy of one session per week with the Adacolumn. After continuous apheresis for 12 wk, 33 of the 84 patients achieved clinical remission, and 47 achieved a clinical response. For patients with previous immunosuppressant and/or biologic failure, the clinical remission rate was 30.0%. Similar results were also found from other reports^[50,57]. These studies indicated that the Adacolumn apheresis is effective in induction of remission in patients with steroid-dependent UC who failed immunosuppressant and/or biological agents.

Apart from these clinical trials aiming at induction of remission, the effectiveness of maintenance in UC patients treated with GMA was also observed. In a multicenter study at 24 medical institutions in Italy^[58], a total of 230 patients (including 194 UC patients) received at least one session of GMA therapy and were then followed up for 1 year. The results showed that 77.7% of UC patients obtained positive outcomes at 3 mo and 87.1% at 12 mo. Similar results were also observed in other studies^[27,41]. Furthermore, it seems to be equally effective in relapsed patients who have achieved remission by previous GMA therapy^[59].

In 2011, a retrospective, observational, multicenter study was conducted for cytomegalovirus (CMV) positive UC patients^[60]. In this study, CMV-positive UC patients were treated with either additional GMA (11 patients) or immunosuppressant (9 patients) after ineffective antiviral treatment. In the GMA group, 9 patients achieved remission and 2 underwent colectomy. In the immunosuppressant group, 4 achieved remission but 5 underwent colectomy. Therefore, it was concluded from this study that GMA was more effective in UC patients with opportunistic CMV infection as compared with conventional drugs like immunosuppressants. Nevertheless, additional studies for GMA therapy for CMV-positive IBD patients need be performed in the future because a conclusion should be reached with care from this retrospective study with a small sample size. However, this study demonstrated that GMA was safer than immunosuppressants in patients with opportunistic infections.

Relapse is an unlucky clinical feature of patients with UC. An open-label, prospective, randomized, controlled study^[61] from the United Kingdom was conducted aiming at prevention of relapse in UC patients by GMA therapy. Sixty UC patients in remission but with fecal calprotectin over 250 mg/g (at high risk of clinical relapse) were enrolled. Twenty-nine patients received five sessions of weekly GMA, and thirty-one patients were kept on maintenance therapy. After 6 mo follow-up, 72.4% of the GMA-treated patients were still in remission, while only 32.3% in the control group were still in remission. Therefore, it was concluded from the studies above that selective leukocytapheresis significantly reduces recurrence rates and delays the time to relapse. Furthermore, GMA in combination with biologics also yielded satisfactory clinical outcomes. In a retrospective study reported by Tanida *et al.*^[62] in 2018, nine refractory UC patients received combination therapy with adalimumab plus intensive GMA. Over half of the nine patients displayed clinical remission at 10 wk, and 33.3% displayed remission at 52 wk under subsequent maintenance monotherapy of adalimumab.

Although GMA therapy has been proven effective and safe in patients with UC, there was a report with contradictory results. In a randomized, prospective, double-blind, sham-controlled trial^[63] conducted at 36 centers in the United States and Canada, 168 patients with moderate to severe active UC were enrolled and assigned randomly to either GMA group (84 patients) or sham-treatment group (84 patients). The results showed that the clinical remission and response rate in the GMA group were 17% and 44%, respectively, while the clinical remission and response rate in the sham-treatment group were 11% and 39%, respectively. No differences between the two groups were found indicating that GMA was unsatisfactory in treating patients with moderate to severe UC. The conclusion from this study contradicts that from the majority of other studies. It is known that the best responders to GMA therapy are UC patients of short disease duration with no previous medications and steroid naïve UC patients^[53,64]. So the possible explanation for this contradiction may be that patients enrolled in this trial did not fall into the category of best responders.

In summary, GMA is an effective and safe therapeutic option for moderate to severe UC patients, particularly for those who are refractory to or dependent on corticosteroids, which can be tapered or avoided. At present, no one can conclude that GMA can be used as a first-line therapy, but at least as an alternative choice for patients with UC (Figures 3 and 4).

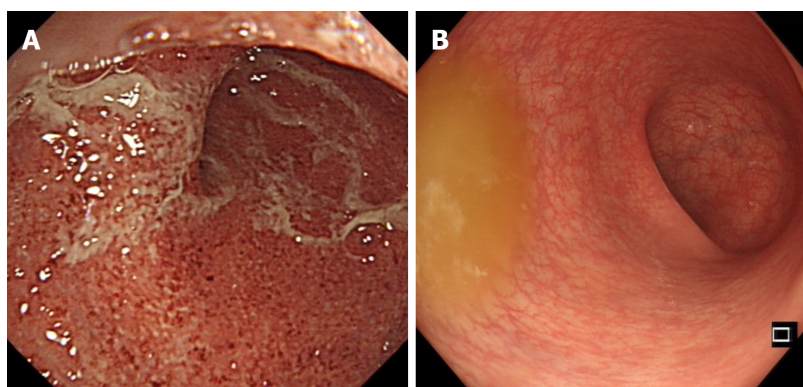


Figure 3 Endoscopic photographs of an ulcerative colitis patient who responded well to selective granulocyte and monocyte apheresis. A: Endoscopic photograph before granulocytes and monocytes apheresis therapy; B: Endoscopic photograph after ten sessions of granulocytes and monocytes apheresis therapy.

GMA for CD: The efficacy of GMA in patients with CD was first reported by Matsui *et al*^[65], who treated 7 CD patients refractory to conventional therapy. The patients received five or six sessions of weekly GMA in combination with the previous conventional therapy. The results showed that 5 patients achieved clinical remission while the other 2 patients did not respond. In another clinical study that enrolled 21 active CD patients, all of the patients achieved significant improvement 7 wk after weekly GMA for 5 consecutive weeks as an adjunct to conventional therapy evaluated by CDAI, IOIBD and IBDQ scores^[66]. Apart from the studies showing the usefulness of GMA, other clinical studies including case reports also verified the effectiveness of GMA in patients with CD^[67-70]. In 2018, Tanida *et al*^[71] reported three patients with refractory CD treated with intensive GMA plus ustekinumab. At wk 10, clinical remission was achieved for all the patients. Therefore, at present it is believed that GMA or in combination with biologics can be used to induce remission in CD patients.

As for maintenance of remission, a clinical case report from Spain showed that one steroid-dependent CD patient experienced no clinical and endoscopic relapse for 12 mo with the combination of infliximab and GMA therapy^[25]. Another CD patient with severe fistula refractory to conventional therapy also achieved sustained remission by GMA therapy^[28]. However, there were a number of CD patients who obtained disappointing outcomes with GMA therapy. In 2013, a randomized, double-blind, sham-controlled study was reported by Sands *et al*^[72]. They enrolled 235 patients with moderate to severe active CD from the United States and Canada, and all the patients finished ten sessions of GMA therapy. The results showed that clinical remission and response rate in the GMA group was 17.8% and 28.0%, respectively, while the clinical remission and response rate in the sham-treatment group was 19.2% and 26.9%, respectively. In another clinical study involving 12 patients with steroid dependent CD who received weekly GMA therapy, only 1 patient experienced no relapse within 6 mo of follow-up in spite of the initial clinical remission in 70% of the patients^[57]. From the above data, it seems that the effectiveness of GMA in CD patients is not as good as in UC as illustrated by a meta-analysis, which concluded that GMA therapy in UC demonstrated a significant higher clinical efficacy than CD^[13]. The possible reasons for the difference of effectiveness of GMA therapy between UC and CD await explanation. One possible reason may lie perhaps in the different intestinal neutrophil infiltration between the small intestine and the colon^[14].

Optimizing GMA for IBD: Based on clinical trials, the efficacy rate was as high as 100% for initial UC patients and over 80% for steroid naïve patients^[52,53]. The best responders to GMA therapy are UC patients with short disease duration and no previous medications and steroid naïve UC patients^[32,52,53,73]. In non-responders, deep colonic lesions or loss of extensive mucosal tissues are usually observed by endoscopic examinations^[52,64]. GMA is a time dependent therapy for IBD patients; several weeks may be needed before achieving favorable clinical outcomes. In addition, five sessions of GMA therapy are generally good for patients with a short course of the disease, while patients with recurrent episodes, especially steroid dependent or refractory, usually require ten sessions to achieve remission^[32,49,56].

Based on its frequency of sessions, GMA therapy is classified into two therapeutic protocols: Regular and intensive GMA. In regular GMA, one session per week is carried out for five to ten sessions, whereas in intensive GMA, two sessions per week

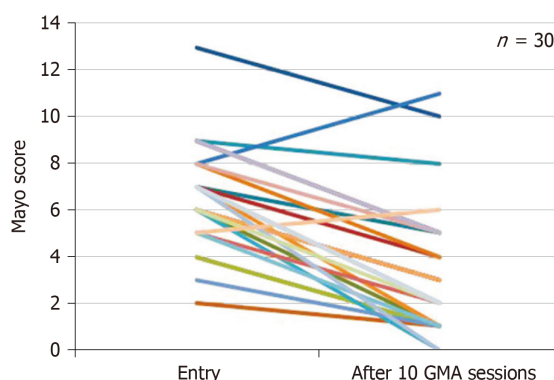


Figure 4 Changes of Mayo scores in 30 ulcerative colitis patients at entry and after ten granulocytes and monocytes apheresis sessions. Mayo scores were significantly decreased after ten granulocytes and monocytes apheresis sessions compared with that at entry^[29]. GMA: Granulocytes and monocytes apheresis.

are required for a total of five to ten sessions^[52,55]. Sakuraba *et al*^[74] reported a prospective multicenter study involving 112 UC patients who were divided into two groups: Regular GMA group and intensive GMA group. The results showed that intensive GMA therapy has a significantly higher remission rate than regular GMA therapy. Treatment duration and volume of blood infusion in the Adacolumn of a single GMA session may also influence the effectiveness of GMA as seen in the study by Kanke *et al*^[51]. They showed that 90 min for each GMA session has a significant better outcome as compared to routine 60 min of treatment. Yoshimura *et al*^[75] attempted to increase the blood volume perfusion from 1800 mL to over 3000 mL per GMA session, which seemed to have yielded a significantly better clinical outcome with no safety concerns.

GMA for special IBD patients

GMA for children and adolescent IBD patients: IBD is featured by its high morbidity in children and adolescents in whom growth and development may be affected by the disease and by the supposed life-long pharmacological treatment as well. Therefore, non-pharmacological treatment appears to bear significant importance for children and adolescent IBD patients^[76].

In 2003, the first clinical report of GMA therapy for pediatric IBD patients was published by Tomomasa *et al*^[77]. They treated 12 steroid-refractory IBD children with an average age of 12 years using GMA therapy (five to ten sessions). Nine of the twelve patients achieved clinical remission, and two patients had no response. The dosage of steroid was tapered in all patients. Four of the nine patients relapsed in an average of 3.5 mo after the last GMA session. The other patients remained in remission until an average of 22.8 mo. Similar results had also been reported by Ruuska *et al*^[78]. In a single center trial^[79], a total of 24 children and adolescents with IBD who failed mesalazine or sulphasalazine were enrolled. After GMA therapy in combination with prednisolone, all the patients obtained remission. Furthermore, in a clinical trial^[80] involving 53 pediatric/adolescent patients, the incidence of adverse events was 18.9%, a figure, however, higher than that in all 437 patients (11.4%).

From most of the above studies, one can see that GMA is effective and well tolerated in children and adolescent IBD patients who have failed conventional drug therapy, and GMA in combination corticosteroids yielded better clinical outcomes. However, there have been no sufficient clinical data to verify the effectiveness of GMA therapy in children and adolescents IBD patients, so more clinical studies are needed to address this question.

GMA for pregnant IBD patients: Clinical and epidemiological studies have shown that the fertility period was usually at the peak incidence of IBD patients, and the disease itself is an important risk factor for pregnancy. The fertility rate of IBD patients is significantly lower than that of healthy people due to disease activity, nutritional status, surgery and drug treatment. Therefore, it is challenging to manage pregnant IBD patients^[81,82]. Theoretically speaking, GMA therapy could be safe and effective for pregnant IBD patients. However, at present most of the published studies were case reports. In 2006, Okada *et al*^[83] reported a 30-year-old pregnant woman of 13 wk gestation with severe steroid-dependent UC who was treated by Cellsorba leukocytapheresis. The patient successfully achieved a rapid improvement after the first GMA session, and clinical remission was obtained 2 wk later. The patient delivered a full-term healthy baby during the remission stage. Another three case

reports involving 5 pregnant UC patients showed satisfactory responses to GMA therapy with smooth delivery and an absence of adverse events^[84-86].

Although evidence of effectiveness of GMA in pregnant IBD patients are based upon case reports, it might become the first-line therapy for pregnant IBD patients due to its safety. Of course, more research is needed before GMA therapy is generally accepted as the first-line therapy for pregnant IBD patients.

Safety of GMA

As a non-pharmacological therapy, GMA is incomparable to other therapies regarding its safety^[87]. The largest clinical study to date was performed by Hibi *et al.*^[55] who followed 656 UC patients treated by GMA from 53 centers in Japan starting in 2009 for 7 years. The results showed that GMA therapy had a very high safety profile with only mild or moderate adverse events related to GMA. In a multicenter study^[21], a total of 53 patients were treated with GMA therapy in combination with prednisone for 5 wk. Only eight mild adverse events were observed in 5 patients, and no patients ceased the treatment due to adverse events. In another study, no significant differences regarding safety were found between patients receiving five and ten GMA sessions as reported by Dignass *et al.*^[88] who divided 196 patients with moderate to severe steroid-dependent or steroid-refractory UC into two groups of five and ten GMA sessions.

In a meta-analysis report involving nine randomized trials of GMA therapy^[13,89], the most common adverse events were headache and flushing, and patients treated with GMA had a significant lower incidence of side effects than conventional therapies, *i.e.* corticosteroids. Besides, no serious adverse reactions had been reported in the children and pregnant women IBD patients who received GMA therapy^[78,84,90].

The use of anticoagulant is indispensable for GMA therapy. Sawada *et al.*^[91] analyzed 832 patients from 116 medical facilities in Japan for safety of anticoagulant use of nafamostat mesylate or heparin. The main side effects in patients using nafamostat mesylate were mild headache (2.2%), nausea (1.3%) and fever (0.9%), while in patient using heparin, the main side effects were decreased platelet count (2.7%), nasal congestion (1.8%) and pain in the vascular access sites (1.8%). Apart from these mild side effects, no serious adverse events were observed in patients using either nafamostat mesylate or heparin. In summary, adverse events of GMA therapy are rare, mild and well tolerated.

FUTURE OF GMA

As a non-pharmacological therapy, GMA has been demonstrated to be effective and safe for patients with IBD. Nevertheless, most of the clinical trials and literature of GMA therapy came from Japan. It has not been used extensively on a global scale, particularly in China, although it was approved by China State Food and Drug Administration for IBD patients in 2013. Furthermore, its effectiveness in IBD patients, particularly in CD patients, was doubted by some authors especially in a biological era when many biologic agents and immunosuppressants have been extensively used for IBD. Besides, price is also one of the main factors limiting the clinical usage of GMA therapy when cost-effectiveness perplexes both doctors and patients. Therefore, much needs to be done before it can be accepted as a therapeutic option for IBD patients worldwide.

Mechanisms of GMA

GMA targets inflammatory immune cells such as granulocytes and monocytes/macrophages to alleviate intestinal inflammation in IBD patients^[14]. In addition, other immune cells such as T cells, B cells and dendritic cells are also involved in the pathogenesis of IBD^[40,42]. At present, although there have been a few studies on the influence of GMA on these immune cells^[40-42], it is unknown how they contribute to the clinical effectiveness of GMA in patients with IBD. It is known that gut microbiota play an important role in the pathogenesis of IBD^[6,92], but research must be done to determine whether GMA therapy influences the gut microbiota. One study (our unpublished data) has shown that the unfavorable gut microbiota could be improved by GMA therapy in patients with UC. It is possible that GMA therapy could exert its therapeutic effect by improving the steady state of gut microbiota in IBD patients. However, more studies are needed before we are able to answer these questions.

Re-evaluation of GMA: Many reports have verified the effectiveness of GMA for induction of remission in IBD especially in UC patients. However, most of them were case reports or clinical trials of small size from Japan and Europe. Therefore, more large-scale, multicenter prospective studies are needed to further verify the

effectiveness of GMA therapy for IBD. Furthermore, the effectiveness of GMA therapy in CD patients is controversial in limited clinical trials to date. Although GMA therapy may be effective for induction of remission in patients with relapsed UC, future studies should be focused on its effectiveness as a maintenance therapy^[76,93]. Besides, it is also worth looking for predictive factors for responders to GMA therapy.

GMA for special IBD patients

The effectiveness of GMA has been verified in children and adolescent IBD patients^[94]. In theory, GMA can be used in IBD patients of any age, but there are limited clinical trials of GMA in children and adolescent IBD patients. Due to the differences in disease characteristics, body weight and circulatory status between children and adults, future studies are needed to determine the appropriate candidates as well as safety of GMA therapy for children and adolescent IBD patients. Fetal safety in pregnant patients with IBD who receive pharmacological treatment during pregnancy has always been a common concern of both doctors and patients. Although most of the drugs for IBD patients are at relatively low risk to fetal safety, there are still insufficient data to prove that various drugs are safe for patients with IBD in pregnancy in terms of miscarriage or malformation. Therefore, the choice of drugs for patients with IBD during pregnancy is difficult for both doctors and patients^[83,84]. Because it is a non-pharmacological technique in which no any medications are involved during the procedures, GMA is much safer for IBD patients in pregnancy as evidenced by several clinical reports^[83-85]. However, future clinical trials and observation of large-scale trials are needed before GMA becomes accepted as a safe and effective therapy for IBD patients during pregnancy.

GMA for other autoimmune diseases

GMA therapy was invented by Japanese scholars for patients with IBD. Due to its effectiveness and safety, especially its effect on anti-inflammatory cytokines, GMA therapy has also been used to treat other autoimmune diseases. In Italy, Morabito *et al.*^[95] treated nine patients with alcoholic hepatitis with GMA. The results showed that GMA therapy could reduce circulating inflammatory markers and improve the patient's clinical status. In addition, GMA also has therapeutic effects on patients with Bechet's disease^[96] and rheumatoid arthritis^[97]. With more clinical applications, it is hopeful that GMA therapy could be used clinically for other autoimmune diseases apart from IBD.

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Non-alcoholic fatty liver disease and Atherosclerosis at a crossroad: The overlap of a theory of change and bioinformatics

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Abstract

Atherosclerosis (ATH) and non-alcoholic fatty liver disease (NAFLD) are medical conditions that straddle a communal epidemiology, underlying mechanism and a clinical syndrome that has protean manifestations, touching every organ in the body. These twin partners, ATH and NAFLD, are seemingly straightforward and relatively simple topics when considered alone, but their interdependence calls for more thought. The study of the mutual relationship of NAFLD and ATH should involve big data analytics approaches, given that they encompass a constellation of diseases and are related to several recognized risk factors and health determinants and calls to an explicit theory of change, to justify intervention. Research studies on the “association between aortic stiffness and liver steatosis in morbidly obese patients”, published recently, sparsely hypothesize new mechanisms of disease, claiming the “long shadow of NAFLD” as a risk factor, if not as a causative factor of arterial stiffness and ATH. This statement is probably overreaching the argument and harmful for the scientific credence of this area of medicine. Despite the verification that NAFLD and cardiovascular disease are strongly interrelated, current evidence is that NAFLD may be a useful indicator for flagging early arteriosclerosis, and not a likely causative factor. Greater sustainable contribution by precision medicine tools, by validated bioinformatics approaches, is needed for substantiating conjectures, assumptions and inferences related to the management of big data and addressed to intervention for behavioral changes within an explicit theory of change.

Key words: Non-alcoholic fatty liver disease; Fatty liver; Arterial stiffness; Bioinformatics; Methodology of research

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Core tip: Atherosclerosis and non-alcoholic fatty liver disease straddle a communal epidemiology, underlying mechanism and a clinical syndrome with protean manifestations, touching every organ in the body. Current therapeutic evidence supports



the recommendation of addressing changes toward healthier lifestyles, including diet and physical exercise, in atherosclerosis and non-alcoholic fatty liver disease, even when defined only by non-invasive methodology. Pathway-based analysis are elucidating key molecular mechanisms underlying complex diseases addressing the joint effect and integrality as function unit of multiple genes, exploring large-scale “-omics” data. No element suggests, apart from naïve statistics, that one condition affects the other directly by any mechanism.

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INTRODUCTION

According to World Health Organization, ICD-11 for Mortality and Morbidity Statistics (Version: 04/2019), non-alcoholic fatty liver disease (NAFLD) is characterized by fatty liver related to insulin resistance in the absence of significant alcohol consumption. It embraces a pathological spectrum from simple steatosis to steatohepatitis. 10%-20% have steatohepatitis (non-alcoholic steatohepatitis), which can progress to cirrhosis and hepatocellular carcinoma^[1]. NAFLD and non-alcoholic steatohepatitis are increasingly a cause of cirrhosis and hepatocellular carcinoma globally. This burden is expected to increase as epidemics of obesity, diabetes and metabolic syndrome continue to grow^[2]. Atherosclerosis (ATH) is a chronic disease of the arterial wall, and a leading cause of death and loss of productive life years worldwide. Its distinctive feature is a hardening of any artery specifically due to atheromatous plaques^[3].

These conditions are increasingly recognized in primary care and specialist practice, which is largely due to the greater availability of non-invasive diagnostic tools. Nonetheless, quantifying future disease burden has always been challenging due to paucity of data in important areas, mandating an international, concerted effort to improve our understanding^[4].

ATH and NAFLD are medical conditions that straddle a communal epidemiology, underlying mechanism and a clinical syndrome that has protean manifestations, touching every organ in the body^[5]. Both entities stem from pathology, which seems certain and unarguable when considered alone, but when considered together, areas of uncertainty arise^[6]. Interrelationship between the two conditions are many, sharing epidemiology and risk factors^[7] which call for behavioral change interventions both in prevention and in therapy, but also challenging multi-factorial mechanisms by the quest of omics approaches^[8], requiring computational biology analysis^[9]. The conceptual milieu implies the overlap of a realistic theory of change, because implies lifestyle interventions focused on risk factors, with bioinformatics tools for the management of big data not comprehensive of all recognized risk components and indexes^[10].

In the real clinical world, easy-to-assess proxies of ATH are frequently preferred: It is needed to have the awareness of using them as assumptions more than as well supported reference standards^[11]. Accordingly, the present overview focuses to briefly delimit the area of the subject matter explicitly addressing which assumptions are still waiting for a demonstration, beyond frail demonstrations of association or correlation.

Arterial stiffness is deemed to occur as a consequence of both biological ageing and arteriosclerosis^[12]. Stiffened arteries require a greater amount of force to permit them to accommodate the stroke volume of the heart. The main consequence is an increase in pulse pressure which damages blood vessels in target organs such as the heart, brain or kidneys^[13].

Measurement of aortic pulse wave velocity (PWV) is conceptually related to arterial stiffness^[13]. By this acknowledgment, PWV, as a proxy of arterial stiffness, is credited to provide statistical evidence concerning the likelihood of occurrence of cardiovascular disease, and in some cases, of all cause mortality^[13]. This notion has some epidemiological support: Regretfully, and surprisingly, no PWV measurement is currently recommended for any practical and accepted general use^[14].

Imaging measures of fat liver content have considerable weaknesses^[15] given that

they do not have complete correspondence to histology: Nonetheless, they have a key role in medical practice, which is extensive and well appreciated, providing a suitable way for monitoring liver fatty content over time and after therapeutic interventions^[16].

CURRENT EVIDENCE

Current therapeutic evidence supports the recommendation of addressing changes toward healthier lifestyles, including diet and physical exercise, in both ATH and NAFLD, even when defined by non-invasive methodology^[15]. Although such lifestyle approaches are acknowledged as effective interventions, they are questionably demonstrated by few clinical trials^[17]. Disappointingly, such studies do not appear to be fully adequate due to limitations of size, duration and methods^[18]. Accordingly, seemingly, none is currently considered suitable for promoting definitive and worldwide shared guidelines^[15].

Research studies on the “association between aortic stiffness and liver steatosis in morbidly obese patients” have been published in several journals recently. One of them^[19], deserves greater consideration because it is based on a realistic anatomic measure of fat content, and because, despite its goal and careful design and development, it recognizes that any independent relationship between aortic stiffness and fatty liver are missing in the results achieved. Both conditions depend, as expected, on better known, and lasting, risk factors: Namely, arterial hypertension, increased body weight and diabetes^[19]. Apart from these points, this study is very valuable because its reference is not only that an appropriate liver histology sampling was available, but also because the measure of aortic stiffness was done by a reliable imaging-dependent tool, using an appropriate distance-based velocity measurement. These relevant features are very well displayed and justified within the article^[19], and its lack is a major flaw of previous studies. Such combined approach is an advance, since previous studies have been performed with similar purposes but with more biased, “qualitative” and “subjective” methods^[20]. The effort made in better understanding and facing the challenge of these two frequently overlapping diseases, even in the specific subset of extremely obese people, as in this investigation, promotes a negative answer to the claim of a causative relationship between ATH and NAFLD, if any, and, more, to the inclusion of NAFLD as a suitable concurrent risk factor for ATH^[19].

Investigations and publications, using very indirect ATH and fatty liver imaging to assess patients' risk, may have also the aim of facilitating future drug trials^[20]. However, there are many pros and cons, and a matter of concern is the disproportionately big and continuous flood of scientific contributions^[20]: This, by itself, may impair an appreciation of the usefulness of these procedures to be used. The core of this valuable debate is mostly concealed by a kind of implicit but not evidence-based acceptance of the diagnostic merit of such very indirect measurement tools^[15]. Nonetheless, several researchers unexpectedly are converging towards some agreement for accepting them as sustainable reference and indexes of outcome^[15].

Even in a relatively limited field of knowledge and science, such as medical approaches to the development of a mostly affluent disease, a warning regarding the misuse of methods, alleged paradigms and unsupported axioms is needed^[21]. Actually, scientific research, both basic and applied, is a dynamic process, constantly evolving and changing perspective, with critical thinking being at the core of the scientific method. In their daily work, scientists of every field are urged to keep pace with a broad spectrum of available data^[22]. Interaction and overlap between these two fields of research, basic and applied, may allow us to solve particular problems or questions, and both may enhance novel knowledge and information^[22]. The twin partners, ATH and NAFLD, are only apparently straightforward and relatively simple topics, and their interdependence calls for definitions without biases. Actually, encompassing many prevalent diseases and being related to several and recognized risk factors and health determinants, the study of the mutual relationship between NAFLD and ATH should involve big data analytics approaches^[23,24]. Regrettably, available studies are not sufficiently comprehensive, not including even part of the data actually available in the specific database. There is a general context that may have effects on both fatty liver- and obesity- and on arteriosclerosis and related heart, brain and kidney diseases. This context encompasses the environment at large^[25], behavioral factors^[26], quantity and quality of dietary intake^[27], with consequent obesity or nutritional-related disturbance^[28], sedentary life, stress, detrimental levels of physical activity, sleep deprivation and night shifts for work or leisure^[29], and certainly many others. Despite these limitations, overall, the effects of well-addressed intervention are reported as beneficial^[30-32].

In brief, regarding the mutual relationship of ATH and NAFLD, no element reasonably suggests, apart from quite naive statistics, that one condition affects the other directly, despite the attractive hypothesis that fatty liver in itself may cause vascular damage. In these matters, it is always unsafe to treat contemporaneous events as causation^[33,34].

IMPLICATIONS, CHALLENGES AND BIASES

Within other backgrounds and perspectives, the scientific community is the silent bystander of determined, even unreasonable, anti-science strain and campaigns^[21]. Rejection of scientific method as an objective tool that can generate universal knowledge is of great concern for scientists and health professionals^[21]. This eschewal may originate from the idea that scientific reductionism is inherently limited in reaching understanding of complex problems and, namely, health and disease topics. The very relevant ongoing debate on the “misuse of statistical significance”^[35], leading sometimes to discarding of genuine discoveries, may add further fuel to mistrust in science and medicine. Reciprocally, suggesting conjectural “mechanisms of disease”, such as claiming the long shadow of NAFLD as a “risk factor”, if not as a causative factor of arterial stiffness and ATH, is probably very much overreaching reality and is harmful for the scientific credence of this area of medicine. “As long as different positions are discussed scientifically, controversy has a chance to move forward”^[22]. This is a very important commitment for the physician, the researcher, the research projects evaluator and the reviewer. No golden rule is available, but the use of “naive” methods, not sufficiently adequate for the purposes of the scientific questions, when detected as inappropriate or frankly biased, should be reasonably discouraged and discarded.

CONCLUSION

Disease study processes are often too rigid, we must agree. In fact, the aim is to collect sufficient evidence, best if coordinated in a convincing architecture, relationships and support, in ways very similar to the theory of change. In fact, the study of diseases often, as in our case, includes the quantitative observation of numerous data and of the changes that are determined with interventions, spontaneous or within clinical trials. In this sense it is acceptable that there are assumptions that are not strongly supported, but it is quite possible that these assumptions are inconsistent and misleading so that, when detected, must be included as biases or mistakes in the proposed rationale of the dependent intervention for change. Despite the verification that NAFLD and cardiovascular disease are strongly interrelated^[36], current evidence is that NAFLD may be only a possibly useful indicator for flagging early arteriosclerosis^[37], and not a likely causative factor. Greater sustainable contributions by precision medicine tools, *i.e.*, by validated bioinformatics approaches and expertise, are needed to allow consideration of any role of big data and how they should be managed^[38]. Very valuable investigations are already available, providing new insights into the relationship of NAFLD, fibrosis and ATH^[39]. Also the shared molecular basis for ATH and vascular disease and its molecular relationships with several related diseases, namely NAFLD and Alzheimer’s disease, are the focus of frontiers research in very well planned and managed investigations^[39,40]. Currently, pathway-based analysis are elucidating key molecular mechanisms underlying complex diseases addressing the joint effect and integrality as function unit of multiple genes by extensive studies exploring large-scale “-omics” data^[41]. In the mind and perception of most people, what matters is if such information and measures may guide the daily choices of physicians and if they may empower the adherence of patients to prescriptions, therapeutic pathways or lifestyle choices. The answer may be yes, but this is more a real world practice affair than the conclusion of any existing or ongoing trial or of any computational statistics analysis and prediction. The methodology described above, as in most more traditional research investigating associations and relationships, alludes implicitly to the assertion that where shadows overlap, they appear darker: Which should mean better visible, if not “clearer”. However, this is not necessarily true. Actually, something is problematic in this assertion, and, more important, we may argue that it is where shadows overlap, that may matter. The need to integrate more omics data with different ones, such as epigenetic or epidemiological data, by bioinformatics, is evident when dealing with mechanisms and processes involving long cascades of multiple biological pathways^[42] (Figure 1).

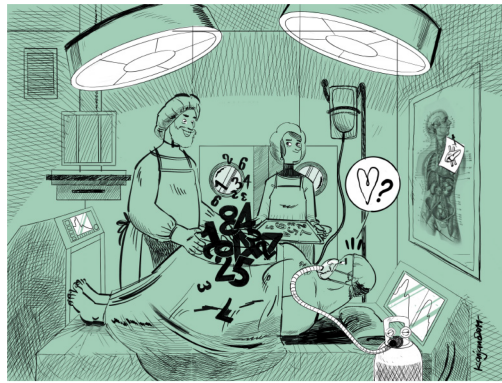


Figure 1 The naive and surreal “vision” of the artist displays in this drawing a tale of bodies and shadows.

The researchers are exploring directly the liver of an obese patient, while the shadows of atherosclerosis and of arterial stiffness, *i.e.*, numbers, amazingly leak out from the liver and graphs of non-invasive measurements appear at the operating table's headboard. The theory that fatty liver in itself may hurt the vessels, as in the drawing of the anatomical drawing behind, lacks currently consistent supporting statistics. In these topics it is always unsafe to treat simultaneity as causation. The use of straightforward diagnostic methods for non-alcoholic fatty liver disease (surgical liver biopsy) and indirect measurements of aortic stiffness, pulse wave velocity, fails to demonstrate a direct independent relationship between the two conditions. The conjectural hypothesis of labeling non-alcoholic fatty liver disease as a novel risk factor for atherosclerosis, defined by the proxy of arterial stiffness, is far away to be demonstrated (drawing by Giuliano Cangiano-Kanjano).

The unpredictability of synergy among different pathways and the possibility of over-fitting, *i.e.*, the production of an analysis that corresponds too closely or exactly to a particular set of data, and may therefore fail to fit additional data or predict future observations reliably, must be appropriately considered when trying to illustrate the genetic, epigenetic, and environmental determinants of trigger and development of any disease^[43]. Conceivably, when dealing with cross-talk of liver with ATH and fat cells^[44], we should take into account what is actually overlapping, when, where, how and why. Mirroring the shadows of concepts, as we do using indirect or surrogate measures of ATH and of fatty liver, respectively, might be an unreliable and even misleading approach. Indeed, we are still waiting for high quality prospective studies in diverse racial-ethnic groups to further elucidate whether or not NAFLD is in fact causally related to ATH, while actual information provides some suggestion of limited associations^[45,46]. The risk and the bias of a this clinical research tale are that, at last, some conclusion, as equally some intervention, based on uncertain assumptions might ultimately succeed in darkening or obscuring, certainly not enlightening concepts and mechanisms. The result will be weakening the strength of consequent warranted intervention which are still based on awareness and participatory behavioral changes^[47] and on intervention models based on a theory of change^[48], which usually is not clearly developed and monitored, as needed.

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Retrospective Cohort Study

Evaluation of bacterial biomarkers to aid in challenging inflammatory bowel diseases diagnostics and subtype classification

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Abstract

BACKGROUND

The challenges for inflammatory bowel disease (IBD) diagnostics are to discriminate it from gut conditions with similar symptoms such as irritable bowel syndrome (IBS), to distinguish IBD subtypes, to predict disease progression, and to establish the risk to develop colorectal cancer (CRC). Alterations in gut microbiota have been proposed as a source of information to assist in IBD diagnostics. *Faecalibacterium prausnitzii* (*F. prausnitzii*), its phylogroups, and *Escherichia coli* (*E. coli*) have been reported as potential biomarkers, but their performance in challenging IBD diagnostic situations remains elusive. We hypothesize that bacterial biomarkers based in these species may help to discriminate these conditions of complex diagnostics.

AIM

To evaluate the usefulness of indices calculated from the quantification of these species as biomarkers to aid in IBD diagnostics.

METHODS

A retrospective study of 131 subjects (31 controls (H); 45 Crohn's disease (CD), 25 ulcerative colitis (UC), 10 IBS, and 20 CRC patients) was performed to assess the usefulness of bacterial biomarkers in biopsies. Further, the performance of biomarkers in faeces was studied in 29 stool samples (19 CD, 10 UC). Relative

Informed consent statement:

Informed consent from the subjects was obtained before enrolment.

Conflict-of-interest statement:

Aldeguer X is a consultant from AbbVie and has received honoraria for lectures, including services on speakers' bureaus, from AbbVie, MSD, Shire and Takeda. Aldeguer X, Serra-Pagès, M and Garcia-Gil J own shares in GoodGut S.L. López-Siles M, Garcia-Gil J, Aldeguer X and Martinez-Medina M own patent WO2017025617A1 concerning a Method for the detection, follow up and/or classification of intestinal diseases. The other authors have nothing to disclose.

Data sharing statement:

Datasets available from the corresponding author at marga.martinez@udg.edu. Consent was not obtained from participants for data sharing, but the presented data are anonymized, and the risk of identification is low. No additional data are available.

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abundances of total *F. prausnitzii* (FP), its phylogroups (PHGI and PHGII), and *E. coli* (E) quantification were determined by qPCR. Loads were combined to calculate the FP-E index, the PHGI-E index and the PHGII-E index. Biomarkers accuracy to discriminate among conditions was measured by the area under the receiver operating characteristic curve (AUC).

RESULTS

In biopsies, FP-E index was good for discriminating IBS from CD (AUC = 0.752) while PHGII-E index was suitable for discriminating IBS from UC (AUC = 0.632). The FP-E index would be the choice to discriminate IBD from CRC, especially from all UC subtypes (AUC \geq 0.875), regardless of the activity status of the patient. Discrimination between UC patients that had the longest disease duration and those with CRC featured slightly lower AUC values. Concerning differentiation in IBD with shared location, PHGI-E index can establish progression from proctitis and left-sided colitis to ulcerative pancolitis (AUC \geq 0.800). PHG I-E index analysis in tissue would be the choice to discriminate within IBD subtypes of shared location (AUC \geq 0.712), while in non-invasive faecal samples FP or PHGI could be good indicators (AUC \geq 0.833).

CONCLUSION

F. prausnitzii phylogroups combined with *E. coli* offer potential to discriminate between IBD and CRC patients and can assist in IBD subtypes classification, which may help in solving IBD diagnostics challenges.

Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Diagnostic tests; *Faecalibacterium prausnitzii*; *Escherichia coli*; Irritable bowel syndrome; Colorectal cancer

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Core tip: This manuscript evaluates the usefulness of new indexes calculated from the quantification of *Faecalibacterium prausnitzii*, its phylogroups, and *Escherichia coli* as biomarkers to assist in challenges of inflammatory bowel disease diagnostics. Firstly, discrimination between inflammatory bowel disease and other intestinal disorders was tested. We present indices to distinguish colorectal cancer from inflammatory bowel disease, especially from subjects with ulcerative colitis. This is of significance given the association between chronic inflammation and the risk of colorectal cancer. In contrast, the proposed indices featured limited performance for discriminating inflammatory bowel disease from irritable bowel syndrome. Secondly, we approach if these biomarkers would be useful to discriminate within inflammatory bowel disease subtypes. We show here good biomarkers to differentiate inflammatory bowel disease subtypes of shared disease location, which may assist in monitoring the risk of progression of the inflamed area. Their application in non-invasive faecal samples is also demonstrated.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory bowel disorders of unknown aetiology that follow a course with periods of activity or flare-ups and periods of remission^[1-4]. Crohn's disease (CD) and ulcerative colitis (UC) are the main idiopathic IBD^[5-7]. Despite these disorders differing in location, histology, and distribution of inflamed areas, sometimes they feature overlapping clinical and pathological characteristics that hamper a distinct classification^[8,9]. It is essential to discriminate both entities to establish an appropriate treatment strategy^[10]. Besides,



there are other intestinal disorders, such as irritable bowel syndrome (IBS), that share symptoms similar to those observed in the early stages of IBD thus increasing its likelihood of misdiagnosis^[11,12]. In contrast, chronic inflammation can lead to tumour formation and promote colorectal cancer (CRC) development. It would, therefore, be interesting to have a biomarker for IBD-progression to CRC, but currently, there is a lack of tools to predict which cases may progress to CRC. Altogether, current IBD diagnostics challenges are to discriminate phenotype variations within IBD accurately, but also to differentiate IBD from other gut conditions with milder or worsening phenotypes.

Given the absence of pathognomonic features, IBD diagnosis currently involves a comprehensive examination of the patient that includes clinical, endoscopic, radiologic, and histological criteria. Besides, as clinical manifestations of IBD are unstable during the disease course, a long monitoring period is needed to classify the disease phenotype accurately^[11,15]. As IBD patients feature an imbalanced gut microbial community in comparison to healthy subjects^[16-25], in the last years the implementation of bacteria representative of this dysbiosis as biomarkers has been started to be explored as a novel strategy to support IBD diagnostics and/or prognostics^[23,26-30].

We and others have pointed out that the abundance of faecal or mucosa-associated *Faecalibacterium prausnitzii* (*F. prausnitzii*) is a potential biomarker to discriminate between gut disorders^[23,26-30]. Moreover, *F. prausnitzii* in conjunction with *Escherichia coli* (*E. coli*) abundance (FP-E index) has been proven to be a better biomarker than total *F. prausnitzii* alone^[26,29]. Besides, the quantification of *F. prausnitzii* phylogroups I (PHGI) and II (PHGII) has been proposed as a source of additional information to discriminate between IBD subtypes. However, the usefulness of an index using the quantification of the phylogroups in conjunction with *E. coli* remains to be explored. Also, there is a lack of comparative studies from a methodological aspect that would allow the establishment of the biomarker of choice.

It is against this background that we examined six options for biomarkers (*F. prausnitzii*, the two phylogroups or the combination of these three with *E. coli*) in a cohort of non-IBD controls (H), IBS, IBD and CRC subjects, to (1) establish which would be the best parameter to discriminate IBD patients from H and IBS subjects; (2) determine which would be the best parameter to discriminate IBD from CRC patients; and (3) identify which would be the most accurate parameter to discriminate within IBD subtypes by location. We hypothesize that bacterial biomarkers based in these species may be of help to discriminate these conditions of complex diagnostics.

MATERIALS AND METHODS

Patients, clinical data and sampling

In this study, data from two groups of subjects were included. Firstly, biomarker performance was tested in biopsy samples. We hypothesized that given the inflammatory nature of IBD, to look for biomarkers in the tissue would be strongly associated with disease course. Secondly, the usefulness of selected biomarkers was assessed in non-invasive samples (*i.e.*, stools).

To test the performance of the mucosa-associated bacterial biomarkers, a re-analysis of the data from a Spanish cohort including IBD, IBS, CRC, and H was performed (Table 1). Subjects were consecutively recruited by the Department of Gastroenterology at the Hospital Universitari Dr. Josep Trueta (Girona, Spain) and the Gastroenterology Unit at the Hospital Santa Caterina (Institut d'Assistència Sanitària of Girona, Salt, Spain) between May 2009 and November 2010. Patients were gender- and age-matched, except CD patients who were significantly younger than those in the H and IBS groups ($P < 0.001$) (Table 1). During routine endoscopy, up to three biopsy samples per patient were taken from different locations along the gut (Table 2) following standard procedures.

To test the performance of bacterial biomarkers in faecal samples, a cohort consisting of 29 IBD (19 CD and 10 UC) patients was recruited by the Gastroenterology Services of the Hospital Universitari Dr. Josep Trueta (Girona, Spain) between March 2014 and May 2015. Subjects were age- and gender-matched for both the groups (Table 1). Participants were asked to collect a stool sample from one bowel movement in a sterile faecal collection container. Subjects brought samples to the hospital, where they were stored at -80°C until DNA extraction was performed.

To control bias between centres, patients with IBD were diagnosed according to standard clinical, pathological, and endoscopic criteria and categorized according to the Montreal classification. Patients with IBS were diagnosed according to Rome III criteria (available at <http://www.romecriteria.org/criteria/>). CRC diagnosis was

Table 1 Sample size and clinical characteristics of subjects

		Healthy ¹	Irritable bowel syndrome	IBD		Colorectal cancer	P value ³
				Ulcerative colitis	Crohn's disease		
Cohort of subjects for biopsies samples collection	<i>n</i> (patients)	31	10	25	45	20	
	Age (mean ± SD, yr)	48.1 ± 16.3	42.4 ± 11.4	40.1 ± 15.8	33.5 ± 11.1	58.6 ± 7.52	< 0.001 ⁴
	Male, <i>n</i> (%)	16 (51.6)	2 (20.0)	16 (64.0)	26 (57.7)	14 (70.0%)	0.605 ⁵
	Active, <i>n</i> (%)	NA	NA	20 (80.0)	28 (62.2)	NA	0.100 ⁵
	Treatment, <i>n</i> (%) ²						
	No treatment	NA	NA	16 (64.0)	17 (37.8)		NA
	Moderate immunosuppressant	NA	NA	3 (12.0)	17 (37.8)		NA
	Anti-TNFα	NA	NA	4 (16.0)	10 (22.2)		NA
	UC location, <i>n</i> (%) ²						NA
	Ulcerative proctitis (E1)	NA	NA	6 (24.0)	NA	NA	
	Distal UC (E2)	NA	NA	11 (44.0)	NA	NA	
	Extensive UC or ulcerative pancolitis (E3)	NA	NA	6 (24.0)	NA	NA	
	CD location, <i>n</i> (%) ²						NA
	Ileal-CD (L1)	NA	NA	NA	19 (42.2)	NA	
	Colonic-CD (L2)	NA	NA	NA	11 (24.4)	NA	
	Ileocolonic-CD (L3)	NA	NA	NA	14 (31.1)	NA	
Cohort of subjects for faecal samples collection	<i>n</i> (patients)			10	19		
	Age (mean ± SD, yr)			47.4 ± 18.3	43.5 ± 18.3		0.429 ⁴
	Male, <i>n</i> (%)			5 (50.0)	10 (52.6)		0.893 ⁶
	Active, <i>n</i> (%) ²			1 (10)	7 (36.8)		0.185 ⁵
	Treatment, <i>n</i> (%) ²	NA	NA	5 (50)	5 (26.3)	NA	
	No treatment	NA	NA	1 (10)	2 (10.5)	NA	
	Moderate immunosuppressant	NA	NA	3 (30)	12 (63.2)	NA	
	Anti-TNFα						
	UC location, <i>n</i> (%) ²						NA
	Distal UC (E2)			3 (30.0)	NA		
	Extensive UC or ulcerative pancolitis (E3)			7 (70.0)	NA		
	CD location, <i>n</i> (%)						
	Ileal-CD (L1)			NA	10 (52.6)		NA
	Colonic-CD (L2)			NA	3 (15.8)		
	Ileocolonic-CD (L3)			NA	6 (31.6)		

¹Controls consisted of subjects who underwent colonoscopy for different reasons: 9/31 rectal bleeding, 11/31 colorectal cancer familial history and 11/31 abdominal pain.

²Maximal disease extent at the time of sampling was available in 23/25 UC patients (cohort for biopsy samples), 4/10 UC patients (cohort for faecal samples), 44/45 CD patients. Activity status in the cohort that provided faecal samples was available for 4/10 UC patients and 11/19 CD patients. Treatment at sampling for the faecal sample's cohort was recorded for 9/10 UC patients, and for biopsy sample's cohort in 23/23 UC and 44/45 CD participants.

³Groups were compared by non-parametric statistical tests, and *P* value ≤ 0.05 was considered significant.

⁴Kruskal-Wallis.

⁵Mann-Whitney *U* test or

⁶ χ^2 test as required. IBD: Inflammatory bowel disease; IBS: Irritable bowel syndrome; CRC: Colorectal cancer; TNF: Tumour necrosis factor; NA: Not

applicable.

established by colonoscopy and biopsy examination, and none of the subjects underwent radiotherapy, chemotherapy or surgery. The control group consisted of subjects with normal colonoscopy who underwent this procedure for different reasons (Table 1). Clinically relevant data of all participants, such as age, gender, and disease activity at sampling, were collected (Table 1). Active CD were defined as those with CDAI > 150 whereas active UC patients had a Mayo score > 3.

Individuals included in this study were > 18 years old, did not have any other intestinal disease, and were not pregnant. Antibiotic treatment within the last month prior to sample collection was the only exclusion criterion. None of the subjects received probiotics before sample collection.

Sample treatment, DNA extraction, and qPCR assays

For biopsies, sample treatment and DNA extraction were performed as reported previously^[26,27]. For faeces, 200-500 mg of faecal material were used for bacterial DNA extraction and purification with the NucleoSpin® Soil (Macherey-Nagel) and following the instructions from the manufacturer.

Previously designed and optimized 16S rRNA gene-targeted primers and probes were used for total *F. prausnitzii*^[26], phylogroups, *E. coli* and total bacterial quantification using quantitative polymerase chain reaction (qPCR). Human cell numbers were determined with the control kit RT-CKFT-18S (Eurogentec, Belgium) according to the manufacturer's instructions.

Amplification reactions were performed as described elsewhere^[26,27,35,36]. In brief, quantifications were performed in a total volume of 20 µL reactions containing: 1× TaqMan Universal PCR Master Mix 2× (Applied Biosystems, Foster City, CA, United States), 900 nmol/L of each primer, 300 nmol/L of each probe, and up to 50 ng of genomic DNA template. Samples were run in duplicate in the same plate. For data analysis, the mean of the duplicate quantifications was used. Duplicates were considered valid if the standard deviation between quantification cycles (C_q) was <0.34 (*i.e.*, a difference of < 10% of the quantity was tolerated). Quantification controls consisting of at least 5 reactions with a known number of target genes were performed to assess inter-run reproducibility. For samples with undetected values during quantification, the number of 16S rRNA gene copies equivalent to the detection limit of each reaction was used. Inhibition of total *F. prausnitzii* quantification was controlled by adding 10^3 copies of an internal amplification control (IAC) template to each reaction. It was considered that there was no inhibition if the obtained C_q was < 0.34 from those obtained when quantifying the IAC alone for any of the replicates. A non-template control (consisting of a reaction without *F. prausnitzii* DNA) and a non-amplification control (which did not contain any DNA template, either bacterial or IAC) were also included in each run. Negative controls resulted in undetectable C_q values in all cases.

All quantitative PCRs were performed using a 7500 Real Time PCR system (Applied Biosystems). The thermal profile was: a first step at 50 °C during 2 min for amperase treatment, followed by a 95 °C hold for 10 min to denature DNA and activate Ampli-Taq Gold polymerase, and a further 40 cycles consisting of a denaturation step at 95 °C for 15 seconds followed by an annealing and extension step at 60 °C (or at 64°C for phylogroups quantification) for 1 min. Data were collected and analysed using the 7500 SDS system software version 1.4 (Applied Biosystems). All quantifications were performed under average PCR efficiencies of $89.51 \pm 7.06\%$.

Sample size, data normalization and statistical analysis

The sample size was defined after the number of patients analysed in similar studies of bacterial abundance in subjects suffering of these conditions^[20,22,26,28].

Relative abundances of total *F. prausnitzii*, phylogroups, and *E. coli* copy numbers were calculated by normalizing each species load for the total bacterial 16S rRNA gene copies. Data are given as the log₁₀ of the ratio between 16S rRNA gene copies of the target microorganism and millions of total bacterial 16S rRNA genes detected in the same sample.

For biopsies, species relative abundances were combined to calculate the FP-E index as previously reported^[26]. Similarly, the PHGI-E index and the PHGII-E index were calculated as follows:

$$\text{PHGI-E index} = [\log_{10} (\text{PHGI}/\text{Hc}) - \log_{10} (\text{E}/\text{Hc})] / [\log_{10} (\text{TB}/\text{Hc})]$$

$$\text{PHGII-E index} = [\log_{10} (\text{PHGII}/\text{Hc}) - \log_{10} (\text{E}/\text{Hc})] / [\log_{10} (\text{TB}/\text{Hc})]$$

Being PHGI and PHGII the 16S rRNA gene copies of *F. prausnitzii* phylogroup I or II respectively; E the 16S rRNA gene copies of *E. coli*; Hc a million of human cells; and

Table 2 Biopsy samples by conditions and locations

	No. Patients	No. biopsies				
		Terminal ileum	Transverse colon	Rectum	Unknown region	Total
H	31	14	24	10	0	48
IBS	10	1	3	3	12	19
CRC	20		3	17	0	20
UC	25	11	23	16	0	50
<i>Location</i>						
Ulcerative proctitis (E1)	6	4	5	5	0	14
Distal UC (E2)	11	3	11	8	0	22
Extensive UC or ulcerative pancolitis (E3)	6	3	6	1	0	10
CD	45	16	31	16	0	63
<i>Location</i>						
Ileal-CD (L1)	19	5	13	7	0	25
Colonic-CD (L2)	11	6	7	4	0	17
Ileocolonic-CD (L3)	14	4	10	4	0	18

H: Healthy controls; IBS: Irritable bowel syndrome; CRC: Colorectal cancer; UC: Ulcerative colitis; CD: Crohn's disease.

TB a million of 16S rRNA gene copies of total bacteria.

For faecal samples, as no Hc quantification was performed to normalize sample size, indexes were calculated as:

$$\text{FP-E index} = \log_{10} (\text{total } F. \text{prausnitzii}/E)$$

$$\text{PHGI-E index} = \log_{10} (\text{PHGI}/E)$$

$$\text{PHGII-E index} = \log_{10} (\text{PHGII}/E)$$

Differences in categorical variables such as gender were assessed by the χ^2 test. For continuous variables such as age or biomarkers load, data normality was assessed through the Kolmogorov-Smirnov test. The non-parametric Kruskal-Wallis test was used to assess differences in variables with more than two categories, such as diagnostics, and CD or UC disease location. Pairwise comparisons of subcategories of these variables were analysed using a Mann-Whitney *U* test. This test was also used to compare, within a subgroup of patients, variables with two categories.

The receiver operating characteristic curve analysis, a plot of the true-positive rate (sensitivity) versus false-positive rate (1-specificity), was applied to establish the usefulness of *F. prausnitzii*, along with each phylogroup, alone or in conjunction to *E. coli* counts (FP-E index, PHGI-E index, and PHGII-E index) to distinguish different intestinal conditions. The accuracy of discrimination was measured by the area under the receiver operating characteristic curve (AUC). An AUC approaching 1 indicates that the test is highly sensitive and highly specific, whereas an AUC approaching 0.5 indicates that the test is neither sensitive nor specific. For the best cut-off value, specificity and sensitivity were established.

All the statistical analyses were performed using the SPSS 15.0 statistical package (LEAD Technologies, Inc.). Significance levels were established for *P* values ≤ 0.05 .

The statistical methods of this study were reviewed by MSc. Oliver Valero Coppin from the Statistical Service at the Universitat Autònoma de Barcelona.

RESULTS

Discrimination of IBD from H and IBS

When considering all biopsy samples (Figure 1A), PHGI quantification was the most discriminative biomarker between H and IBD patients (AUC > 0.75). This can be attributed to higher load of PHGI in H in comparison to the other groups of subjects (Supplementary Table 1). Notably, discrimination was especially good between H and subjects with CD, which achieved 73% specificity and 91% sensitivity at the best cut-off value ($\log_{10}[\text{16S rRNA phylogroup I}/10^6 \text{ 16S rRNA total bacteria}] = 2.3$). This discrimination was particularly accurate when analysing ileal samples (AUC > 0.9) (Figure 1B). Besides, discrimination between H and IBD subjects achieved greater AUC values when considering only active IBD patients (Figure 1C). However, the discrimination was still good (AUC > 0.75) when taking into account only those with inactive disease. Therefore, our results support PHGI as an indicator of healthy gut

status.

Regarding discrimination between IBD and IBS patients, different biomarkers performed best to distinguish IBS from UC or CD. When pooling all biopsy samples (Figure 1A), PHGII-E index was suitable to discriminate IBS from UC. This discrimination was excellent when considering ileal or rectal biopsies and suitable for colonic biopsies analyses (Figure 1B). It is of note that the PHGII-E index allowed good discrimination between these two conditions, even in the inactive cohort of patients (Figure 1C). In contrast, FP-E index was good for discriminating IBS from CD when pooling all samples, although this was not sustained for all sampled locations, probably due to the effect of the location of inflammation in CD. In contrast, FP was the best biomarker to discriminate IBS and CD in colonic and rectal samples, whereas PHGI counts discriminated best at the ileum (Figure 1B). This biomarker was good to discriminate IBS from active CD patients, whereas the PHGII-E index provided the best discrimination between IBS and inactive CD. Overall, to select a general biomarker to discriminate IBS from IBD, useful in all kinds of samples and conditions was challenging. However, FP could be an interesting candidate as performed in the suitable-excellent AUC range for all comparisons, regardless of the intestinal region selected for analysis.

Discrimination between IBD with colonic inflammation and CRC

In general, the FP-E index was the most discriminatory between CRC and IBD patients when taking into account all biopsy samples together (Figure 2A), because lower FP-E values were associated with CRC subjects (Supplementary Table 1). Notably, discrimination was especially good between CRC and UC patients, which achieved 85% specificity and 94% of sensitivity at the best cut-off value (FP-E index = 0.009). This discrimination was excellent between CRC and patients with ulcerative proctitis (E1) and ulcerative pancolitis (E3) with 85% specificity and 100% sensitivity, while for patients with extensive UC (E2) sensitivity was reduced to 86% with the same specificity rate. Although good discrimination was achieved (AUC > 0.870) we observed that discern between E2 patients and those with CRC featured slightly lower AUC values, and in turn, these groups of patients had the longest disease duration (mean years of disease duration \pm SD by UC subtype was: E1 = 0.93 ± 1.69 ; E2 = 7.10 ± 4.27 ; E3 = 2.63 ± 2.20).

In addition, this excellent discrimination was sustained regardless of the activity status of the patients. Regarding the location of sample (Figure 2B), for our particular cohort, colonic biopsies were the most discriminatory between CRC and those patients with E1 and E3, although good separation of groups was also achieved with rectal samples. In turn, rectal samples performed better to discriminate between E2 subjects and those with CRC.

The FP-E index was also suitable to classify CRC patients and those with CD of colonic location (*i.e.*, C-CD and IC-CD). Interestingly, better AUC values were obtained for PHGI and when considering rectal samples alone, which needs further confirmation given the low number of samples analysed at this location for CD patients.

Discrimination within IBD with shared location

Biomarkers analyses in biopsy samples: The FP-E index was the best biomarker to differentiate UC from CD patients considering all locations (Figure 3A), given that UC patients had higher FP-E index values than CD patients (Supplementary Table 1). However, no consensus could be reached about the biomarker that performed best when comparing IBD subtypes with shared location of the inflammation. PHGI-E index was good to differentiate E1 and E2 from E3, particularly in ileal samples (AUC ≥ 0.875) although suitable discrimination was also obtained when analysing colonic biopsies. In contrast, the PHGII-E index was the most accurate to discriminate C-CD from all UC locations when considering all samples together, and this was sustained in colonic samples (Figure 3B).

As regards discrimination between CD locations, in general, all the biomarkers showed AUC ≤ 0.75 except for the PHGII-E index in ileal samples, which allowed for good discrimination between IC-CD from both C-CD and I-CD.

Interestingly, when considering only active patients, the PHGI-E index was the most discriminatory for all the comparisons when pooling samples, and when considering only those from the ileum and colon. Except for CD with ileal involvement, also suitable discrimination was obtained with the PHGI-E index from rectal samples. Analyses in inactive patients are not shown because, in most cases, they could not be conducted given the low number of samples with these characteristics when separating by IBD subtype.

Biomarkers analyses in faecal samples: Analyses of IBD faecal samples showed that

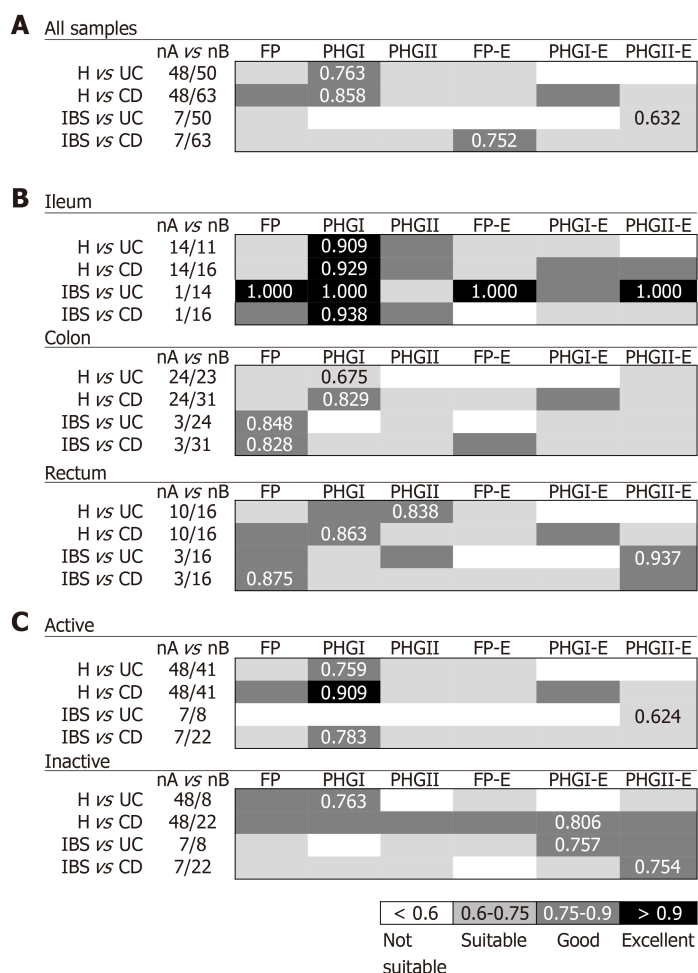


Figure 1 Usefulness of *Faecalibacterium prausnitzii*, its phylogroups (PHGI and PHGII) and their index in conjunction to *Escherichia coli* to discriminate between milder gut conditions [Healthy controls (H) and irritable bowel syndrome] and inflammatory bowel disease (ulcerative colitis and Crohn's disease) by pooling all biopsy samples together (A), by location of sampling (B) and by activity status (C). Best area under the receiver operating characteristic curve (AUC) values for each comparison are shown. FP: *Faecalibacterium prausnitzii*; UC: Ulcerative colitis; CD: Crohn's disease; IBS: Irritable bowel syndrome.

the most suitable biomarker to discriminate between UC and CD conditions was PHGI (Figure 3C), whose load was higher in the former, regardless of the disease extent (Supplementary Table 2). This biomarker was different from that found in biopsies, and the AUC was 1.4 times lower than that obtained in tissue samples.

In contrast, better AUC values were achieved in faecal samples for PHGI and PHGI-E compared to those in biopsies to discriminate C-CD from E2, E3 and IC-CD, although corroboration by engaging more C-CD subjects is needed. It is of note that the results obtained for FP as a biomarker to distinguish IC-CD from I-CD, which substantially improved the biopsy results.

DISCUSSION

Quantification of bacterial biomarkers may be a valuable tool to assist in the diagnosis of intestinal disorders. In this work, we explored the usefulness of two species (*E. coli* and *F. prausnitzii*), extensively reported as dysbiosis representatives of IBD^[16,18-20,22,23,26-30], to discriminate between different gastrointestinal disorders.

Firstly, we explored whether or not these bacterial biomarkers could assist in discriminating IBD from IBS, where symptoms can be similar at early stages of the disease. It was observed that a general biomarker to discriminate IBS from IBD could not be established, and therefore two biomarkers should be used. While FP-E index allowed discrimination between IBS and CD, PHGII-E index was the most appropriate to discriminate between IBS and UC. Our cohort of IBS patients was limited and not classified by IBS subtypes. As differences in gut microbiota

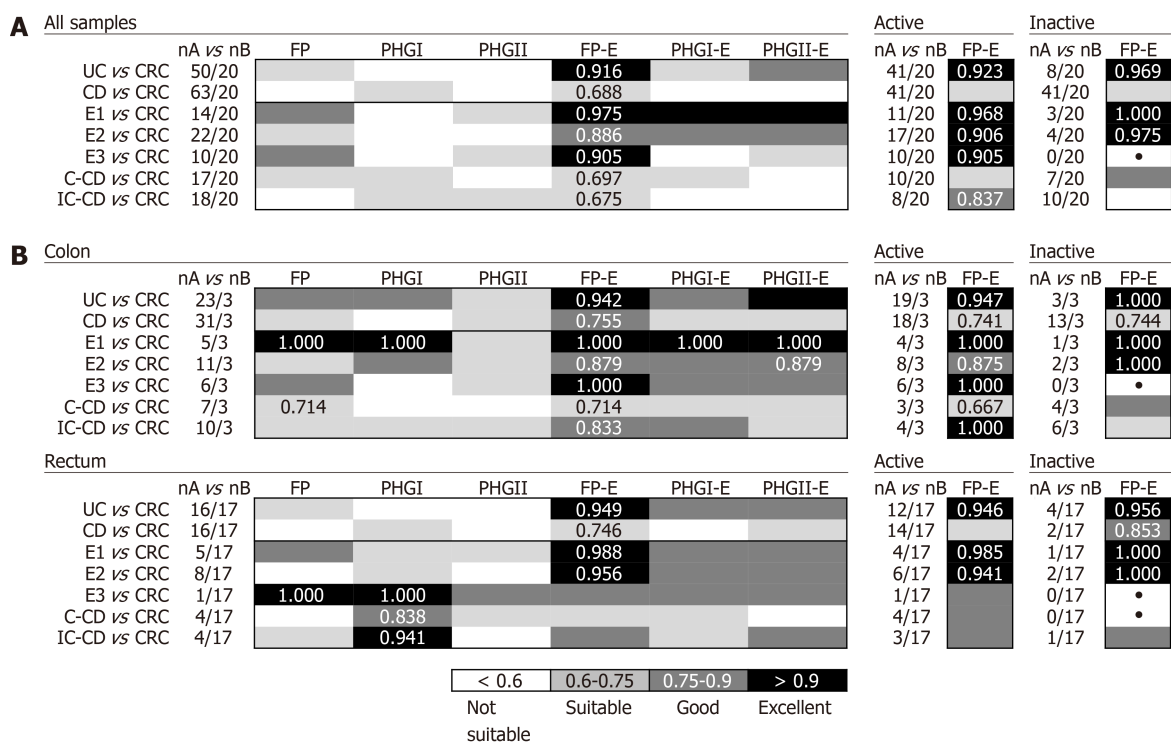


Figure 2 Usefulness of *Faecalibacterium prausnitzii*, its phylogroups (PHGI and PHGII) and their index in conjunction to *Escherichia coli* to discriminate inflammatory bowel disease with colon inflammation and colorectal cancer by pooling all biopsy samples together (A) and by location of sampling (B). For the best biomarker, results depicted by activity status of the patients are shown in the right panels. Best area under the receiver operating characteristic curve (AUC) values for each comparison are shown. : AUC not calculated (comparisons with one empty group of subjects). E1: Ulcerative proctitis; E2: Distal UC; E3: Extensive UC or ulcerative pancolitis; C-CD: Colonic-CD; IC-CD: Ileocolonic-CD; FP: *Faecalibacterium prausnitzii*; UC: Ulcerative colitis; CRC: Colorectal cancer; CD: Crohn's disease.

composition have been found between patients with diarrhoea-predominant IBS and those with constipation-predominant IBS^[37], we propose that in further studies aiming to define a biomarker between IBS and IBD, phenotype should be taken into account. Besides, the inclusion of newly diagnosed patients would be of interest to establish whether these biomarkers would be of assistance to discriminate between conditions at an early stage of the disease, particularly when symptoms are overlapping.

Secondly, as there is an association between IBD (especially those involving colonic inflammation) and risk of CRC^[13,38], the usefulness of the six biomarkers to tell apart CRC and IBD patients with colonic inflammation was explored. Among the six options of biomarkers considered, the FP-E index was the most discriminatory between CRC and IBD patients, especially from UC, regardless of the activity status of the patient and irrespective of whether colonic or rectal samples were used. This observation is of particular relevance because it has been demonstrated that the extent and duration of the disease increase the risk of patients with UC developing CRC. Future follow-up studies to establish if this index would be useful to predict the risk of CRC development associated with IBD are needed. In contrast, discrimination for CD patients was somewhat limited. Therefore it would be of interest to determine if the combination of *F. prausnitzii* or its phylogroups with other representatives of CRC dysbiosis enriched in CRC patients^[39,40] such as some phylotypes related to *Bacteroides*, *P. stomatidis* or *G. morbillorum* could provide a clearer diagnostic test.

Finally, the usefulness of these biomarkers to discriminate IBD subtypes with shared location of inflammation was assessed. The PHGI-E index was a good parameter to discriminate UC subtypes, which is of interest for clinicians to monitor the risk of progression of the inflamed area. From our data, this index allowed the best discrimination within UC subtypes with active disease in ileal and colonic samples. However, a deeper analysis to decipher which sample is the best to analyse is required, as in our study, not all the subjects provided samples from all locations, and interindividual variability may be affecting our observations. Also, further confirmation is required concerning inactive patients since our cohort was limited.

In contrast, we have observed that PHGII load, in conjunction with *E. coli* counts, can distinguish with suitable accuracy between all UC patients regardless of their disease subtypes and patients with colonic CD (C-CD). The capacity to discriminate between patients with C-CD and E3 is noteworthy because inflammation in these two

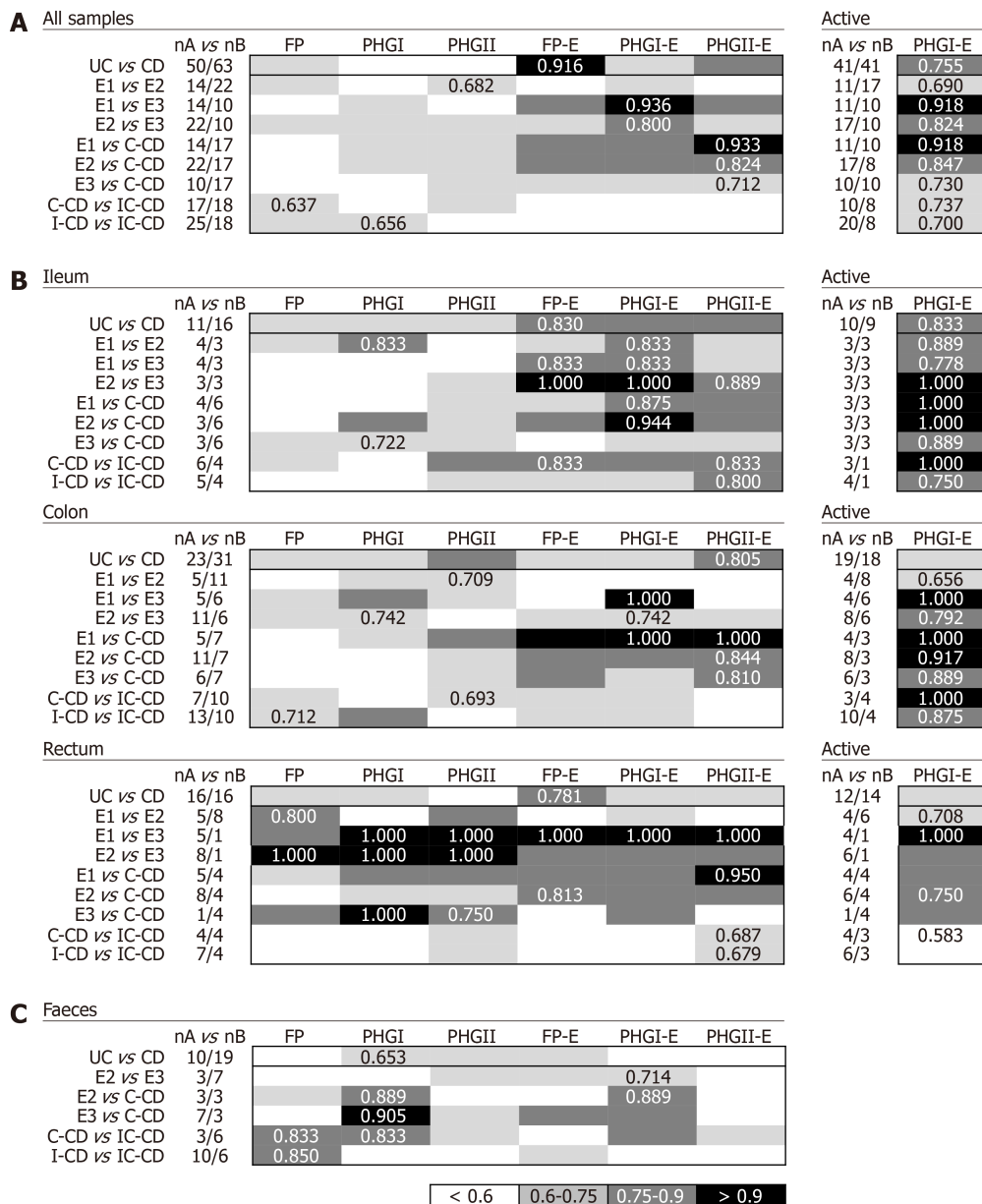


Figure 3 Usefulness of *Faecalibacterium prausnitzii*, its phylogroups (PHGI and PHGII) and their index in conjunction to *Escherichia coli* to discriminate within inflammatory bowel disease with colon inflammation taking into account all biopsy samples together (A), by location of sampling (B) and faeces (C). For tissue samples, selected results for PHGI- *Escherichia coli* of active patients are shown in the right panels. Data for inactive patients is not included because of the small cohort engaged. Best area under the receiver operating characteristic curve (AUC) values for each comparison are shown. UC: Ulcerative colitis; CD: Crohn's disease; E1: Ulcerative proctitis; E2: Distal UC; E3: Extensive UC or ulcerative pancolitis; C-CD: Colonic-CD; IC-CD: Ileocolonic-CD; FP: *Faecalibacterium prausnitzii*; UC: Ulcerative colitis; CRC: Colorectal cancer; CD: Crohn's disease.

disorders affects a wide area of the colon and may present similar clinical manifestations, thus hampering a clear classification. Due to differences in treatment and management between UC and CD^[10] it is extremely important to discriminate between these two entities accurately.

The best discrimination for CD vs UC was obtained for patients without shared inflamed area (data not shown), but the discrimination needs to be improved to differentiate IBDs with shared disease location, particularly within CD. The combination of *F. prausnitzii* or its phylogroups with other representatives of IBD dysbiosis may be a way to improve discrimination between IBD subtypes. For instance, depletion of *Roseburia hominis* has been reported as representative of UC dysbiosis, while the depletion of *Ruminococcus gnavus* and *Ruminococcus torques*, with a concomitant increase in *Dialister invisus* or *Bifidobacterium adolescentis* have been reported as signatures of CD dysbiosis^[16,18]. In addition, the identification of novel species whose abundance differs between IBD subtypes sharing the inflamed location may be of assistance in this regard.

Overall, we observed that the FP-E index would be the selected biomarker to

discriminate IBD from CRC while PHG I-E index would be the choice to discriminate within IBD subtypes, and yet no general biomarker of preference could be established to discriminate IBS from IBD. PHGII-E index would be suitable to tell apart IBS and UC patients, whereas the FP-E index could be of assistance to discriminate between IBS and CD although further confirmation on its usefulness for inactive patients is required. It has been reported that active CD and UC can be specifically diagnosed monitoring the faecal bacterial community in conjunction with leukocyte counts. Although in this previous study location of disease has not been considered, it demonstrates that serologic biomarkers may be a source of additional information. A recent study suggested that anti-*E. coli*, anti-*Fusobacterium nucleatum*, and anti-*F. prausnitzii* antibodies did not possess diagnostic value for CD or UC. However, it would be worth testing if discrimination between gut conditions is enhanced when these bacterial indicators are combined with other previously reported serologic biomarkers of intestinal disease [such as calprotectin, lactoferrin, C-reactive protein, Perinuclear Anti-Neutrophil Cytoplasmic Antibodies (p-ANCA), and Anti-*Saccharomyces cerevisiae* antibodies (ASCA)].

In order to establish if the proposed indices are suitable for discriminating conditions in less invasive samples, data from the quantification of these species in faeces of IBD subjects was used. We restricted the proof of concept to IBD subjects as these are the conditions more similar in clinical traits and therefore more difficult to discriminate. Our results allowed to demonstrate that, despite our initial selection of biomarkers was based in tissue samples, they are also valuable in faeces. However, differences in which biomarkers performed the best were found. These differences could be because the cohorts used for faecal and biopsy analyses involved different subjects or may reflect the fact that gut microbiota composition is different between faeces and biopsies. Thus, in the future, if biomarkers are selected from tissue, it is crucial to test performance in faecal samples, ideally including the two kinds of samples from the same subject. We have observed that whereas quantification of *E. coli* in biopsies improved discrimination, the role in improving discrimination when faecal samples are used remained more limited.

On the one hand, this may be explained by the fact that this species may be directly involved in host-interaction during diseases. On the other hand, this information leads us to hypothesize that for future applications, other biomarkers selected from faecal samples analysis could also be included. Concerning *F. prausnitzii*, the observed differences on which subpopulation should be used as a biomarker, may be related to the distribution of phylogroups along with the gastrointestinal tract, each one with specific metabolic features^[43,44].

To robustly validate our observations would require a larger cohort of completely independent patients, including volunteers from different ethnicities, to test these biomarkers as a tool for gut disease diagnostics. Moreover, it would be of interest to test whether *F. prausnitzii* or its phylogroups, in conjunction with *E. coli* as biomarkers could discriminate other intestinal disorders within IBD such as indeterminate colitis, unclassified IBD, pouchitis, microscopic colitis, and diverticulosis as these can also be possible confounding conditions.

ARTICLE HIGHLIGHTS

Research background

Currently, inflammatory bowel diseases (IBD) diagnostics features several challenges mainly related to its accurate differentiation from other disease with similar symptoms. In the last years, some studies have shown that the abundance of *Faecalibacterium prausnitzii* (*F. prausnitzii*) is a potential biomarker to discriminate between gut disorders. This species load in conjunction with *Escherichia coli* (*E. coli*) abundance (F-E index) has been proven to be a better biomarker than total *F. prausnitzii* alone. Besides, the quantification of *F. prausnitzii* phylogroup I and phylogroup II has been proposed as a source of additional information to discriminate within IBD. However, the usefulness of an index including the quantification of the phylogroups in conjunction with *E. coli* remains to be explored, and also its applicability to tell apart these conditions from other gut disorders with milder or worsen phenotypes.

Research motivation

Currently, IBD diagnosis involves a comprehensive examination of the patient that includes clinical, endoscopic, radiologic, and histological criteria. In addition, as clinical manifestations of IBD are unstable during the disease course, a long monitoring period is needed to classify the disease phenotype accurately. As IBD patients feature an imbalanced gut microbial community in comparison to healthy subjects, in the last years the implementation of bacteria representative of this dysbiosis as biomarkers has been started to be explored as a novel strategy to support IBD diagnostics and/or prognostics.

Research objectives

The main objective of this study was to evaluate six options of bacterial biomarkers in terms of their capability to discriminate IBD from other gut disorders and within IBD subtypes.

Research methods

Adult males and females undergoing routine colonoscopy at the Hospital Dr. Josep Trueta and Parc Hospitalari Martí i Julià in Girona (Spain) were asked to participate, providing either biopsy and/or faecal samples. Subjects included healthy controls as well as patients with IBD, CRC or irritable bowel syndrome (IBS). Genomic DNA extracts of samples were used to assess the load of bacterial markers candidates (total *F. prausnitzii*, phylogroup I and II of this species and *E. coli*) by qPCR using specific primers previously reported. Relative abundances to total Bacteria present in the sample, and indices combining *F. prausnitzii* and *E. coli* were calculated. Biomarkers accuracy to discriminate conditions was measured by the area under the receiver operating characteristic curve (AUC). To the best of our knowledge, this is the first study that tests combination of *F. prausnitzii* phylogroups and *E. coli* application to assist in discriminating challenging IBD diagnostic conditions, compares their performance with previously reported biomarkers and further corroborates results in non-invasive samples.

Research results

This study reveals that the F-E index would be the choice to discriminate IBD from colorectal cancer (CRC), especially from ulcerative colitis (UC), regardless of the activity status of the patient and irrespectively if a colonic or a rectal sample was used. This observation is of particular relevance because there is an association between IBD (especially those involving colonic inflammation) and the risk of CRC. Besides, we have observed that PHG I-E index is a good parameter to differentiate pancolitis from other UC subtypes, which is of interest for clinicians to monitor risk of progression of the inflamed area. The application of bacterial biomarkers in feces is also demonstrated, which is a non-invasive method and may represent a step forward to implement these biomarkers in clinical practice to support IBD diagnostics.

Research conclusions

This study corroborates that *F. prausnitzii* combined with *E. coli* can help to discriminate within IBD subtypes both in tissue and fecal samples, as well as offer potential to differentiate IBD and CRC patients. Use of biopsy samples presented better performance, but we confirmed that suitable results in fecal samples were shown too. The comparison of the performance of new indices with those previously reported in the literature has allowed establishing the biomarker of choice to select depending on the conditions to discriminate. From these comparisons, we hypothesize that given the complexity of the disease in terms of multiple subtypes and phenotypes during the disease course, it would be complicated the establishment of a universal biomarker using only two species and total microbiota composition could be a more informative approximation in this regard. However, given the outcome obtained only with the biomarkers evaluated here, we envisage that implementation of bacterial load assessment in clinical routine may ease IBD diagnostics in the future, for example for initial screening.

Research perspectives

This study contributes to providing evidence that bacterial biomarkers assessment may help in solving intestinal disorders diagnostic challenges. Because differences in performance were observed between tissue and faecal samples, attention should be paid to this issue in similar studies. Future directions of research could assess if discrimination between gut conditions is enhanced when these bacterial indicators are combined with other bacterial or serologic biomarkers of intestinal disease. Also, validation in a larger cohort of completely independent patients, including volunteers from different regions would be required to define a tool with worldwide application in clinical routine.

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