Ig-Profiling to Identify Crohn's Disease-Driving Bacteria

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Abstract

Crohn's disease (CD) is an autoimmune disorder that is widely investigated, but the aetiology is not yet fully elucidated. In the present study, it was investigated if the profile of bacteria-coating by IgA and the four IgG subclasses, in faecal samples, can be used as a biomarker to differentiate healthy individuals and patients suffering from CD, and to distinguish CD patients in remission and active disease. In addition, were studied possible correlations to currently used disease markers. In total were used faecal samples obtained from 62 CD patients and 20 healthy individuals. 53 CD patients donated two samples representing two-time points referred to as visit 1 and visit 2. Bacterial coating by IgA and the four IgG subclasses was determined by a quantitative flow cytometric assay. CD showed to be related with an increase level of IgA, IgG2 and IgG4. However, the results concerning IgG2 and IgG4 are ambiguous. The Bristol Stool Scale is the used disease marker that shows more correlations with active disease. The profile of bacteria coating found for all the antibodies, neither shows potential to distinguish disease state, nor to distinguish between healthy individuals and CD patients. However, the treatment of patients might have influenced the results, and thus in future studies, patients that did not receive treatment should be used.

Keywords: Pathogenesis; Crohn's disease; Antibodies; Gut Microbiota; Immunopathogenesis

Crohn's disease is an autoimmune disorder that can affect any part of the gastrointestinal tract and can be transmural and discontinuous being associated with periods of active inflammation with symptoms such as abdominal pain and (bloody) diarrhoea, alternated with periods of remission. Treatment is merely symptom-based and focuses in inducing or maintaining remission, showing limit long-term efficacy. There is no sex-specific distribution in adult Crohn's disease and the onset of the disease usually occurs in the second to fourth decade of life. Although the exact aetiology of CD is still unknown, the disease was associated with risk factors such as smoking, gene predisposition, gene mutation, defects in the immune system and above all, the gut microbiota. Genetic factors only explains 23% of the CD cases. Many environmental factors have been associated to CD, the most studied include smoking which proved to increase the risk of CD and exacerbating the disease. Furthermore, CD is characterised by an imbalance between pro-inflammatory and anti-inflammatory cytokines which is related to an exacerbated and uncontrolled Th1 and Th17 response that results in disease progression and tissue damage[1,2]. In addition, several studies reported that a change in gut microbiota is present in both mucosal and faecal samples of IBD patients. Dysbiosis in CD is associated with a decrease in *Bacteroidetes* and *Firmicutes* phyla in particular, *Clostridium* clusters XIVa and IV and with an increase of certain species such as *Gammaproteobacteria* and *Actinobacteria*,

but mainly adherent invasive *Escherichia coli* (AIEC). Dysbiosis in gut microbiota is pointed as the most important factor in the disease development being associated with mucosal inflammation. Since it is accepted that microbiota could be the main responsible for CD development and progression it would be of great interest to find the specific microorganisms responsible for CD. Thus, monitoring mucosal inflammation is crucial to limit the complications that can arise from extended inflammation. Currently, CD progression is studied through endoscopy and use of clinical activity scores and non-invasive biochemical markers. The endoscopy is an invasive procedure, the clinical activity scores do not correlate well with mucosal inflammation, and the biochemical markers available are not specific for Crohn's Disease. It is believed that CD results of a complex interplay between the host gut microbiota and environmental factors, in humans that have a genetic predisposition. A continuing inflammation in the gut will disrupt the intestinal barrier function and will lead to an uncontrolled and exacerbating response by the mucosal immune system that may be related to the production of antibodies. Antibodies are part of the humoral immune response that are related to an immunological response against microorganisms. Studies on the intestinal profile of Immunoglobulin (Ig)G-producing cells in the mucosa, revealed that patients with CD had an increase of total IgG compared to healthy individuals[3–5]. IgG is associated with activation of complement components, initiation of phagocytosis and induction of cell-mediated cytotoxicity. As a result, IgG could be involved in CD once it could be responsible for the destruction of intestinal epithelial cells. The level of IgG2 producing cells was observed to be increased in CD patients compared do healthy individuals[6,7]. Some studies reported that Crohn's disease patients secrete decreased amounts of total IgA[3,8] while others mentioned an increased level of IgA in serum of CD Patients[9]. IgA exists in high quantitates in intestinal mucosa being part of the first line of defence against pathogens by preventing bacteria from colonizing and toxins or antigens from being absorbed. Previous studies associated CD to IgG and IgA increase, but most of the research was based in serum samples that do not necessarily correlate with gut inflammation. Thus, the use of faecal samples, that are easy to obtain, is a better alternative since it is representative of mucosal inflammation in CD patients. Finding a new biomarker, specific for Crohn's disease and non-invasive, would enable the prediction of relapse, the monitoring of the effect of therapy and asses the disease activity and risk of complications. Therefore, the present study aimed to investigate the profile of bacteria coating by IgA and IgG subclasses in faecal samples of CD patients to understand if this profile could be used as a biomarker for Crohn's Disease. Specifically, it was studied if the profile could be used to distinguish healthy individuals from CD patients and CD patients in remission from CD patients with active disease.

MATERIALS AND METHODS

Samples: Faecal samples from 62 CD patients, and 20 healthy individuals were

obtained from collaborators at Maastricht University (The Netherlands). 53 patients presented two samples that represented two-time points from each patient. At the first visit (T1) all patients were classified as being in remission, whereas at the second visit (T2) 19 patients had entered active disease. 8 patients only had a samples regarding an active state and one patient had only a samples in remission state. These 8 samples were analysed together with the ones from patients that presented active disease in the second visit (T2 All the patients were under the effects of medication in the time of sampling or at least 3 months prior.

α-antibodies and Buffer Solutions: α-IgG antibodies were purchased from Southern Biotech and α-IgA was purchased from Miltenyi Biotec, each labelled by a specific fluorochrome. BIMA (Biotin Maleimide)-conjugated α-IgG1 (clone HP6001), AF647 (Alexa Fluor® 647)-conjugated α-IgG2 (clone HP6002), AF488-conjugated α-IgG3 (clone HP6050) and phycoerythrin (PE)-conjugated α-IgG4 (clone HP6025). AF647 and AF488 are analogous to allophycocyanin (APC) and fluorescein isothiocyanate (FITC), respectively. Allophycocyanin/Cyanine 7 (APC/Cy7)-conjugated streptavidin, that binds to the BIMA-conjugated α-IgG1, was also purchased from Southern Biotech.

PE-Vio770-conjugated α-IgA (clone REA1014); Vio 770 is analogous to Cyanine 7. Staining buffer 1 consisted of phosphate-buffered saline (PBS, 20 mM phosphate, pH 7.2, Lonza) with 1% bovine serum albumin (BSA, Sigma-Aldrich). Staining buffer 2 was PBS containing 1 mM EDTA (Sigma-Aldrich), 0.01% Tween (Sigma-Aldrich) and 1% BSA. To prevent non-specific antibody binding a blocking buffer of PBS containing 1% BSA (Bovine Serum Albumin, pH 7, Sigma-Aldrich) and 20% normal mouse serum (Sigma-Aldrich) was prepared.

Samples preparation and Flow cytometry Eppendorfs with 110-150 mg of faecal matter were prepared. PBS was added in the ratio of 1 mL PBS/100 mg faeces and left at 4°C with rotation for one hour followed by centrifugation at 50 *g* for 15 min at 4°C to remove large particles. Firstly, it was calculated the amount of sample needed to obtain 20×10^6 DAPI+ cells for further preparation. In these step the samples were staining with 50 µL 0.07 mM DAPI (Sigma-Aldrich) at room temperature for 15 min in the dark followed by addition of 10 µL BD counting beads (1024000 eBeads/mL, BD Biosciences). The samples were analysed on a FACSCanto II flow cytometer (BD Biosciences). The appropriate volume was transferred to a new 1.5 mL Eppendorf tube. Upon a 5-minute centrifugation (8000xg, 4°C) the bacterial pellet was resuspended and left to incubate with 50 µl blocking buffer for 20 minutes at 4°C. The samples were stained with α-IgG1 ($12.5\cdot10^{-3}$ µg/µL), α-IgG2 ($12.5\cdot10^{-3}$ µg/µL), α-IgG3 ($12.5\cdot10^{-3}$ µg/µL), α-IgG4 ($25\cdot10^{-3}$ µg/µL), α-IgA (2500 cells/ µL), and $25\cdot10^{-3}$ µg/µL streptavidin. Staining buffer 1 was added to a final volume of 100 µL. Upon incubation at 4°C in the dark at rotation for 30 min samples were centrifuged at 8000 *g* for 5 min at 4°C. The bacterial pellets were washed twice in staining buffer 1 and resuspended in 500 µL staining buffer 2. 120 µL of all stained samples were transferred to FACS tubes

and incubated with 50 µL 0.07 mM DAPI and 80 µL staining buffer 2 to a final volume of 250 µL. After incubation at room temperature for 15 min the samples were analysed on a FACSCanto II with the PMT settings discovered through a compensation assay.

Data and Statistical analysis: FlowJo (version 10.2, LLC) was used to perform the gating of flow cytometric data that was later exported to Excel. The statistical analysis was performed as indicated in figure legends using GraphPad Prism 5 and 7.

RESULTS AND DISCUSSION

The samples were divided into four groups to allow a comparison study. The Control group is composed by the samples of the 20 healthy individuals. The patients whose disease was in remission state in the first sampling are part of the Remission (T1) (54 patients) and the ones that presented the same state in the second visit are part of the Remission (T2) group (34 patients). The Active (T2) group includes the patients whose disease switch from remission to active state (19 patients) and 8 more patients that only had one sample- corresponding to active disease.

IgG2 decrease and IgG4 and IgA increase in CD patients Besides the difference between antibody coatings in each group, it was also analysed the difference within the group of patients whose disease state was followed up over time (53 patients). The amount of bacteria/g faeces and the patient-specific difference between the first and second time were analysed. The results are presented in Figure 1 and 2.

In general, the amount of bacteria/g faeces was the same in healthy individuals and CD patients (no significant difference was found between patients in remission and the ones presenting exacerbate disease) with approximately 1×10^{10} bacteria/g faeces. In contrast, Vandeputte *et al*.[10] reported that the level of bacteria in CD patients and healthy individuals differs, being 3×10^{10} bacteria/g faeces and 1×10^{11} bacteria/g faeces, respectively. Although, in the present work, the amount of bacteria in healthy individuals is lower than expected, the number of bacteria in CD patients is in the range reported by Vandeputte *et al.* In all the groups, the distribution of the bacteria coating was found to be IgA>IgG4>IgG1>IgG2>IgG3. The present work showed that IgA is the immunoglobulin present in higher percentage as expected since IgA is part of the first line of defence against pathogens and commensals[11,12]. In previous studies[13,14], the percentage of IgG-subclass producing cells, in CD patients, followed the distribution IgG1>IgG2>IgG4>IgG3 in serum and mucosa, which is in contrast to findings in this work. However, the studies are relative to samples from serum and mucosa which do not necessarily correlate to the faecal samples. Since Philipsen *et al.* reported low amounts of IgG4 in the blood[15] and in the present study it was the subclass to present the higher amount, it may suggest that IgG4-producing cells are in higher number in intestines. However, it is known that only a fraction of the intestinal induced IgG-producing cells returns to lamina propria in the gut, after recirculation, which can explain the differences between serum and mucosal

samples. The high amount of IgG4-coated bacteria in faecal samples must be related to an increased transepithelial transport of this subclass in comparison to others. In addition, it is known that IgG4 is not able to perform strong effector functions which could be an advantage to mediate sampling of antigens from the lumen, once this mechanism occurs trough transepithelial transport as well. However, it is not known if, in the case of CD, it is necessary the intervention of FcRn transport for antibody crossing, since CD is characterised by a transmural inflammation. On the other hand, the presence of IgG4 in high quantities may be normal since this subclass is characterised by a non-inflammatory nature that can be responsible for the coating of several commensals involved in a tolerogenic response, in equilibrium conditions. The low levels of IgG3-coated bacteria could be related to the presence of IgG1 since it is known that IgG1 presence inhibits the FcRn binding of IgG3 leading to a decrease in IgG3 transport[16].

The slight increase in the level of IgA-coated bacteria/g faeces, that was observed between patients in remission in first visit and patients that had a change in disease state between visits (from remission to active), showed to be significant. The comparison between patients that remained

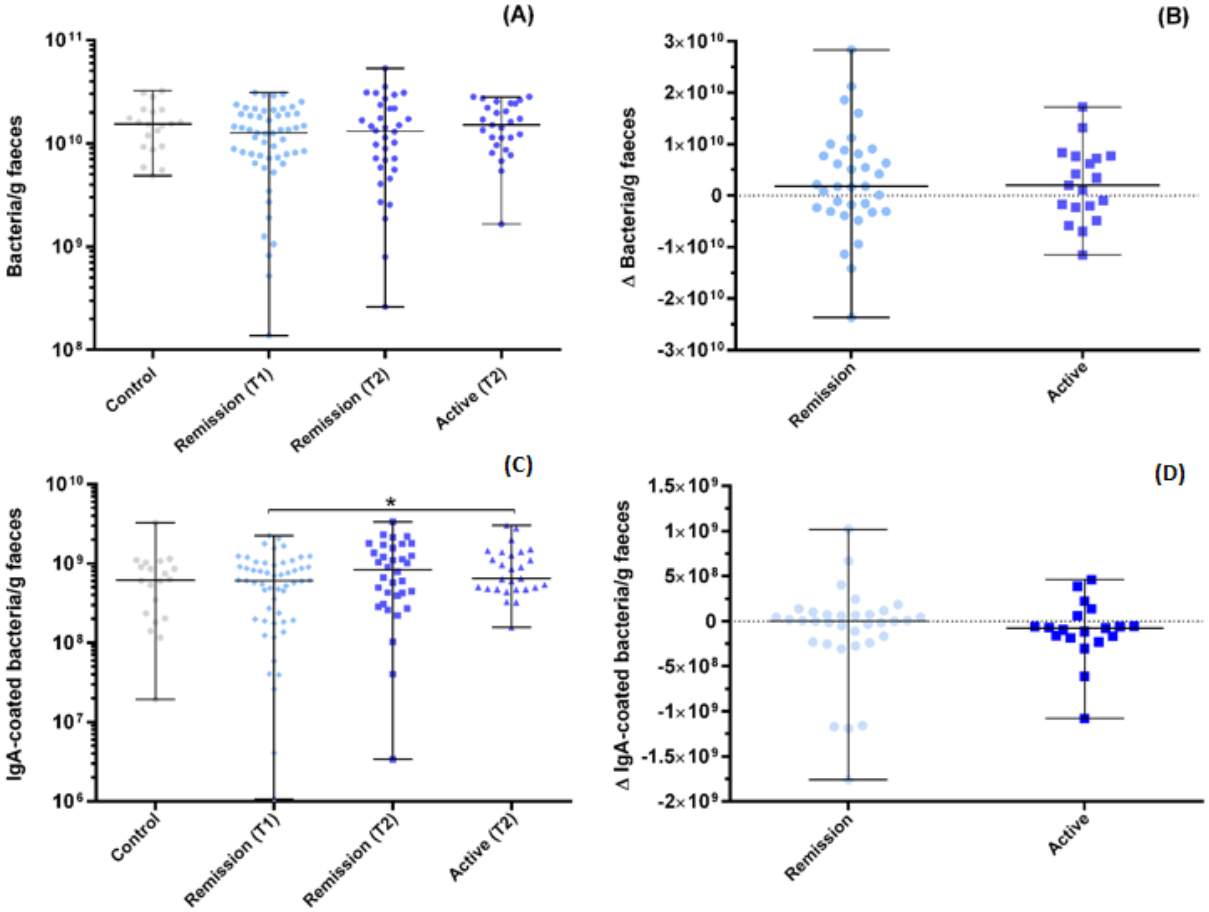

Figure 1. Bacterial density and profile of IgA-coated bacteria in Control and CD Patients with active disease or in remission.

*(A) and (C) the quantitative amount of bacteria and IgA coated bacteria/g faeces in samples from CD patients at visit 1 (T1), where 54 patients were in remission (Remission T1), and visit 2 (T2), where 19 patient presented active disease (Active (T2)) and 34 remained in remission (Remission (T2)). In addition, the quantitative amount of IgA and IgG coated bacteria/g faeces is shown for 20 healthy control individuals (Control) and 8 samples of CD patients that were in active disease were analysed with the patients that presented active disease in the second time. The significant finding is based on a Wilcoxon signed-rank test between pairs. *P-value < 0.05. (B) and (D) the patient-specific difference (Δ) between the quantitative amount of bacteria coated and IgA coated bacteria/g faeces at visit 1 and visit 2. For each patient, the amount coated at visit 1 (T1) was subtracting from the amount measured at visit 2 (T2). The significant finding is based in a Wilcoxon signed-rank test. 0.01 < *P-value < 0.05. Indicated are median ± ranges. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

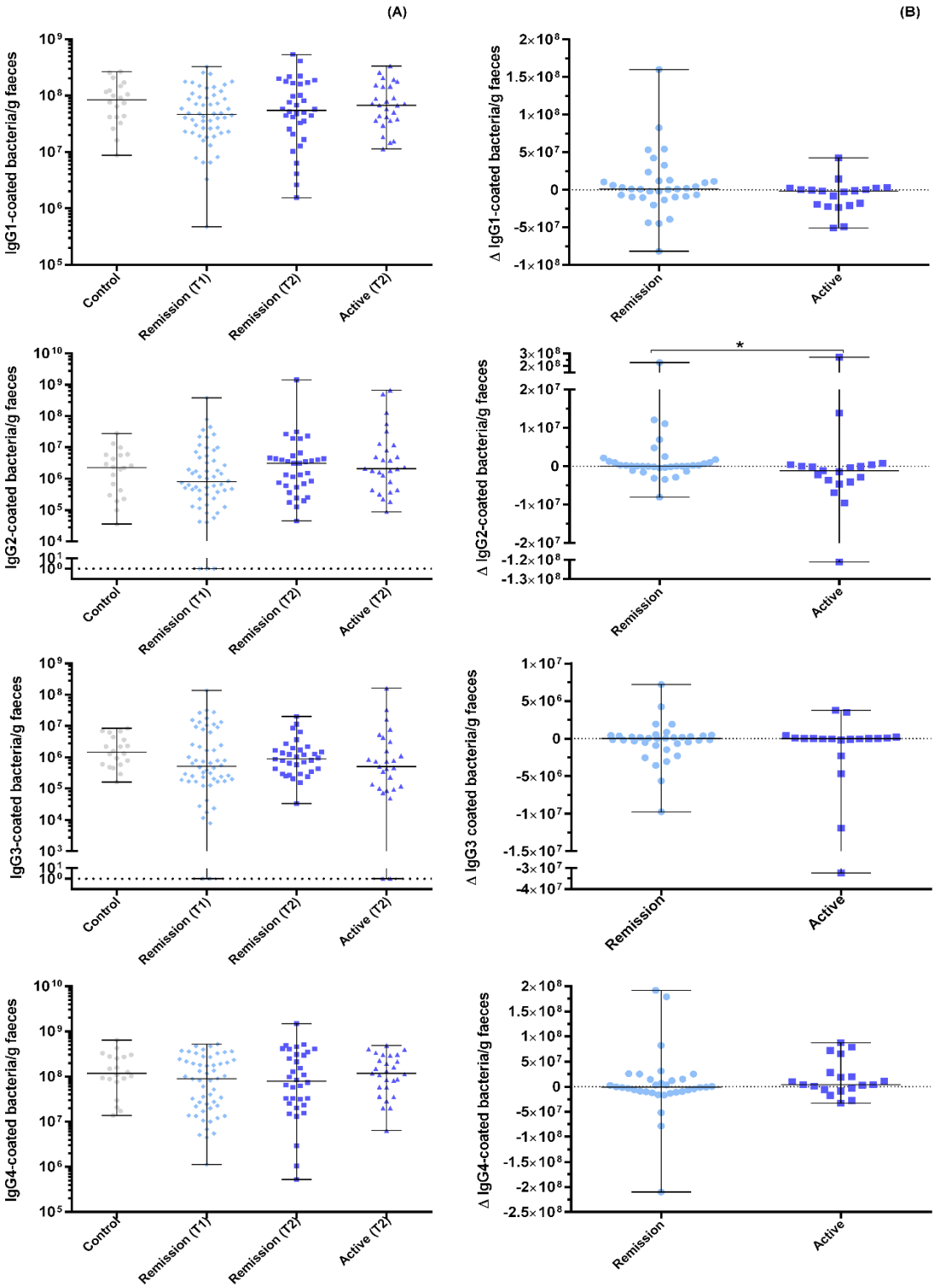

Figure 2. Comparison of the profile of bacteria-coating by IgG subclasses in CD patients with active disease or in remission.

*(A) The quantitative amount of IgG1-4 coated bacteria/g faeces in samples from CD patients at visit 1 (Remission (T1)), where 54 patients were in in remission, and visit 2, where 19 patient presented active disease (Active (T2)) and 34 remained in remission (Remission (T2)). In addition, the quantitative amount of IgG1-4 coated bacteria/g faeces is shown for 20 healthy control individuals (Control) and 8 samples of CD patients that were in active disease were analysed with the patients that change the state of disease in the second time point. For samples in which no bacteria are observed to be coated by a specific IgG subclass, the data point is set to 1 to enable the presentation of the results on a logarithmic axis. (B) The patient-specific difference (Δ) between the quantitative amount of IgG1-4 coated bacteria/g faeces at visit 1 and visit 2. For each patient, the amount of IgG1-4 coated bacteria at visit 1 was subtracting from the amount measured at visit 2. The significant finding is based on a Mann-Whitney test. *P-value < 0.05. Indicated are median ± ranges. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

in remission and patients whose disease developed to an active state, demonstrate that active patients have a non-significant decrease of the amount of IgA-coated

bacteria/g faeces. The same was observed in the patient-specific difference between the two visits which is in line with Philipsen *et al.* that did not found differences in the level of IgA between patients in remission and with disease in an exacerbating state[15]. However, no significant differences were found between healthy individuals and CD patients which contrasts with Palm *et al*.[17] and Peterson *et al.*[18] who reported that the proportion of IgA-coated bacteria was increased in CD patients comparing to healthy individuals.

In contrast to what was expected, IgG-coated bacteria/g faeces presented similar profiles in healthy individuals and CD patients. Although no significant difference was found, it appears that active state is related to a slight increase in the level of IgG. In accordance with ours, the study of Waaij *et al.* showed that IBD patients have an increased percentage of IgG-coated faecal anaerobic bacteria compared to controls, [19]. In addition, Harmsen *et al.* found that CD patients had more IgG binding gut bacteria than healthy individuals[20] and Baklien *et al.* reported that the IgG cell fraction is increased up to 30 times in CD patients, depending on the severity of the lesion[21].

In line with Wang *et al.*[22], it was observed that IgG4, when comparing the patient-specific difference between the visits, presented a minor increased in patients whose disease turned active. Additionally, for IgG2, the patient-specific data shows a decrease for active patients which suggests

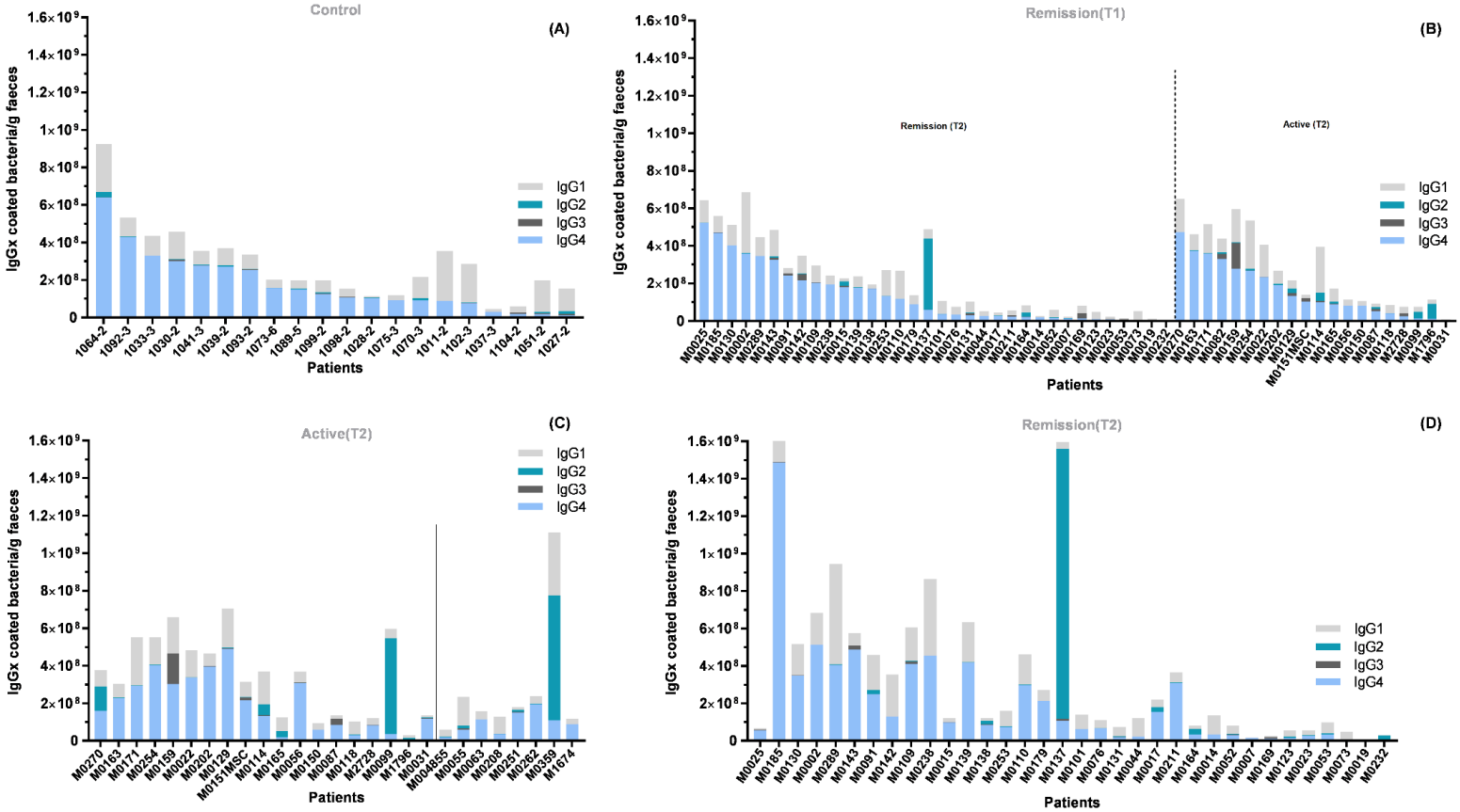

Figure 3. Comparison of the profile of bacteria-coating by IgG subclasses in CD patients in the different groups.

The quantitative amount of IgG1-4 coated bacteria/g faeces in samples from ***(A):*** *healthy individuals;* ***(B)*** *54 CD patients at visit 1 (Remission (T1)), where all patients were in in remission.* ***(C)*** *19 CD patients that disease change from remission to active at visit 2 and 8 CD patients that disease state was active (Active (T2)) and* ***(D)*** *34 patients whose disease remained in remission in the second visit (Remission (T2)). (- - - -) divides the patients that remained in remission in the second visit from the patients that presented active disease. (——) divides the patients that only had one sample from the other. The patients appear in a descending order of IgG4 in the plot concerning Remission (T1). In Active (T2) and Remission (T2) plots, the patients appear in the same order as in Remission (T1) plot. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

that active disease is related to a decrease in the level of IgG2, contrasting to several studies that correlated CD with an increase of IgG2[14,15,23]. Specifically, Philipsed *et al.* related active disease with an increased concentration of IgG2 in patient's serum. On the other hand, Helgeland *et al.* reported a reduced proportion of IgG2 in serum of healthy individuals and affected Crohn's disease twins[13]. Similarly, Rai *et al.* noted that IBD patients display low levels of Ig in serum[24]. Again, the level of Ig in blood may not express the level of Ig in faecal samples. No significant differences were found for IgG1 and IgG3 between controls and CD patients which is in agreement to Philipsen *et al.*[15]. Overall, the profile of IgA and IgG subclass-coated bacteria are not optimal to distinguish between healthy individuals and CD patients. Furthermore, the profile is also not able to differentiate patients in remission and patients in active disease and cannot be use to predict disease relapses, since only small, insignificant, differences were observed.

Patient-specific production of Ig

The antibody production showed to be patient specific (Figure 3) since the level of coating by all subclasses was different between patients that were part of the same group, thus suggesting that some patients produce more IgA and IgG than others. Furthermore, the relative quantity of each antibody varies with the patient, not following the order suggested by the profiles (IgG4>IgG1>IgG2>IgG3). Palm *et al.* reported that each individual displayed a distinct pattern of IgA coating which is in accordance with the results of the present work[17].Even though the profile changes a lot

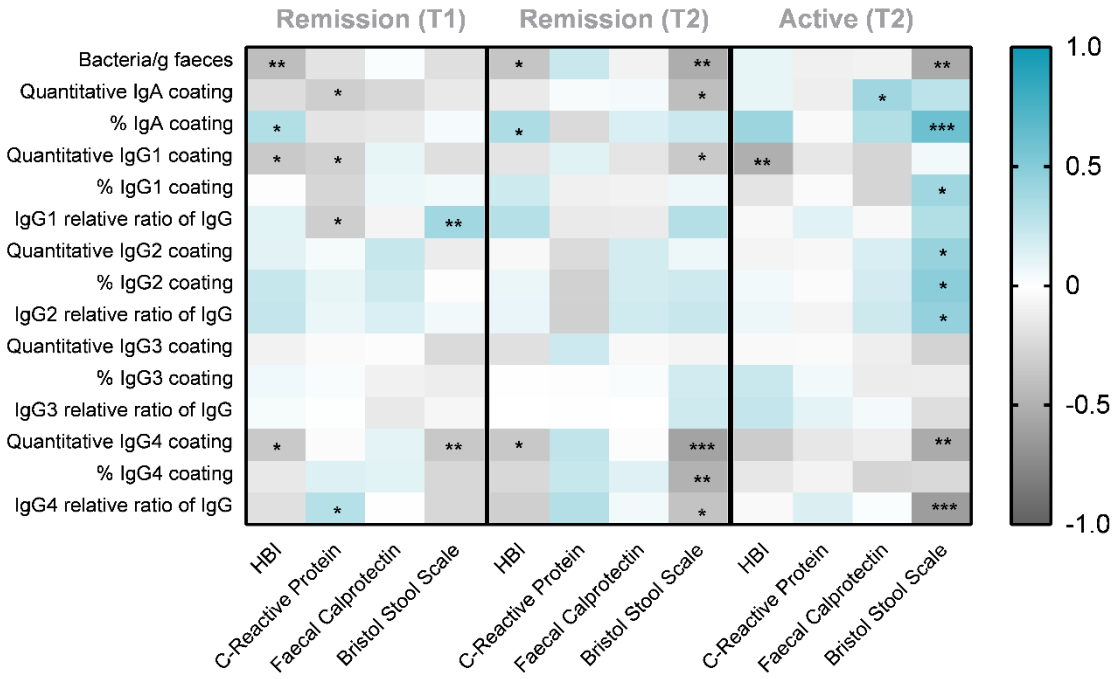

Figure 4. Correlations between bacteria-coating by IgA and IgG subclasses and currently used disease markers in CD patients.

Heat map showing correlation coefficients between disease markers and the amount of bacteria/g faeces, and the quantitative amount of IgA and IgG1-4 coated bacteria/g faeces. The amount of bacteria coated by IgA and IgG1-4 is given as a percentage and a quantitative measure based on the coating/g faeces. Furthermore, the amount of bacteria coated by IgG1-4 is also given as a relative ratio of total IgG coating. The heat map is divided into Remission (T1), that includes all patients in a state of remission, Remission (T2) that includes patients that were still in remission on visit 2 and Active (T2) concerning to patients that have entered a state of active disease. Negative correlation coefficients (grey) represent invers associations between the tested parameters, whereas positive correlation coefficients (blue) represent positive associations. The correlation coefficients were obtained by a Spearman Rank analysis. 0.01< P-value < 0.05, ** P-value < 0.01, *** P-value < 0.001. HBI: Harvey-Bradshaw Index. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

between patients, when the samples donated in visit 1 were divided in two groups, one for patients who later enter a state of active disease and another for patients in remission, a decrease of IgG4 and an increase of IgG1 were occurring between visits for most of the patients. The decrease of IgG4 found in the patient-specific analysis is opposite to the increase found in the IgG4 bacteria coating profile which supports the idea that antibody coating is patient-specific and, consequently, CD treatment should be patient-specific as well.

Bristol Scale- disease marker for CD

A correlation analysis was performed to identify correlations between the antibodies and the followed disease markers: Harvey-Bradshaw Index (HBI), the level of C-reactive protein (CRP) in blood samples, calprotectin in faecal samples and the Bristol Stool Scale (BS). The several correlations found for BS (Figure 4) demonstrated the importance and sensitivity of this biomarker to CD since several correlations were found. In all the groups, it was observed that BS is negatively correlated to IgG4 and to the amount of bacteria coated. Since higher classification in BS means faeces with more liquid, thus less faecal content it makes sense that the amount of bacteria shows a decrease with BS. The correlation found for IgG4 may be, due to lower regulatory response that is reported to be associated to CD. On the other hand, active disease shows to be positively correlated with IgG2 which, which is in agreement with the reports that associate CD to a IgG2 increase[14,15,23]. The correlations found for IgA are ambiguous but for active disease it is observed a positive correlation between the percentage of bacteria coated by IgA and the BS which was expected since active disease showed to be related with an IgA increase.

Collectively, in contrast with the previous findings, the correlations found between the used disease markers and Ig levels indicate that CD is associated to increased levels of IgG2 and decreased levels of IgG4.

CONCLUSIONS

The present study, demonstrate that the profile of bacteria coating by IgA and IgG subclasses do not provide a tool do distinction healthy individuals from CD patients. Furthermore, the profile is not optimal to distinguish the state of the disease and/or a possible relapse. However, the results show that Crohn's disease may be associated with an increased IgA, IgG2 and IgG4 although the results are ambiguous. Some of the results showed an increase of IgG2 and a decrease in IgG4 in CD patients whereas, other results show the opposite. Thus, it is reasonable to believe that those antibodies are related to Crohn's disease and that future work should be focused in those subclasses. The treatment of the patients appears to influence the results thus, future studies should consider the use of non-treated patients.

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