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

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Preface

The work presented in this thesis was performed at the Department of Biotechnology and Biomedicine of Denmark Technical University (Lyngby, DK), during the period February-July 2018, under the supervision of Prof. Susanne Brix Pedersen, and within the frame of the Erasmus programme. The thesis was co-supervised at Instituto Superior Técnico by Prof. Leonilde Moreira.

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Resumo

A Doença de Crohn (DC) é um distúrbio autoimune que apesar de ser amplamente investigado, ainda não tem origem conhecida. Neste estudo, investigou-se a possibilidade de usar o perfil de bactérias revestidas por immunoglobulina A (IgA) e pelas subclasses de IgG, em amostras fecais de pacientes com DC, como um biomarcador que diferenciasse indivíduos saudáveis de pacientes com DC, e em particular, para distinguir pacientes que tivessem a doença ativa daqueles que estão em fase remissiva. Para além disso, foram investigadas possíveis correlações e associações com marcadores usados para a doença, bem como com características dos pacientes como por exemplo a localização da doença

As amostras fecais foram obtidas de 53 pacientes, sendo que cada paciente doou duas amostras que foram recolhidas em dias diferentes que correspondem a visita 1 e visita 2. Na primeira visita, todos os pacientes estavam em remissão enquanto que, na segunda, 34 permaneciam em remissão e 19 apresentavam doença ativa. Foram ainda analisadas 20 amostras de indivíduos saudáveis e mais 9 amostras de pacientes com DC que só doaram uma amostra. A quantidade de bactérias revestidas por cada um dos anticorpos foi obtida através de citometria de fluxo.

A DC demonstrou estar associada a um valor elevado de IgA, IgG2 e IgG4. Contudo, os resultados referentes a IgG2 e IgG4 foram ambíguos. A Escala de fezes de *Bristol* foi o marcador que apresentou mais correlações com a fase ativa da doença.

O perfil de revestimento das bactérias pelos anticorpos, não demonstrou potencial para ser usado para distinguir entre estados da doença, e entre pacientes e indivíduos saudáveis. Contudo, o tratamento dos doentes pode ter influenciado os resultados obtidos e por isso, em estudos futuros, devem ser usados pacientes que nunca tenham recebido tratamento.

Palavras-chave:

Patogénese; Doença de Crohn; Anticorpos; Microbiota Intestinal; Immunopatogénese

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 and to patient's characteristics such as disease location or age at diagnosis.

Faecal samples were obtained from 53 CD patients each donating two samples representing two-time points referred to as visit 1 and visit 2. At the first visit all patients were classified as being in remission, whereas at the second visit 34 remained in remission and 19 patients had entered active disease. For a comparison study, faecal samples from 20 healthy individuals and another 9 CD patients were obtained. 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

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List of Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity
AID: activation-induced cytidine deaminase
AIEC: adherent invasive *E. coli*
AMPs: antimicrobial peptides
APC: allophycocyanin
APRIL: proliferation-inducing ligand
BAFF: B cell activating factor
BCR: B cell receptor
BS: Bristol stool scale
BSA: bovine serum albumin
CARD: caspase recruitment domain
CD: Crohn's disease
CD40L: CD40 ligand
CDI: *Clostridium difficile* infection
CRP: C-Reactive Protein
CSR: class-switch recombination
CTLs: cytotoxic T cells
Cy: cyanine
DCs: dendritic cells
E. coli: *Escherichia coli*
ERK: extracellular signal regulated
Fab: antigen-binding fragment
Fc: crystallizable fragment
FC: faecal calprotectin
FDC: follicular dendritic cells
FITC: fluorescein isothiocyanate
FMT: faecal microbiota transplantation
FSC: forward scatter channel
GALT: gut-associated lymphoid tissue
HBI: Harvey-Bradshaw index
IBD: inflammatory Bowel disease
IECs: intestinal epithelial cells
IFN: interferon
IFN-γ: interferon gamma
Ig: immunoglobulin
IL: interleukin
ILCs: innate lymphoid cells
ILFs: isolated lymphoid follicles
IRGs: immunity related GTPases
LRR: leucine-rich repeat
LTi: lymphoid tissue inducer
M cells: microfold cells
MAP: *Mycobacterium avium subspecies paratuberculosis*
MAPK: mitogen activated protein kinase
MDP: muramyl dipeptide
MHC: major histocompatibility complex
MLNs: mesenteric lymph nodes
NBD: nucleotide-binding domain
NF-kB: nuclear factor-kappa-B
NK: natural killer
NO: nitric oxide
PAMP: pathogen-associated molecular patterns
PBS: phosphate-buffered saline
PE: phycoerythrin
PIgR: polymeric Ig receptor
PPs: Peyer's patches
PRR: pattern recognition receptors
RORγt: retinoic acid receptor-related orphan nuclear receptor gamma
SCC: side scatter channel
SCFA: short chain fatty acids
sIgA: secretory IgA
STAT: signal transducer and activator transcriptor
TGF: transforming growth factor
TLR: toll like receptors
TNF: tumor necrosis factor
Treg: regulatory T cells
UC: ulcerative colitis

I. Introduction

In the past decades, the incidence of CD has been increasing in industrialised countries in Europe and North America. Currently, a rise has also been reported in Asian countries in line with westernization.

CD 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. The disease can also express extraintestinal inflammation.

Treatment is merely symptom-based and focuses in inducing or maintaining remission, showing limit long-term efficacy. About 50% of patients undergo surgery within 10 years of diagnosis, this is due to the lack of biomarkers or methods that allow to predicate relapse.

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 is pointed as the most important factor in the disease development being associated with mucosal inflammation. Thus, monitoring mucosal inflammation is crucial to limit the complications that can arise from extended inflammation. Endoscopy is the current standard but is expensive and, most of all, an invasive procedure for the patients that most of times is associated with relapses. In addition, several disease markers such as FC (Faecal Calprotectin) and CRP (C-Reactive Protein) are used to evaluate disease activity, but they lack specificity for CD. 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.

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. Furthermore, it would decrease the costs associated with the disease once it could predict the complications before surgery being needed.

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. Additionally, it was intended to evaluate possible associations and correlations of this profile with the currently used disease markers and with the patient characteristics namely, disease location, disease phenotype, age at diagnosis, gender and smoking history.

II. Theoretical background

II.1. The Mucosal Immune System in the Intestines

The gastrointestinal tract is crucial for the proper functioning of the human body once it is responsible for the nutrients absorption. Intestines are the part of the gastrointestinal tract where the intake of nutrients occurs, named the lower gastrointestinal tract. The human gut is a result of a symbiotic interaction between host and commensal bacteria.

A mucus layer covers the intestines, working as a barrier for pathogens, but also providing a good environment for commensal microorganisms. The mucus is produced by goblet cells[1]. The mucosal immune system can distinguish commensal microbiota from pathogens through pattern recognition receptors (PRR) and among those toll like receptors (TLR) that are able to recognise pathogen-associated molecular patterns (PAMP)[2].

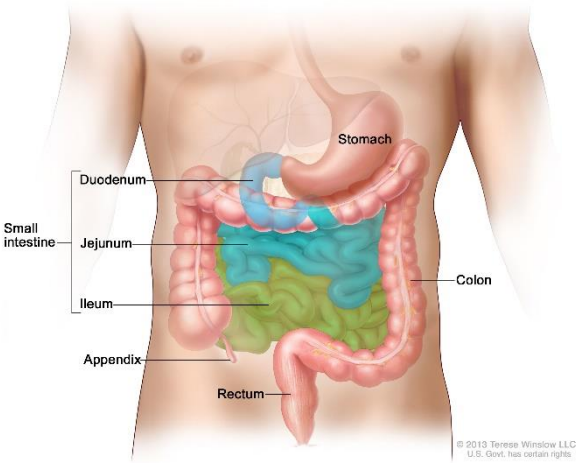

Figure 1. Overview of human gut. (reproduced from Martinez-Guryn et al., 2018).
Small intestine is divided in duodenum, jejunum and ileum.

The small intestine is divided in three parts: duodenum, jejunum and ileum (Figure 1) and is responsible for the production of several enzymes needed for food degradation[3]. Villi, finger-like projections that exist in the small intestine (mostly composed by enterocytes), increase the surface area available for an efficient nutrient absorption[4]. The high superficial area makes the small intestine vulnerable to infections. Therefore, this region is known for having high presence of lymphoid tissues allowing better protection of the epithelium barrier[5]. Molecules such as immunoglobulin A (IgA) and antimicrobial peptides (AMPs), such as the defensin and cathelicidins family of proteins, are involved in the protection against non-commensal bacteria[1]. Part of the antimicrobial peptides are synthetized by Paneth cells that are present in the small intestine but absent from the large intestine [6].

The gut-associated lymphoid tissue (GALT), present throughout the intestine, is also associated with gut protection. Epithelial cells separate the GALT from the lumen intestine and its components. Peyer's patches (PPs), mesenteric lymph nodes (MLNs) and Isolated lymphoid follicles (ILFs) are part of the GALT that is associated with adaptive responses[7]. The adaptive responses are carried out by T and B cells. Besides T and B cells, the Peyer's patches also include dendritic cells (DCs) and the epithelium above is covered by microfold (M) cells. M cells allow the contents from lumen to cross the epithelial barrier which is crucial for nutrient intake, but increases the gut vulnerability to infectious[8] (Figure 2[9]).

The large intestine, known as colon, receives the unabsorbed contents from the small intestine, and is the environment of an elevated number of commensal bacteria. The main function of the large intestinal tract is to protect epithelial tissue from invasion and due to that, the mucus layer in the colon is very thick and the number of goblet cells is greater than in the small intestine.

Unlike the small intestine, the large intestine does not contain villi but only crypts that expedite the water intake[6].

Although the presence of certain types of microbionts is adverse for the gut, the majority of the microbiota is fundamental for intestinal homeostasis preventing dysbiosis, an adverse change of the composition and function of the gut microbiota. Studies showed that germ-free animals had defects on GALT and in antibodies production which corroborates the importance of microbiota to mucosal immune system. Besides that, several diseases have been associated to an inappropriate inflammatory response that is related with dysbiosis in the immune interaction between microbiota and immune system[2]. The mechanisms responsible for these diseases are still unknown. Therefore is crucial to acquire insight on the mucosal immune system in the human intestines.

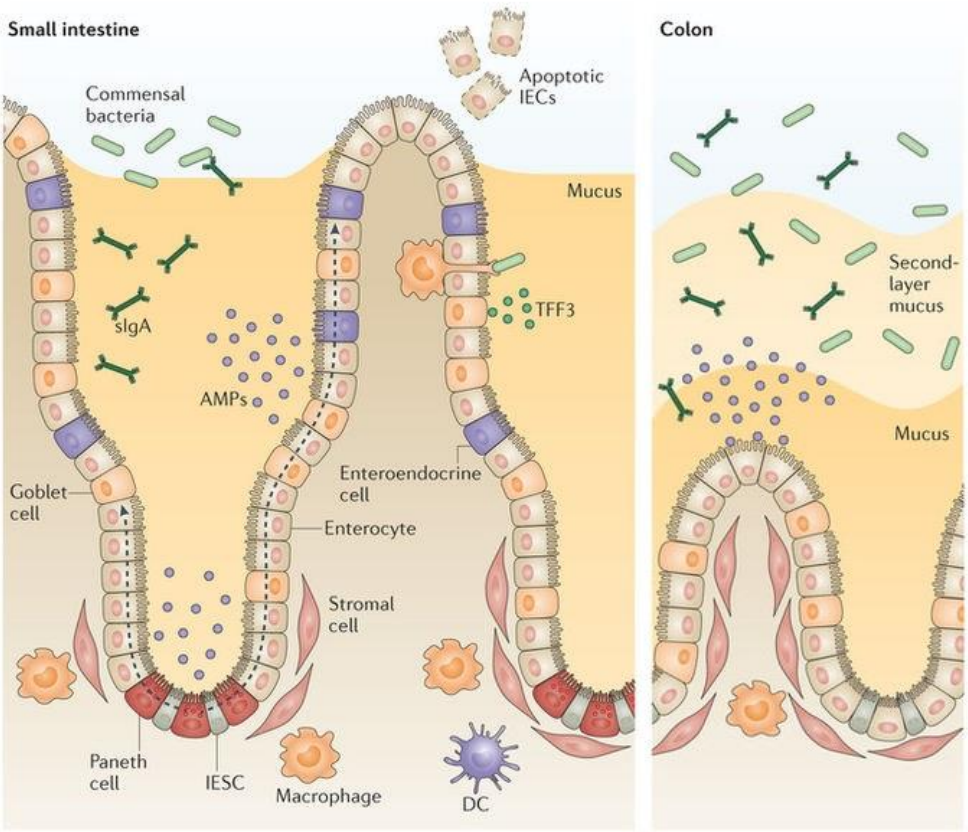

Figure 2. Overview of immune-related tissue in the human gut (reproduced from Peterson et al., 2014).

Intestinal epithelial cells (IECs) act as a barrier between lumen and the mucosal immune system. Epithelial, stromal and haematopoietic cells are part of the IECs. The pathogens are excluded from the epithelial surface through the action of mucus and antimicrobial proteins (AMPs) secreted by goblet cells and Paneth cells. The secretory IgA (sIgA) further interact with bacteria to also protect the mucosal immune system. Microfold cells (M cells) and goblet cells mediate transport of luminal antigens and live bacteria across the epithelial barrier to dendritic cells (DCs), and intestine-resident macrophages sample the lumen through transepithelial dendrites. In the colon, a second layer of mucus is present to ensure higher protection once the number of bacteria is higher.

II.2. Crohn's Disease

Crohn's disease is a major chronic inflammatory bowel disease (IBD) recognised in 1932. IBD is thought to be driven by an abnormal immune response to intestinal microbiota in genetically predisposed individuals, comprising CD and ulcerative colitis (UC)[10]. 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. Additionally, there seems to be a smaller peak that has been described from 50 to 60 years of age[11].

Incidence and prevalence of Crohn's disease are greater in developed countries than in developing countries and in urban areas than in rural areas. Prevalence is highest in Europe (322 per 100 000), Canada (319 per 100 000) and the USA (214 per 100 000)[12]. Curiously, areas of low incidence and prevalence, such as Asia, have observed a steady increase in annual incidence of CD (0.54 per 100 000)[13].

The CD patients are classified according to their age at diagnosis, disease phenotype and location based on the Montreal classification[14] (Figure 3).

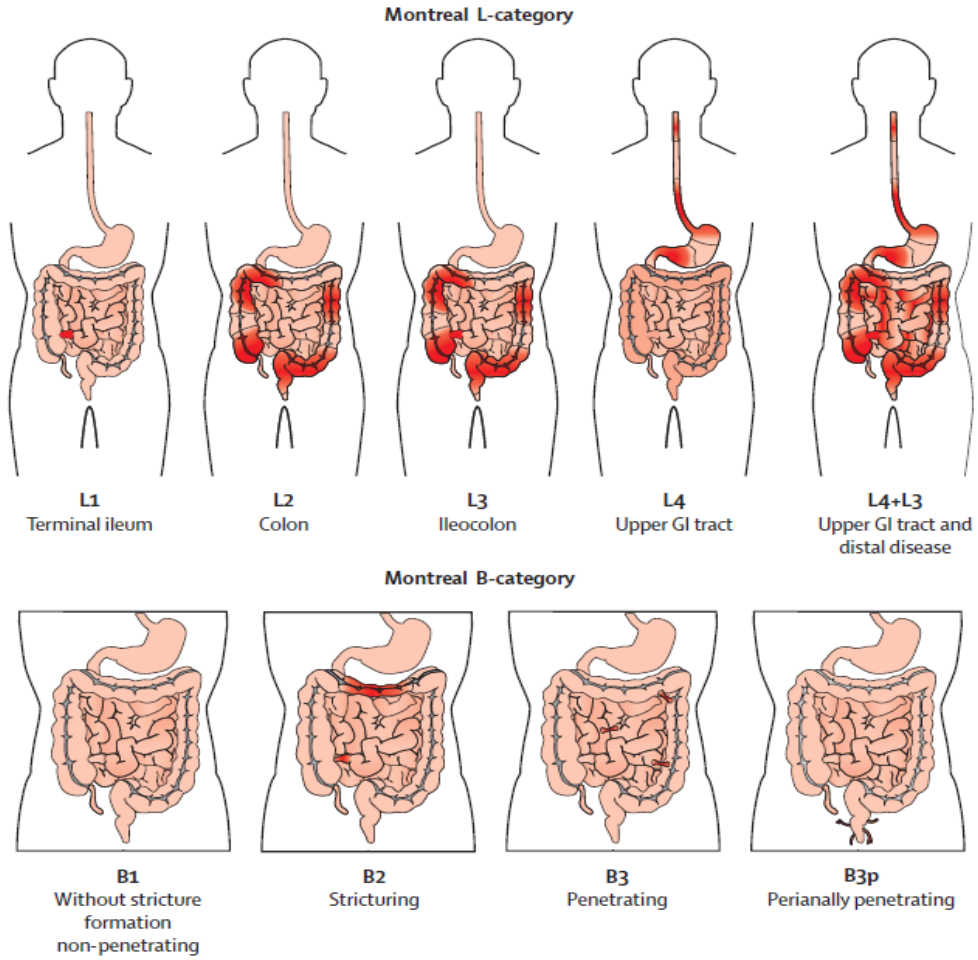

Figure 3. Montreal classification for Crohn's Disease (reproduced from Baumgart and Sandborn, 2012).

Classification by age is A1 <16 years, A2 17–40 years, A3 >40 years. CD can affect the terminal ileum (L1); colon (L2); ileum and colon (L3) and upper GI tract (L4). L4 is also used as a modifier that can be added to L1–L3 when concomitant upper GI disease is present. Disease behaviour can be described as non-stricturing and non-penetrating (B1); stricturing (B2) and penetrating (B3). p is added to B1–3 when concomitant perianal disease is present. GI=gastrointestinal. p=perianal disease modifier.

CD is mostly manifested in the terminal ileum (30% of CD patients) and colon (20% of CD patients) although inflammation can be present anywhere in the gastrointestinal tract from the mouth to the anus[14,15]. The disease is characterized by a discontinuous pattern of inflammation since bowel segments with active inflammation alternate with normal segments. The location of the affected bowel segments usually remains stable during the course of CD, and only about 15% of patients have changes in disease location over a period of 10 years[16]. Same patients have relapses of active disease but also experience periods of remission with less severe symptoms. On the other hand, some patients have constantly active disease with no spontaneous remissions[17].

The symptoms can vary accordingly to the site, the age of the patient, the duration of the disease, and the disease severity, but the most common ones include abdominal cramping, pain, rectal bleeding, diarrhea, loss of appetite, weight loss, and fatigue. The disease progression may lead to complications such as the onset of fistulas, abscesses and strictures. Although CD is a disease of the gastrointestinal tract, inflammation in CD is not restricted to it and extraintestinal manifestations of the disease may occur. Frequently patients present skin and eye problems, joint paint, liver inflammation, mouth ulcers, anemia and are more propitious to develop osteoporosis. These complications frequently require surgery, and in spite of modern medical therapy, 40–71% of patients need surgical therapy within 10 years after diagnosis[18].

Currently, the diagnosis is based on a combination of methods including for instance endoscopy, histology on colonic biopsies and tests for biomarkers such as CRP and FC and lactoferrin. Immunologic biomarkers in serological samples are used to recognise self-antigens or cross-reacting with several bacterial and fungal antigens. Distinguish between UC and CD is often a hard task due to the substantial group of IBD patients with ambiguous colonic diseases. Cross-sectional imaging using, for example ultrasonography, computed tomography, magnetic resonance imaging and barium contrast radiology are helpful to detect small bowel inflammation and lesions indicating the diagnosis of CD. As opposed to endoscopy, cross-sectional imaging is a non-invasive method that readily can detect the complications of strictures, abscesses and fistulas and in general assess the thickness of the bowel[19]. Disease activity, location and severity dictate the choice of treatment. If the patient suffers from mild to moderate disease without complications, the first line medicaments are corticosteroids like budesonide to induce clinical remission, mixed with immunosuppressants such as thiopurines in order to maintain the remission. If the disease is severe with extensive bowel damage and complications, and the patient is unresponsive to previous treatments, administration of anti-TNF-α agents, such as infliximab is proposed[11].

Several CD patients undergo surgery during their life, often more than one surgery, either because of complications that exacerbate disease state, unresponsiveness to treatment and intolerance to the medicaments[20]. Although the precise aetiology of CD is still unknown, it is believed that the disease results from a complex interplay between genetic susceptibility, environmental factors, and altered gut microbiota, leading to dysregulated innate and adaptive immune responses[11]. Several animal models of intestinal inflammation have been developed

using chemical induction, immune cell transfer, or genetic manipulations. More recently, murine models of chronic ileal inflammation that spontaneously develop and closely resemble human CD with regard to disease location, histologic features and clinical response to therapy have been characterized[21]. Although these models allowed significant progress in our understanding of the mechanisms involved in CD, to mimic the complex inflammatory state observed in CD is still a challenge.

In the following sections, a review concerning the risk factors for CD is presented.

II.2.1. *Genetic Susceptibility*

The role of genetic factors in CD has always been considered relevant. Even though genetic susceptibility is related with CD arising, it only explains 23% of the CD cases[22].

Some studies pointed out that having a relative with a history positive for IBD is a risk factor and that the disease pattern of the relatives could be of great help to predict the phenotype and disease course of the patient. Nevertheless, the lifetime risk for developing CD when a first degree relative has IBD is only up to 5%[23].

Genetic studies with twins support the idea that CD is related with genetics. Monozygotic twins showed a phenotypic similarity of 36% regarding to the location of the disease thus providing an evidence for a genetic contribution to CD susceptibility[24].

Over the years, more than 140 different genetic *loci* in 17 chromosomes were found to be implicated in CD genetic susceptibility[25]. These *loci* encode proteins related with homeostatic mechanism such as innate pattern recognition receptors, Th17-lymphocytes differentiation, maintenance of epithelial barrier integrity, autophagy and secondary immune response[26].

In 1996, Hugot and colleges discovered a susceptibility *locus* for CD in chromosome 16, *IBD1,* which in 2001 lead to the identification of the gene most strongly associated with CD, *NOD2*/CARD15[27,28]. The *NOD2* gene product, previously known as CARD15, is composed by two caspase recruitment domains (CARD), a nucleotide-binding domain (NBD) and a leucine-rich repeat region (LRR region). *NOD2* is highly expressed in ileal Paneth cells thus several studies revealed that mutations in *NOD2* are associated with ileal CD[29]. *NOD2* plays a crucial role in immune response once it recognizes muramyl dipeptide (MDP), that is present in peptidoglycan of bacteria[30].

In presence of MDP, *NOD2* protein activates the nuclear factor-kappa-B (NF-kB) which leads to the production of a variety of cytokines, chemokines, and antimicrobial peptides (TNF-α, interleukin (IL)-6, IFN-γ, IL-1β, IL-10, IL-8/ CXCL8, and α-defensin) depending on cell types[31]. Polymorphisms in *NOD2* gene located within or close to the LRR domain that recognizes MDP suggests that *NOD2*-mediated susceptibility to disease may be caused by a failure to activate NF-kB pathway[30]. On the contrary, CD patients showed elevated levels of pro-inflammatory cytokines related with an increased NF-kB activation[32] which is the reason for the disease treatment to be based on sulphasalazine and glucocorticoids- NF-kB inhibitors[33]. One possible explanation to this contradiction was suggested by the discovery that *NOD2* could negatively attenuate NF-kB signalling in response to TLR2 activation[34]. Combining all the different studies

leads to the suggestion that *NOD2*'s function may positively or negatively influence NF-kB surrounding according to different conditions[35].

Several studies reported that *NOD2* is also involved in the production of a subset of α-defensins that are produced in Paneth cells located in the crypts of the small intestine[29,36]. These studies revealed an altered expression of α-defensins in ileal CD patients and supported the idea that *NOD2*, once again, confers genetic susceptibility[37]. On the other hand, Simms and colleges[38] (2008) report that the reduction on α-defensins in ileal CD patients are associated with inflammation and not to *NOD2* mutations. Further studies are needed to clarify if *NOD2* is related or not with the decrease of α-defensins.

Defects in autophagy, the process by which cells surround and destroy foreign invaders such as viruses and bacteria, was also related with CD genetic susceptibility. *ATG16L1* and *IRGM* were two of the genes associated with malfunctions of this process.

IRGM gene codes for a protein, belonging to a family of interferin-inducible immunity related GTPases (IRGs), that activates autophagy in infected cells, for example, with bacteria that causes tuberculosis[39].

ATG16L1 is part of a large protein complex crucial for autophagy and plays a role on Paneth cells function. Patients with deficiencies in *ATG16L1* gene presented Paneth cells with notable abnormalities in the granule exocytosis pathway, compromising the phagocytosis process[40].

Both *ATG16L1* and *IRGM* deficiency showed increased adherent invasive *Escherichia coli* (AIEC). *ATG16L1* deficiency abolishes the ability of cells to form autophagosomes, which allows the growth of AIEC[41]. AIEC is a pathogenic group that has the ability to invade epithelial cells and survive and replicate within macrophages and is associated with ileal mucosal CD pathogenesis[42].

NOD2 (as well as his homolog, *NOD1*) is also involved in the autophagy process once it recruits *ATG16L1* gene product to the plasma membrane at the bacterial entry site. Deficient NOD2 is not able to recruit ATG16L1 having implications on autophagy. Therefore, both ATGL16 and *NOD2* have to be intact for the proper functioning of the autophagy pathway[43]. Mutation in any of these genes will lead to an insufficient antigen presentation due to effects in major histocompatibility complex (MHC) class II processing, affecting T cell response[44].

In spite of the large number of *loci* that have been discovered, genetic factors explain only about 20% of the heritability of CD[17]. Besides that, it seems that it is not a specific mutation that causes the disease but instead a complex interplay between genetics and gut microbiota.

II.2.2. *Environmental Factors*

Many environmental factors have been associated to CD, the most studied include smoking, diet, drugs, geographical and social status, stress, microbial agents and intestinal permeability[17]. These are related with developed and urbanised countries which are the most affected by CD[45].

Cigarette smoking, which is the best studied environmental factor, proved to increase the risk of CD and exacerbating the disease[17]. In addition, smoking appears to increase the risk for

surgery and boosts the relapse rate of CD. Furthermore, smoking cessation seems to improve CD[46].

Contrary to what it is though, stress is not an initiating factor but acts as a modulator of the disease. A reduction in dietary fibre and an increase in saturated fat intake have also been associated with increased risk[11].

Antibiotic exposure is also related with CD, in particular, antibiotic exposure in childhood increases the risk of Crohn's disease suggesting that antibiotic intake has an important role in CD evolution[11].

Infectious agents were also pointed as relevant for CD, supported by the observation that CD frequently occurs after infectious gastroenteritis[47]. Furthermore, dysbiosis and augmented translocation of commensal bacteria into the bowel wall have been described in CD, suggesting that bacterial antigens may cause immune cell activation in this disease[48–50].

II.2.3. *Gut Microbiota*

The intestines are composed by more than 100 trillion different microbial organism that are collectively referred as the gut microbiota[51]. Bacteria is the major group of gut microbiota, with 99% of the bacteria present in the intestines belonging to one of these *phyla*: *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*[52]. Bacteria populations composition and number vary with the location in the gastrointestinal tract, increasing from the jejunum to the colon[53].

As previously mentioned, the gut microbiota has functions related with the immune system, metabolism, defence of the host and nutrition. Intestinal microbes are capable of degrading certain carbohydrates that human digestive enzymes are not (for example starch and cellulose) through fermentation that gives rise to short chain fatty acids (SCFA). The gut microbiota is also involved in the production of vitamin K, some components of vitamin B and some amino acids[54,55]. Furthermore, gut microbiota has a crucial role in the gut development once the maturation of GALT is due to a post-natal interaction with the microbes that leads to the expansion of lymphoid tissue[56]. The development of T cells is also associated with microorganism presents in the gut[57]. Hence, the production of IL-17A, IL-17F, IL-21, and IL-22 is also mediated by the gut microbiota once those interleukin are produced by Th17 cells, a subtype of effector T cells[51]. The presence of beneficial bacteria is fundamental to protect the gut from pathogenic organisms once they compete for mucosal attachment sites[58]. Although bacteria can act as protective agent, they are also associated with the pathogenesis of IBD.

Several studies reported that a change in gut microbiota is present in both mucosal and faecal samples of IBD patients[59]. Dysbiosis in CD is associated with a decrease in *Bacteroidetes* and *Firmicutes phyla* in particular, *Clostridium* clusters XIVa and IV[60]. These *phyla* are responsible for the production of SCFAs such as butyrate that has an anti-inflammatory role in the gut and is a primary energy source for colonic epithelial cells[11]. The decrease of SCFAs and, consequently, the reduction in butyrate availability compromises the differentiation and expansion of T regulatory cells (Treg) and the growth of epithelial cells which is reflected in intestinal homeostasis[61,62]. For

example, *Faecalibacterium prausnitzi (F. prausnitzii),* that belongs to the *Clostridium* cluster IV, is known for butyrate production and its decrease is related with the risk of relapse of ileal CD after surgery. The anti-inflammatory nature associated with *F. prausnitzii* was also enlighten by Sokol and colleagues[63] (2008) who discovered that human peripheral blood mononuclear cells stimulated with *F. prausnitzii* induced the production of IL-10 (an anti-inflammatory cytokine) and inhibited the synthesis of IL-12 and IFN-γ (inflammatory cytokines).

Dysbiosis in CD patients has also been associated with an increase of certain species such as *Gammaproteobacteria* and *Actinobacteria*, but mainly *Escherichia coli.* AIEC associated with CD has pro-inflammatory properties. It is reported that 1/3 of CD patients have an increased AIEC. These bacteria are capable of crossing the mucosal barrier, surviving and leading to the secretion of a large number of TNF-α (pro-inflammatory cytokine) that induces intestinal inflammation[64]. Besides the secretion of TNF-α, some strains of AIEC appear to be involved in granuloma formation, that is also an inflammatory response that commonly occur in CD patients[65].

Another specific microbe that has been studied is *Mycobacterium avium* subspecies *paratuberculosis* (MAP). MAP is related with chronic intestinal inflammation in dogs, nonhuman primates and cattle. Some CD patients showed a higher level of MAP/MAP-DNA; antibodies to MAP; and MAP-reactive T cells in mucosal tissues[66,67]. Moreover, genetic studies showed that CD patients that have *NOD2* mutation also show defective MAP recognition[68]. Furthermore, mutations in a gene associated with autophagy (*ATG16L1*) is related with MAP[69]. However, several studies have contradictory results. For example, Elguezabal *et al.*[70] and Juste *et al.*[71] found higher MAP-DNA content in healthy individuals comparing to CD patients. Additionally, CD patients treated with anti-mycobacterial drugs didn't show any improvement which suggests that MAP does not play a role in CD[72]. More data is needed to assess the role of MAP in CD pathogenesis.

Several strategies to manipulate the gut microbiota have been studied, including antibiotic and probiotic use and faecal transplantation.

Concerning the use of antibiotics, they have an important role in managing complicated CD. Several studies have been conducted to understand how they can be used to maintain or induce disease remission. They are used to ameliorate the disease status and provide evidence that gut microbiota have a role in CD pathogenesis once treatment with antibiotics show an improvement of the disease symptoms.

The efficacy and relevance of probiotics in CD has not been demonstrated. Although there is very few studies one this subject, Butterworth reported that probiotics have no advantage over placebo in the maintenance and induction of CD[73].

Faecal microbiota transplantation (FMT) has the purpose of converting an unhealthy intestinal microbiota to a healthy one by transferring intestinal microbiota of healthy donors. FMT has been performed since 1958[74], being often related with *Clostridium difficile* infection (CDI). In a dysbiotic gut, the risk of *C. difficile* overgrowth is high, and transplantation of a non-dysbiotic intestinal flora could reverse this alteration. Several studies present a cure rate higher than 90% for FMT in

patients with ileal CD[75,76]. In CD studies, FMT appear to induce remission in more than 60% of CD patients[77,78].

The sum of these data indicates that gut microbiota plays a major role in CD pathogenesis. Understanding which microbial populations have important roles in CD and the mechanism that they use is crucial to target and reduce the pathogenic microbes and increase the number of beneficial commensals.

II.2.4. *Immunological pathogenesis*

CD is known to be an autoimmune disease that is related to an abnormal function of the intestinal mucosal immune system in the presence of food or bacterial antigens. Although several hypotheses about CD pathogenesis have been presented, the specific mechanism that leads to the immune imbalance causing the damage of intestinal mucosa and the source of the inflammation is still unclear.

Several studies reported that CD is a consequence of chronic inflammation of T lymphocytes, in particular CD4+ T cells, being related to a Th1 response. The Th1 response is responsible for the pro-inflammatory responses that kill the intracellular parasites where interferon gamma (IFN-γ) and IL-12 are the principal Th1 cytokines. Th1 cells are an activator of macrophages via IFN-γ secretion that activates the microbial activity of these cells resulting in TNF-α production[79]. However, in CD patients TNF amount is low which leads to an ineffective pathogen killing by macrophages that results in granuloma formation[80]. IL-12 induces the production of IFN-γ, favours Th1 differentiation, and forms a link between innate and adaptive immunity.[81] The Th2-type cytokines include IL-4, -5, -10 and -13 will counteract the Th1 mediated microbial action. The production of one type of cytokine inhibits the other type, *i.e.*, if Th1-cytokines are produced earlier or in greater extent than Th2-cytokine, Th2-cytokine production is inihibited[82]. Although the two types of response should not be coincident, evidences show that IL-4 and IL-13, related to Th2 cells, are involved in ileal CD. The initial stage of inflammation in CD is composed by the induction phase and effector phase that occur in simultaneous. Th1 and Th2 may participate in the two phases, simultaneously or sequentially[82].

Regulatory T cells are a subtype of T cells capable of producing anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β). IL-10 induces Treg cells proliferation through signal transducer and activator transcriptor 3 (STAT3) activation while TGF-β supresses pro-inflammatory responses by macrophages and effector T cells via activation of SMAD3 and SMAD4 proteins. By down regulating, for example, IL-10, Treg cells inhibit the intestinal inflammatory response. In CD patients, it was found that Treg cells were decreased and effector T cells were increased comparing to healthy patients thus suggesting that Treg cells are suppressors of Th1 responses[83].

Although CD has previously been related to a Th1 response it was found that Th17 cells can be the main contributor for the disease. Th17 cells are a type of T cells that have in common with the Treg cells the fact that both are subsets of CD4+ T cells. Treg cells inhibit intestinal mucosal inflammation whereas Th17 cells promotes intestinal inflammation induced by autoimmune

diseases[82]. The two types of cells have opposite functions and modulate the proliferation of each other in a way that allows a balanced immune response[84]. The presence of pathogens can induce CD4+ T cells to differentiate into cytotoxic T cells (CTLs) that release IL-17 to stimulate Th17 cells to produce TGF-β and IFN-α. A study found that TGF- β can induce the differentiation of naïve T lymphocytes to Th17 cells which immunosuppress Treg cells[85]. Some of the cytokines related to the Th17 exacerbated response are mentioned below.

The Th17 differentiation is induced by IL-1β and IL-23 and enhanced by IL-6, acting through the STAT3 which mediates the expression of retinoic acid receptor-related orphan nuclear receptor gamma (RORγt). Th17 cells activated by TGF-β and IL-6 promote mucosal defense, barrier tissue integrity and curtail immunopathogenic responses, whereas IL-23-activated Th17 cells promote chronic tissue inflammation during infection, granuloma formation and autoimmunity[86]. Th17 cells that are not expose to IL-23 are not inflammatory and have as main function the production of IL-10 whereas, when cells are exposed to IL-23 they became pro-inflammatory reaching their full pathogenic function[87].

Increased levels of IL-23 in CD patients suggested that this cytokine could be a key drive of CD because it promotes the proliferation and accumulation of Th17 cells which leads to the maintenance of the inflammatory response. Besides, IL-23 also suppresses Treg expansion and reduces the production of IL-10, an anti-inflammatory cytokine[86]. IL-12, previously mentioned, and IL-23 are both part of IL-12 cytokine family sharing subunit IL-12p40. In the past, it was though that CD was a Th1 response due to the increase presence of IL-12 in CD patients but is now believed that what was reported as IL-12 was actually IL-23[88,89]. The use of briakinumab and ustekinumab in CD patients, which are human monoclonal antibodies against IL-12/IL-23p40 induced clinical response and remission, thus clarifying the importance of these cytokines in CD pathogenesis[90,91].

Kinugasa *et al.* found that IL-17 plays a role in the regulation of intestinal epithelial barrier function regarding the extracellular signal regulated (ERK)-mitogen activated protein kinase (MAPK) pathway, which could be responsible for intestinal inflammation[92].

IL-6 not only plays a role in mediating expression of RORγt, but it also induces the expression of IL-21 that activates STAT3 thus facilitating Th17 cells expansion. Both IL-6 and IL-21 induce upregulation of the IL-23 receptor (IL-23R) which, once more, amplifies Th17 cells differentiation[93].

Collectively, effector cytokines such as IL-17A (also known as IL-17), IL-17F, IL-22 IL-21, and IFN-γ produced by Th17 cells promote the production of pro-inflammatory cytokines that contribute to inflammation and tissue damage[94]. IL-17A and IL-17F are pro-inflammatory cytokines that play important roles in combating infections and mediating autoimmunity by recruiting and activating neutrophils through IL-8. Some studies[95] reported an increased amount of neutrophil infiltration in CD patients whereas others demonstrated an impaired recruitment of these cells[79]. A defect in the recruitment of neutrophils leads to inefficient clearance of pathogens which results in exacerbated and chronic inflammation. Furthermore, as previously mentioned, polymorphism in *NOD2* and *ATG16L1* genes is related to defective autophagy which leads to

lower bacterial clearance in CD[43]. As a result, could be hypothesised that CD is related to an ineffective clearance of pathogens by the immune system.

Innate lymphoid cells (ILCs) are present in the immune system and are known to express RORγt that is important for Th17 cells development[96]. Several studies reported that ILCs play an important role in CD pathogenesis through numerous pathways. The imbalance in the regulation of ILC diminish tolerance to food and bacterial antigens in the gut leading to CD, being ILC3 the most important ILCs type[97–99]. ILC3 cells include all the ILCs able to produce IL-17 or IL-22 which can promote Paneth cells to secrete antimicrobial peptides (such as Reg Ⅲβ and Reg Ⅲγ). In CD patients, it was observed an increased amount of ILC3s which may disturb the homeostatic conditions. Studies found that a reduction in IL-22+ ILC3 in the intestinal mucosa in CD leads to an increased exposure of intestinal tissue to many antigens which caused an abnormal immune response[100]. Hutnick *et al.*[101] attributed the occurrence of CD to the deletion of IL-22+ ILC3 once it caused the spread of *Alcaligenes sp*. in the intestinal lymph tissue and induced a systemic immune response. Furthermore, it was found that in IBD patients, expression of MHCII by ILC3 was reduced, thus damaging the intestinal mucosa due to an immune response against commensal bacteria [102].

Besides ILC3s, also ILC1s were found to be increased in CD patients when comparing to healthy individuals. A possible plasticity between ILC3 and ILC1 could be the explanation[100,103].

The imbalance between T effector cells (such as Th17 cells and ILCs) and Treg cells that occurs in CD leads to 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.

II.3. Antibodies

The present study focuses on the humoral immune response. In particular, it was investigated the profile of bacteria coating by IgA and IgG subclasses on faecal samples of CD patients. The following section will describe the mechanisms related to antibodies production as well their characteristics.

The humoral immune response represents immunologic responses that are mediated by antibody molecules produced by plasma cells. Antibodies contribute to immunity by binding to pathogens neutralizing them and facilitating their uptake by phagocytic cells that are specialized to destroy ingested bacteria through opsonisation or by the complement system[104]. The link between the adaptive and the innate immune systems is accomplished by the antibodies which combine antigen-binding sites and binding sites for different innate receptors and adaptor molecules[105]. The antibodies possess two functional domains where antigen-binding fragment (Fab) confers antigen specificity whereas the crystallizable fragment (Fc) drives the antibody function[106]. In humans are known five classes of antibodies, namely immunoglobulin A; D; E; G; and M, which differ in heavy-chain structure.

II.3.1. *Immunoglobulin A*

Immunoglobulin A (IgA) is the most abundant antibody in humans, constituting more than 80% of all the antibodies secreted by plasma cells. 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[107]. IgA exists in two forms, namely serum IgA and secretory IgA. There are two subclasses of IgA with IgA1 having a longer hinge region than IgA2 resulting in higher sensitivity to bacterial proteases (Figure 4[108]). On the other hand, IgA2 presents an increased protection against protease digestion which may explain why it is the predominant subclass in the mucosal secretions. In contrast, IgA1 accounts for more than 90% of serum IgA[109]. IgA1 exists primarily as a monomeric molecule in the serum, whereas, IgA2 is a polymer when secreted[110].

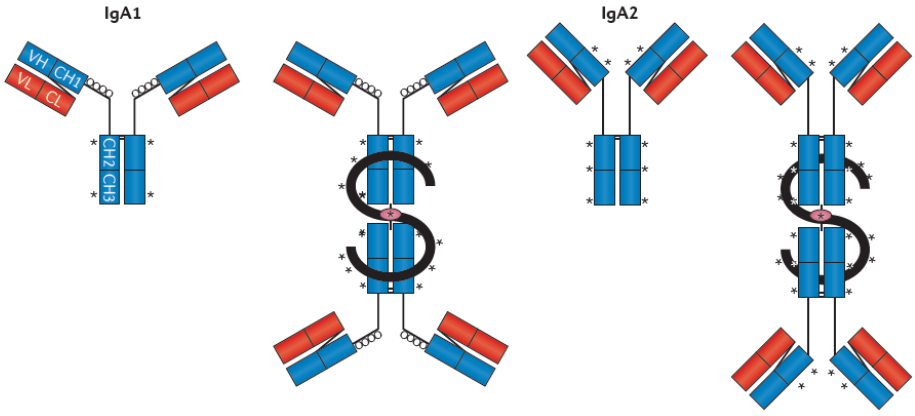

Figure 4. Isotypes of immunoglobulin A (reproduced from Lu et al., 2017).
IgA1 is the predominant IgA in serum whereas IgA2 is the most secreted in mucosal membranes. IgA1 has a longer hinge that turns it more susceptible to proteolytic cleavage by pathogens. By contrast, IgA2 presents higher resistance to proteolytic lysis.

IgA synthesis is accomplished with class-switch recombination (CSR[1]) which requires the enzyme activation-induced cytidine deaminase (AID). The main places involved in the generation of IgA-secreting plasma cells are the gut-draining mesenteric lymph nodes (MLNs), PPs and ILFs-part of the intestinal lamina and GALT[111].

The IgA induction in PPs and MLN'S can be T-cell dependent or T-cell independent whereas in ILFs and diffuse lamina propria appear to occur in the absence of segregated T cell zones, thus being T-cell independent.

The presence of pathogens is recognised by the intestinal immune system that produces high-affinity, T cell dependent, pathogen-specific IgA. Several studies suggested that IgA induced by pathogens yield high-affinity and is termed "classical" IgA. By contrast, commensal-induced IgA is believed to be derived from T-independent responses which generates IgA of relatively low-affinity, referred as "innate", "natural" or "primitive" IgA[111]. "Natural" IgA secretion is thought to represent an innate-like mechanism that is evolutionarily ancient. Although natural IgA is considered to be polyreactive[112] - being able of naturally bind and neutralize multiple targets with

[1] CSR-Process that causes a switch in the class of antibodies that are displayed in the cell surface of a B cell. Thus, an IgG-secreting plasma cell can be converted in a IgA-secreting plasma cell.

low affinity - it is observed that it binds to a broad, but defined, subset of microbiota. This finding suggests that evolutionary pressure may have led same bacteria to express a specific cell surface that attracts or repeal IgA[107].

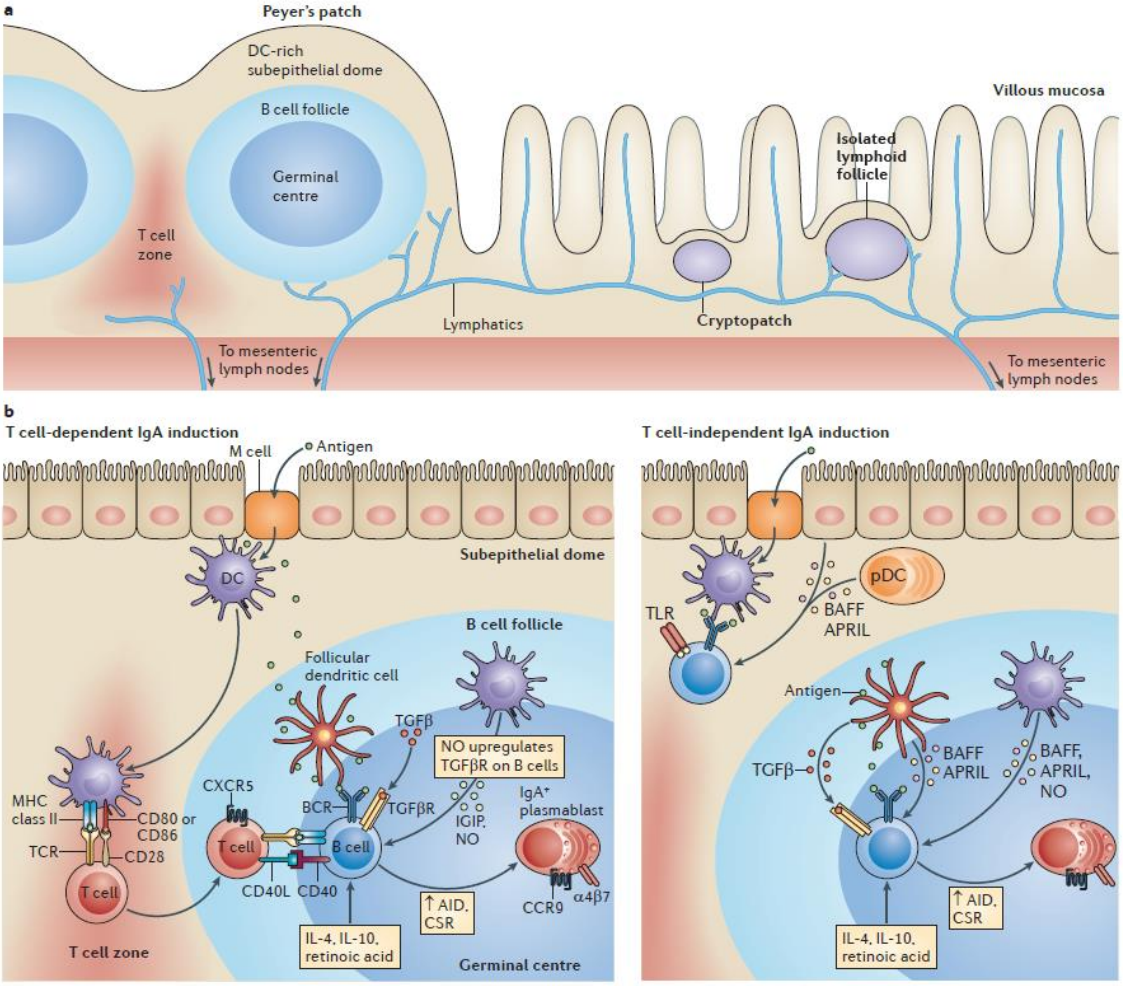

Figure 5. Induction of Immunoglobulin A (reproduced from Pabst, 2012).

a. IgA induction occurs in Peyer's patches (PPs), the cryptopatch–isolated lymphoid follicle continuum, the villous lamina propria and the gut-draining mesenteric lymph nodes (not shown).

b. The induction of IgA in PPs can be T cell-dependent (left) and T cell-independent (right). Antigens are transcytosed by microfold (M) cells and delivered to dendritic cells (DCs). In T cell-dependent IgA generation, DCs enter interfollicular T cell zones to activate naive T cells, which differentiate into effector T cells, enter B cell follicles and release IgA-inducing cytokines. B cells are stimulated by effector T cells, which results in the expression of activation-induced cytidine deaminase (AID) in B cells and enabling class-switch recombination (CSR). The presence of nitric oxide (NO) upregulates the expression of the transforming growth factor-β receptor (TGFβR). In T cell-independent IgA induction, the expression of AID is induced through innate mechanisms, including Toll-like receptor (TLR) signalling and the CD40L-related cytokines B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL). Those cytokines are produced by DCs, plasmacytoid DCs (pDCs) and follicular dendritic cells. BCR, B cell receptor; CCR9, CC-chemokine receptor 9; CXCR5, CXC-chemokine receptor 5; IGIP, IgA-inducing protein; IL, interleukin; TCR, T cell receptor.

Peyer's patches are the main site of IgA induction. In the PPs, the T cell-dependent mechanism involves B cell activation and AID expression through B cell receptor (BCR) and CD40 ligand (CD40L). The M cells, located in the epithelial barrier, transport the antigen from the lumen into the sub-epithelial dome. There, DCs take and transport the antigen to the inductive sites of PPs or the MLNs, called T cell zones, where naïve antigen-specific T cells are activated via the interaction with dendritic MHC class II molecules and differentiate into effector T cells that enter

B cell follicles. B cells are activated on the boundary between T cell zone and B cell follicle where are stimulated by T cells that express CD40L and cytokines that induce the expression of AID, enabling the CSR. In the germinal centre, which is separated in a light and a dark zone, the B cell will undergo somatic hypermutation in the dark zone and isotype switching in the light zone[111]. The somatic hypermutation is a mechanism that involves AID and introduces point mutations in the variable region of immunoglobulin genes. This mechanism leads to an elevated amount of B cells with differences in BCR affinity for the specific antigen. In the light zone are present the follicular dendritic cells (FDC) that present intact antigen. In case B cells possess a low BCR affinity, the cells will suffer apoptosis once they are not able to bind efficiently to the antigen. On the other hand, B cells with high affinity to BCR will acquire the antigen from FDC, process it and present the antigen peptide on MHC class II molecules[113]. Lastly, the activated lymphocytes will enter the bloodstream, but will return to the lamina propria as mature IgA producing plasma cells due to the expression of gut-specific homing receptors.

T-independent mechanism

The T cell-independent IgA induction is performed by mechanisms that include TLR signalling, the B cell-activating factor (BAFF) and the proliferation-inducing ligand (APRIL). The CD40L-related cytokines, BAFF and APRIL are able to trigger AID expression and consequently CSR. These cytokines are produced by DCs and FDCs. The T-independent mechanism appears to be related to the production of low affinity antibodies once the affinity maturation cannot exist without T cells. Nevertheless, the T cell-independent mechanism is very important for IgA production once this antibody is needed for the protection of epithelium against a broadly of microorganism and not to specific species that require high affinity[111].

Isolated lymphoid follicles were also associated with the induction of IgA due to the ability to express AID in germinal centres. Although ILCs contain B cell population with BCR repertoires, they do not have T cell zones which only enable T cell-independent mechanism instead. The ILFs contribution for IgA induction, in healthy individuals, is not yet known but owing that lymphoid tissue inducer cells (LTi, part of the ILCs) present T cell-like effector functions it is thought that they may compensate for the lack of T cells and thus promote the induction of IgA[111].

The studies related to IgA induction in lamina propria are contradictory. Some studies reported that AID is expressed in cells of the lamina propria[114,115] whereas others mentioned that AID expression only occurs in PP's and ILFs[116,117]. Despite this, the observation of cells in lamina propria that induce TGF-β, BAFF and APRIL may be an evidence of the importance of this site for the IgA induction.

MLN's were also associated to IgA induction once it receives lymph from the intestinal lamina propria, but also from Peyer's patches and ILFs. It was suggested that B cells undergoing CSR in PPs and ILFs can enter the MLNs to further differentiate before their release into the circulation[111].

The induction of IgA was associated to TGF-β and the cytokines IL-4, IL-6, IL-10, and IL-21. IL-6 is known to enhance the production of all the Ig thus it also enhances the IgA class switching. In addition, the presence of both retinoic acid and IL-6 can induce IgA secretion[111].

IL-21 induces naive B cells to IgA class switching where IgA1 accounts for most of this response.[118] While IL-4 concomitantly attenuates the IL-21-induced IgA, the IL-10 increases the secretion of Il-21 induce IgA[118]. It was also reported that the combination of IL-10 and TGF-β is need for switching to IgA1 and IgA2[119].

The release of IL-6, IL-10, TGF-β, retinoic acid and nitric oxide (NO) enhances the IgA production in PPs. The stimulation of APRIL and BAFF by the presence of NO can enhance the T-cell independent IgA production in lamina propria and enhance the T cell-dependent IgA production in PPs by upregulating TGFβR[120]. The levels of APRIL and BAFF are augmented in the presence of IL-4 and IL-10[118].

As referred above, Treg, Tr1, and Th17 cells have the ability to release TGF-β and IL-10, which promotes intestinal B cell production of IgA. Thus, the production of IgA is reported to be induced by Treg and Th17 responses. On the other hand, IgA-producing B cells and their precursors might, through the release of IL-6, IL-10, and TGF-β, enhance the generation of Treg, Tr1, and Th17[121–124].

II.3.2. *Immunoglobulin G and subclasses*

Immunoglobulin G (IgG) is the most abundant class in blood and can be divided in four subclasses, designated as IgG1, IgG2, IgG3, and IgG4, in order of decreasing abundance (Figure 6[125]). Although the four subclasses present an amino acid sequence homology over 90%, differences in constant region, particularly in their hinges and upper CH2 domains leads to important functional difference between the subclasses[125]. The four subclasses present differences regarding to antigen binding, immune complex formation, complement activation, triggering of effector cells, placental transport and half-life[105]. Different types of antigens lead to different IgG responses that can result to marked skewing toward one of the subclasses. A subclass deficiency is usually not detrimental to the individual but could result in an enhanced susceptibly toward specific pathogens. The subclasses 1 and 3 are related to the activation of effector mechanisms, whereas subclasses 2 and 4 may induce responses only in certain conditions[105]. Immunoglobulin G 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[126].

The mechanisms by which IgG induction occurs is similar to the T-cell dependent presented for IgA. The dependent pathway is thought to be the dominant in the IgG induction once this antibody is produced against a specific target. In spite of this, is also possible that some IgG could be produced in PPs by the T cell-independent pathway.

The following sections will describe some of the cytokines related to the regulation of each of the four IgG subclasses. However, it is important to enhance that most of the studies regarding this theme report to 1990 or earlier thus further investigation should be made to reveal the exact

mechanisms that affect the production of these subclasses. Due to the lack of research in this area the assumptions made are based in few studies.

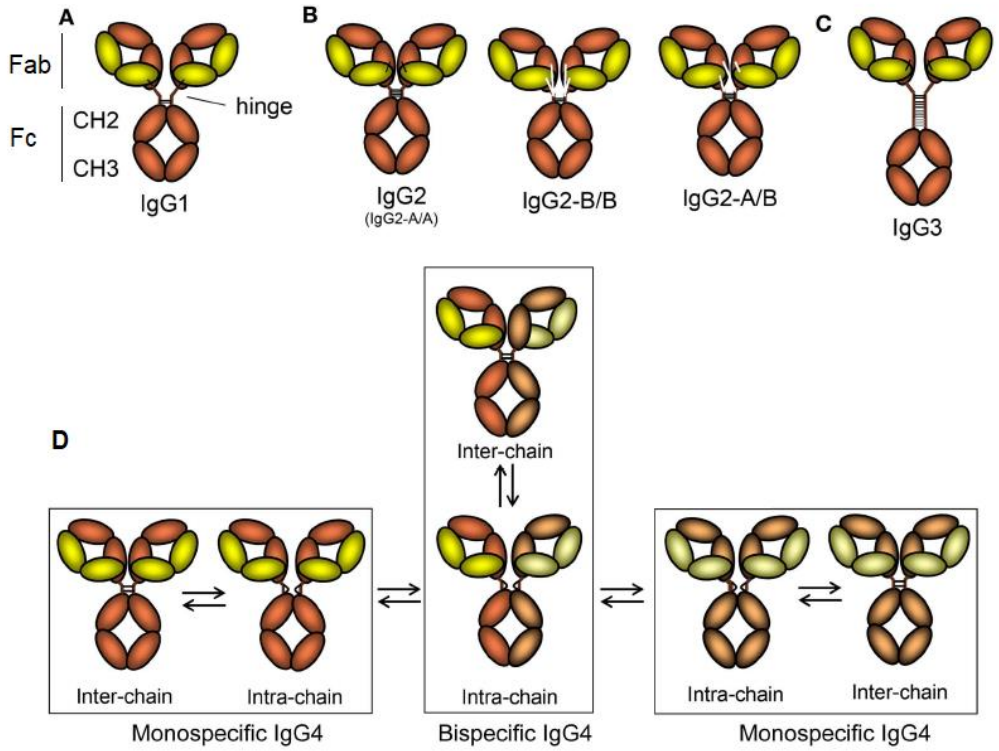

Figure 6. The schematic layout of the IgG subclasses and respective isotypes (reproduced from Vidarsson et al., 2014).

A.*IgG1 only presents one form where the two heavy chains are linked by two disulfide bridges. The fragment antigen binding (Fab) region is responsible to binding interactions with antigens, and the fragment crystallizable region (Fc) mediates indirect effector.* ***B.*** *The classical A/A isoform of IgG2 has four different disulfide bridges between the two heavy chains is followed by the B/B form, with only two disulfide bridges and alternative linkages of the light chain to the heavy chain form. Lastly, the intermediate form A/B is shown.* ***C****. Immunoglobulin G3 only exists in one form.* ***D****. Isomers of IgG4 resulting in half-molecule exchange. On the far left and far right are depicted two IgG4 which are connected with two inter-chain disulfide bridges. Those clones are in equilibrium and can either revert back to covalently linked form or swap heavy chains in a stochastic process with that of neighboring IgG4 molecule creating an asymmetric bispecific IgG4 (bottom middle) that is also influx, reverting into covalently linked IgG4 (top, middle). This process results in IgG4 monovalent-bispecific molecules.*

The IgG1 Subclass

IgG1 subclass is the most predominant serum subclass displaying important effector functions, representing 60-65% of total IgG (Figure 6A)[127]. As a result, deficiencies in IgG1 (usually associated with recurrent infections) leads to decreased levels of total IgG[128]. IgG1 responses are often, but not always, formed together with IgG3 and sometimes IgG4. IgG1 is correlated to the activation of the classical pathway and to the binding to all the Fc receptor in different effector cells[129]. The binding to Fc receptors includes antibody-dependent cell-mediated cytotoxicity (ADCC), which is a mechanism through which natural killer cells (NK) bind to the antibody resulting in the release of cytokines such as IFN-γ[106].

Although the accurate mechanism of IgG1 production is still unknown, several cytokines were suggested to play a role namely IL-4, IL-10, IL-21, IFN-γ, IL-6 and IL-27. In the presence of IFN-γ, IgG1 production is suppressed whereas in the presence of IL-6 the opposite occurs[130].

Studies reported that IL-4 is involved in the induction of IgG1, IgG2, IgG3 and little IgG4 whereas others studies observed that IL-4 alone has a small capacity to direct the isotype switching towards IgG1 production[119]. IL-21 jointly with IL-4 was found to enhanced IgG1 production. However, IL-21 is known to enhance the proliferation of activated B cells which may increase the amount of total antibody instead of only the amount of IgG1[131].

IL-10 was also associated with IgG1, being reported that influences isotype switching towards this subclass. Furthermore, IL-4 and IL-10 together appear to have a synergetic effect, enhancing IgG1 production[119]. In addition, IL-27 was found to induce IgG1 production although to less extent than the other cytokines[132].

The cytokine that is mentioned in more articles is IL-4 which may suggest that IgG1 is associated with a Th2 response once this cytokine is a Th2-related cytokine. A Th2 response is mainly related to a parasite infection[133] which is in line with the fact that IgG1 is associated to parasitic antigens[134].

The IgG2 Subclass

IgG2 accounts for 22 % of total IgG and is associated to antibody responses regarding bacterial capsular polysaccharide antigens, being almost the only subclass involved on it[135,136]. Similarly to IgG1, several studies related IgG2 deficiency to an increased susceptibility to bacterial infections. The IgG2 subclass can exist in two isoforms namely, IgG2-A (the most common) and IgG2-B that differ in the hinge configuration and disulphide bond (Figure 6B).

Low amounts of IgG2 are often associated to deficiency's in IgG4 and IgA1 and IgA2[137]. In contrast to IgG1, IgG2 has low capacity to induce effector functions mainly because the only receptor of fragment crystallizable of IgG (Fcγ receptor) that binds to IgG2 is the FcγRIIa[125]. The FcyRIIa receptor is expressed on phagocytes and NK cells and mediates opsonisation[138].

The production of IgG2 is enhanced in the presence of IFN-γ and IL-6. Although the presence of IFN-γ stimulates the production of IgG2, it is not required for the generation of IgG2 responses[130]. The cytokines IL-18 and IL-12 that, jointly, stimulate the IFN-γ, were also found to enhanced IgG2 production[139]. IFN-γ is a Th1 cytokine thus, it is hypothesized that IgG2 inductive mechanism is due to a Th1 response. As previously mentioned, IL-4 was also associated to IgG2 induction, although it is unclear if this cytokine alone is sufficient to induce IgG2[119].

A Th1 response occurs, usually, against intracellular pathogens such as bacteria. Studies reported that IgG2 deficiency leads to absence of IgG carbohydrate antibodies[140] that are associated to antigens from polysaccharides produced by bacteria thus supporting the idea that IgG2 is Th1-associated.

The IgG3 Subclass

IgG3 is the subclass with the strongest capacity to mediate effector functions, thus it is considered the most important pro-inflammatory IgG subclass[106]. IgG3 subclass is the first to appear in case of viral infections and is able to promote ADCC, opsonisation and strongly activate the classical pathway of complement system[141]. This subclass has the capacity to bind to all the

Fcγ receptors which may be associated to the much longer hinge region that possesses in comparison to the other subclasses. The relative long hinge makes the C1q binding site more accessible which leads to a more efficient complement activation[125]. Nevertheless, IgG3 has a shorter half-life than the other subclasses, possibly due to the long hinge (Figure 6C) that makes IgG3 more susceptible to proteolysis. This might be a result of a mechanism that limits the potential excessive inflammatory response[106].

The production of IgG3 was reported to be linked to the presence of cytokines IL-21 and IL-10. IL-21 was found to be a switch factor for both IgG1 and IgG3[131], however, it appear that predominantly activates IgG3 production[118]. As previously mentioned, IL-21 is associated to a Th17 response whereas it could be hypothesised that IgG3 is associated to this response. In contrast, it was found that IgG3 plays a role in the defence against *Chlamydia trachomatis*, which is an intracellular bacterium. In general, a Th17 response is related to responses against extracellular bacteria and fungi[136]. IL-10, an anti-inflammatory cytokine, was also reported to favour the isotype switching towards IgG3 production[122,142,143] even though IgG3 is known to perform pro-inflammatory effector functions.

The IgG4 Subclass

IgG4 antibodies are thought to be induced after exposure to antigens in a non-infectious setting or by allergens, in addition to IgG1 and IgE. In patients that suffer from allergies and underwent immunotherapy, IgG4 induction was correlated to a relief of symptoms. As result, it was suggested that IgG4 induction is a result of repeat or long-term exposure to antigens in a non-infectious setting[105].

IgG4 has the ability to exist as half-molecules that randomly recombine with other IgG4 molecules resulting in monovalent bispecific antibodies (Figure 6D). Although the monovalent bispecific allows IgG4 molecule to bind two different antigens it also reduces the binding capacity once identical antigen can only bind to one of the Fab arms[125]. Regarding to the effector functions, IgG4 is poor activator of the classical complement system due to the reduced or absent ability to bind to C1q. Furthermore, comparing to the other subclasses, IgG4 binding to most of the Fc receptor is low or inexistent. The FcγRIIb receptor is the exception once IgG4 binds strongly to this receptor that is inhibitory instead of activator which may contribute to the low pro-inflammatory capacity of IgG4[144]. Overall, IgG4 seems to be related to an anti-inflammatory response.

The production of IgG4 was associated to the presence of Treg cells and cytokines IL-10, TGF-β, IL-4 and IL-13. Furthermore, it was reported that IL-4 and IL-13, Th2 cytokines, and IL-10, a Treg cytokine, can be involved in the IgG4 and IgE class switch[145].

Several studied reported that IL-10 enhances IgG4 production by potentiating IL-4-induced IgG4 switching since IL-10 alone is not an IgG4-inducer. IL-4 is an inducer of IgE switching thus, the co-current presence of IL-10 and IL-4 suppress the IgE production[146]. The relation between IgE and IgG4 is supported by studies that reported that in patients with allergies, allergen-specific

IgE is switch to allergen-specific IgG4 due to immunotherapy. Thus, IgG4 is suggested to contribute to a regulatory and tolerogenic[2] response[147,148].

II.3.3. *Transepithelial Transport of IgA and IgG*

Secretory IgA is transported across the epithelial cell layer into the intestinal lumen through an active unidirectional process that is mediated by the polymeric Ig receptor (pIgR). The pIgR is an antibody transporter that is expressed on the basolateral surface of epithelial cells[149]. Polymeric IgA, IgA secreted by intestinal B cells, binds to pIgR on the epithelial cell. It is then translocated to the surface of epithelial cells, leading to the generation of secretory IgA (sIgA) complexes. These complexes comprise a secretory component derived from the association of the extracellular portion of pIgR with the polymeric IgA and that confers mucolytic properties to sIgA[109,120]. The secretory IgA plays different roles regarding the protection against microbial agents. sIgA promotes the exclusion of microorganisms and their products by entrapping them in the mucus; downmodulates the expression of pro-inflammatory bacterial epitopes on commensal bacteria; and, in general, promotes the establishment of host-microbe relationships[150]. In additions, sIgA prevents the access of microbial components involved in epithelial attachments, mediates neutralization of pathogens and facilitates antigen sampling needed to produce antigen-specific IgA[150].

Likewise, it was found that the transepithelial transport of IgG is also mediated by a receptor-mediated mechanism, the human neonatal Fc receptor (FcRn). The FcRn is present in human adults and is expressed by enterocytes and antigen-presenting cells, being mostly distributed in the distal intestine[151]. The main difference between pIgR and FcRn is that FcRn-mediated IgG transport exhibits bidirectionality thus, the antibody can be transported from the lumen to the lamina propria and *vice versa*[152].

The FcRn binds to the four IgG subclasses with comparable affinity so it is expected that the transport is similar in all the subclasses[153]. Nevertheless, it was found that FcRn-mediated transport and rescue of IgG3 is inhibited in the presence of IgG1 due to intracellular competition between IgG1 and IgG3[141]. This finding may be related to the IgG3 shorter half-life once FcRn is known to protect the antibodies against degradation and thus, a less effective binding can lead to a lower protection.

II.4. Aim of studies

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[154]. To prevent complications and disease progression is crucial to monitor mucosal inflammation. Furthermore, the treatment is merely symptom-based and performed to ensure disease remission but not the cure. Therefore, CD patients have a

[2] Immunological tolerance

significant decrease of quality of life and the disease accounts for substantial costs to the health care system, especially during exacerbations that could imply surgery. Consequently, new non-invasive markers that can follow the progression of disease for patients with CD are needed.

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. Based on that, Tedjo *et al.,* found out that faecal microbiota can be used to distinguish adult CD patients based on disease activity[154]. Nevertheless, the method is not able to identify specific taxa that are involved in disease development and progression. Conversely, Palm *et al.* discovered that IgA coating identifies inflammatory commensals that can drive or exacerbate intestinal inflammation in a mousse model of IBD[155].

In the present study, it was thus aimed to investigate:

- The humoral response against intestinal bacteria by profiling the bacteria-coating by the IgA and IgG subclasses in faecal samples of CD patients;
- The possibility of using the profile to differentiate from healthy individuals and CD patients;
- The possibility to differentiate patients in remission and active disease based on the profile;
- The associations between used disease markers and the profile of antibodies;
- The associations between the patient's characteristics-namely, disease phenotype and location; age at diagnosis; gender; and smoking history- with the profile of bacteria coated by the antibodies.

The overall principle behind the experimental assay is depicted in Figure 7 but the present work will only be focused in studying the profile of bacteria coated by the different antibodies mentioned above. The profile of bacteria coated by IgA and IgG subclasses was conducted using a flow cytometry assay. The assay was based on the principle that IgA- and IgG-coated bacteria, bind to the specific anti-antibody that is labelled by a fluorophoro (Figure 7). A fluorescent label was used to each antibody, being five in total, one four each antibody (IgA, IgG1, IgG2, IgG3, IgG4). The detailed description of the experimental procedure is found in the section Material and Methods.

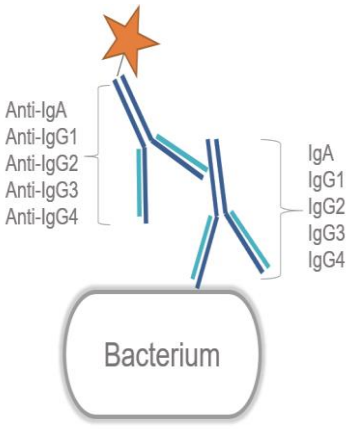

Figure 7. Principle used in the experimental assay.
Faecal IgA- and IgG-coated bacteria are detected using fluorescent labelled anti-IgA and IgG through flow cytometry.

Although there are few studies regarding this subject, it was reported an increased level of IgG-coated bacteria in faecal samples from CD Patients[156,157]. Moreover, studies on the intestinal profile of IgG-producing cells in the mucosa, revealed that patients with CD had an increase of

total IgG compared to healthy individuals[158–160]. Furthermore, the level of IgG2 producing cells was observed to be increased in CD patients compared do healthy individuals[161,162].
Regarding the production of IgA, some studies reported that Crohn's disease patients secrete decreased amounts of total IgA[126,158] while others mentioned an increased level of IgA in serum of CD Patients[163].

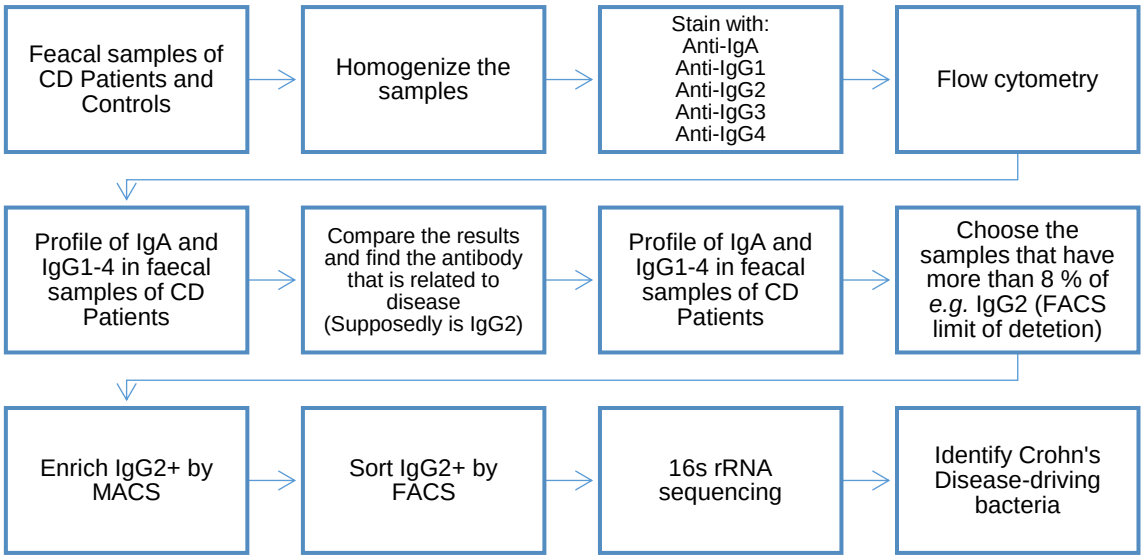

Figure 8. Overall procedure to identify Crohn's Disease-driving bacteria.

Since CD is proposed to be related to a Th1 and Th17 response, it can be hypothesised that CD patients will have increased levels of IgA, IgG2 and IgG3-coated bacteria. The findings concerning IgG2, mentioned above, appear to support this hypothesis.

In the following section, will be described the materials and methods used in the present work.

III. Materials and Methods

III.1. Materials

III.1.1. *Samples*

Faecal samples from 54 CD patients, and 20 healthy individuals were obtained from collaborators at Maastricht University (The Netherlands). The control subjects and CD patients were age and gender matched. The samples represented two-time points from each patient, therefore 107 faecal samples from the 54 CD patients were analysed, since one of the patients only had a sample regarding one-time point. 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.

Of the 54 patients, 26 used to smoke; 19 have never smoked and 9 were currently smokers (at the time of sampling). Patients age was in the range of 17-68 years old, whereas 42 years old was the mean.

Furthermore, faecal samples used in a CD-UC cohort were also analysed. The samples were of 8 CD patients obtained, once more, from collaborators at Maastricht University. These samples were analysed together with the ones from patients that presented active disease in the second visit (T2).

The percentage of patients diagnosed before 16 years old (A1), between 17 and 40 (A2, including) and after 40 years old (A3), was 3.23 %; 74.19 % and 22.58 %, respectively (for n=62 patients). The disease was located on the ileum (L1); colon (L2); ileum and colon (L3); ileum and upper GI (L1 & L4); and ileum, colon and upper GI (L3 & L4) in 19; 18; 22; 1 and 1 patients, respectively.

Regarding to disease phenotype: 65.57 % of patients had non-stricturing and non-penetrating; 21.31% stricturing and 13.11 % had penetrating (for n=61 patients). Of the 62 patients, 49 never undergone surgery; 4 removed part of ileocaecal (removing the terminal ileum and the caecum); 3 undergone hemicoloectomy (removal of a segment of the colon), 1 had surgery on the rectum and sigmoid (the S-shaped part of the large intestine just before the rectum); 1 had surgery on the sigmoid; 3 on colon and 1 had an unknown type of surgery.

The mean time between the first and second sample collection was 19 weeks. The maximum time was of fifty-nine weeks and the minimum was of four weeks.

In the study, samples of 55 women and 27 men were analysed; in the patients group 42 were women and 20 were men.

In total, 135 samples were analysed. Information of all patients and healthy control individuals can be found in Appendix B. Table 1 presents a summary of patient's characteristics that were studied in this work. Note that all the patients were under the effects of medication in the time of sampling or at least 3 months prior.

Table 1. Characteristics of the patients used in the study.

Number of CD Patients (%)			
Single sample	9		
2 Samples	53		
Disease state at second visit (n=53)			
Remission	34 (64.15 %)		
Active	19 (35.85 %)		
		Remission Patients	***Active Patients***
Gender (n=62)		**n=35**	**n=27**
Female	42 (67.72 %)	25 (71.43 %)	17 (62.96 %)
Male	20 (32.26 %)	10 (28.57 %)	10 (37.04 %)
Location (n=61)		**n=34**	**n=26**
L1	19 (31.15 %)	12 (35.29 %)	7 (26.92 %)
L2	18 (29.51 %)	9 (26.47 %)	9 (34.61 %)
L3	22 (36.07 %)	13 (38.23 %)	9 (34.61 %)
L1&L4	1 (1.64 %)	-	1 (3.85 %)
L3&L34	1 (1.64 %)	1 (2.94 %)	-
Phenotype (n=61)		**n=34**	**n=27**
B1	40 (65.57 %)	25 (73.53 %)	15 (55.56 %)
B2	13 (21.31 %)	6 (17.65 %)	7 (25.93 %)
B3	8 (13.11 %)	3 (8.82 %)	5 (18.52 %)
Age at diagnosis (n=62)		**n=34**	**n=27**
A1	2 (3.23 %)	1 (2.94 %)	1 (3.70 %)
A2	45 (74.19 %)	28 (82.35 %)	17 (62.96 %)
A3	14 (22.58 %)	5 (14.71 %)	9 (33.34 %)
Smoking (n=54)		**n=35**	**n=19**
Currently	9 (16.66 %)	7 (20% %)	2 (10.53 %)
Ex	26 (48.15 %)	18 (51.43 %)	8 (42.10 %)
Never	19 (35.18 %)	10 (28.57 %)	9 (47.37 %)

III.1.2. *α-antibodies and Buffer Solutions*

α-IgG antibodies were purchased from Southern Biotech, Birmingham:

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) was purchased from Miltenyi Biotec, Germany. Vio 770 is analogous to Cyanine 7.

Two different staining buffers that were previously prepared and sterile filtrated (0.2 µm Cellulose Acetate Membrane, VWR International) were used. Staining buffer 1 consisted of phosphate-buffered saline (PBS, 20 mM phosphate, pH 7.2, Lonza- Switzerland) with 1% bovine

serum albumin (BSA, Sigma-Aldrich- USA). 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. To staining the samples a DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) solution was prepared, solubilizing 1 mg DAPI in Milli-Q (Merck- USA, 7.052×10^{-4} M).

III.2. Methods

III.2.1. *General Procedure for Sample Preparation for Flow Cytometry*

The following procedure was performed in samples that were kept at -80 ºC and was based on results obtained through a titration and compensation previously performed.

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. For each sample, 3 Eppendorfs with 200 µL supernatant were kept in -80 ºC.

For further preparation one of the Eppendorf tubes were used. The bacterial pellet was washed twice with staining buffer 1 and then resuspended in 200 µL with this buffer as well (centrifugation at 8000 *g* for 5 min at 4°C). For cell counting a 150 times dilution of the sample was prepared in a FACS tube using staining buffer 2. 440 µL sample suspension was incubated 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- USA), in a FACS tube. The rest of the dilution was also analysed to minimize the false negatives. The samples were analysed on a FACSCanto II flow cytometer (BD Biosciences), and the amount of sample needed to obtain 20×10^6 DAPI+ cells for further preparation was calculated using formulas 1 and 2:

1) $$\frac{\text{Events in DAPI}^+\text{region}}{\text{Events in bead region}} \times \frac{\text{Beads/test}}{\text{Test volume}} \times 150 \times \frac{500}{440} = \text{Cell/µL in sample}$$

2) $$\frac{20\times10^6 \text{ cells}}{\text{cell/µL in sample}} = \text{volume to take from 200 µL Eppendorf [µL]}$$

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 of:

	FSC	SSC	PE (IgG4)	APC (IgG2)	FITC (IgG3)	APC-Cy7 (IgG1)	Pe-Cy7 (IgA)	Pacific-Blue
Voltage	475	400	525	615	575	675	500	380

Setting for channels not employed:

	PerCP-Cy5.5	AmCyan
Voltage	350	374

When needed a dilution of the samples was performed to allow the Flow Cytometry analysis.

To make sure that the staining was properly performed a negative control of all samples was performed. 7 µL of each sample (of the same suspension that was transferred to a new 1.5 mL Eppendorf tube) was transferred to FACS tubes and incubated with 50 µL 0.07 mM DAPI and 193 µL staining buffer 2 to a final volume of 250 µL. After incubation at room temperature, for 15 minutes, the samples were analysed on a FACSCanto II with the PMT settings previously mentioned.

III.2.2. *Flow cytometry*

Flow cytometry is a method for analysing multiple physic characteristics of cells and other particles in suspensions including internal complexity; proteins; membranes and size. Specialized cytometers can detect cells between 0.2 -150 microns although most of them only detected 1-15 microns in diameter[164]. Overall, the method is based on moving thousands of cells (per second) through a laser beam and then capturing the light that emerges after each pass.

A flow cytometer is composed by three subsystems: fluidics, optics and electronics. The fluidic subsystem is responsible for transporting the cells from the sample tube to the laser beam. The cells are transported in a fluid stream, called sheath fluid, which allows that only one cell at a time passes through the light sources (lasers)- interrogation point. Upon arrival to the interrogation point, the optic subsystem is responsible for the excitation of the cells (by lasers) as they pass through the flow chamber. Thereafter, the signals of scatter and/or fluorescence light are captured; filtered and converted to a signal through photodetectors. Lastly, the electronic system is used to convert the electrical signals into data[164].

Light scattered in the same path as the laser beam is correlated to the size of the cell in a proportional manner corresponding to light scatter in a forward direction (FSC). Light scattered to the side (SSC) is correlated to the cell granularity (90º angle to the laser beam path).

To allow the detection of cell surface proteins and other components, are used fluorochrome-conjugated molecules. The numbers of fluorochromes that can be used are limited by the numbers of lasers available in the flow cytometer. FACSCanto II, the flow cytometer used in the current study, has three lasers violet (405 nm), blue (488 nm) and red laser (633 nm). As previously mentioned, the lasers are separated to ensure that the cell is only passing one laser

beam at a time. The application of several fluorochromes can lead to a phenomenon called *spillover*[165], described in the following section.

III.2.3. *Compensation*

Spillover is a phenomenon that occurs whenever the fluorescence emission of one fluorochrome is detected in a detector designed to measure signal from another fluorochrome. To eliminate false positive signals caused by *spillover,* a compensation was performed to avoid that.

A mix of faecal matter of different samples was used to perform the compensation.

A drop of OneCompeBeads™ Compensation Beads (eBioscience) was add to FACS tubes (one tube for each fluorophore that was used) containing an amount of a specific antibody conjugate. Anti-IgG1 ($3.1 \cdot 10^{-4}$ µg/µL), anti-IgG2 ($5 \cdot 10^{-3}$ µg/µL), anti-IgG3 ($5 \cdot 10^{-3}$ µg/µL), anti-IgG4 ($2.5 \cdot 10^{-4}$ µg/µL), anti-IgA (1000 cells/ µL) and $6.3 \cdot 10^{-4}$ µg/µL anti-streptavidin staining buffer 1 was added to a final volume of 100 µL. Anti-IgG1 and conjugated-streptavidin were added to the same FACS tube.

After a 30 minutes incubation, at 4ºC, the suspension was washed two times with 1 mL staining buffer 1 (500 *g* for 5 minutes).

The supernatant was decanted and 0.3 mL of staining buffer 1 was added to each tube that was analysed separately on a FACSCanto II.

III.2.4. *Data Analysis*

FlowJo (version 10.2, LLC) was used to perform the gating of flow cytometric data. An example of the gating strategy used can be found in Appendix A., Figures 28 and 29. The gating strategy was based on the comparison of samples stained with only DAPI with the samples stained with the. First the gates are placed on the $DAPI^+$ stained samples, then are copied to the stained samples; the final adjusted gates are then copied to the $DAPI^+$ stained samples. The first gate to be placed is the $DAPI^+$ vs FSC (forward side scatter) to choose only the $DAPI^+$ events and then the Noise gate is placed to minimize the noise that is a false positive signal. Finally, the gates to all the antibodies channels are placed. The gating is sample specific. Data from FlowJo_V10 was exported to Excel where the following calculations were performed.

The values obtained from the normalisation step were used to calculate the amount of bacteria per gram of faeces. Since a ratio of 1000 µL PBS/0.1 g faeces was used for the initial faeces suspension, the number of bacteria per gram faeces was calculated by the formula:

$$3) \quad \text{Bacteria/ µL} \times \frac{1000\ \mu L}{0.1\ g\ faeces} = \text{Bacteria/g faeces}$$

The relative amount of Ig-coated bacteria was given by the gating performed in FlowJo_V10. The quantitative value of Ig-coated bacteria per gram of faeces was calculated as follows:

$$4) \quad \frac{\text{Bacteria/g faeces}}{100\ \%} \times \text{Relative Ig coating [\%]} = \text{Quantitative IgG coating}$$

The relative ratio of total IgG, namely, the percentage that each IgG subclass-coating comprises of the total amount of IgG coated bacteria was also calculated.

III.2.5. *Statistical Analysis*

Statistical analyses and graphic presentation were conducted in **GraphPad Prism 5** and **7**.

The statistical approach used was as follow: A normality test was performed in all the data sets to test whether the data was Gaussian distributed. For comparison of two Gaussian distributed groups of the same variable, a student's t test was performed and for more than two groups a one-way ANOVA with a Tukey post hoc test was made. In the case of data sets that were not Gaussian distributed, a log transformation and a new normality test was performed. For transformed subsets, in which all the groups were Gaussian distributed, the same statistical approach was applied for the comparison of the groups. For data subsets that were still not Gaussian distributed, for two groups comparison, a non-parametric Wilcoxon test (for paired data) and a Mann-Whitney test for unpaired data (using the non-transformed values) were performed. For more than two groups, a nonparametric Kruskal-Wallis test with a Dunn's post hoc test was conducted.

To compare two different variables/factors, a correlation analysis between factors was performed using Pearson Correlation for Gaussian distributed parameters and a Spearman Rank Correlation for non-Gaussian distributed. To use a Pearson Correlation, the two factors have to be Gaussian distributed. In the case that one or two of the factors were not Gaussian distributed a log transformation was performed. If after the log transformation the two factors followed a normal distribution, the Pearson Correlation was performed otherwise a Spearman Rank Correlation was used.

The following section will display the results found for the present study.

IV. Results

IV.1.Profile of IgA- and IgG1-4-Coated bacteria in Control and CD Patients

To investigate the profile of bacteria coated by IgA and IgG subclasses, faecal samples from 54 patients were analysed using the flow cytometry assay. The patients donated samples at two different time points, referred to hereafter as time 1 (T1) and time 2 (T2), which allowed the comparison of different parameters over time. All patients were in remission at the time of the first sampling, whereas in the second sampling, 19 presented active disease and 34 remained in remission. One of the patients only had one sample from the first-time point. Besides those patients, 20 controls and 8 samples of CD patients used in a previous CD-UC cohort were also analysed. Since the 8 CD patients presented active disease, they were analysed together with the samples from patients that displayed an active state in T2.

The samples were provided by Maastricht University that divided patients in active and remission disease based on the following criteria:

I. Remission
   a. Faecal calprotectin (FC) level of < 100 µg/g and a C-reactive protein level (CRP) of < 5 mg/L;
   b. Faecal calprotectin level of < 100 µg/g, a C-reactive protein level of < 10 mg/L, and a Harvey-Bradshaw Index (HBI) score of ≤ 4;

II. Active
   a. Faecal calprotectin level of > 250 µg/g;
   b. Faecal calprotectin level was increased at least five times from the first sample and had reached a level > 100 µg/g.

For the statistical analysis, the samples were divided into four groups. 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. 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 9.

The amount of bacteria/g faeces is slightly higher in the patients with active disease comparing to the ones in remission in T1 (1.97×10^8 bacteria/g faeces higher) and T2 (2.49×10^8 bacteria/g faeces higher). The Control group also presented higher amount of bacteria/g faeces comparing to the remission groups, but no significant differences were observed for the active disease group (Figure 9A). Regarding patient-specific difference between the two sampling times, patients that switch to active disease tend to have an increased amount of bacteria/g faeces comparing to

patients remaining in remission (2.00×10^8 bacteria/g faeces), although the difference is not significant (Figure 9B).

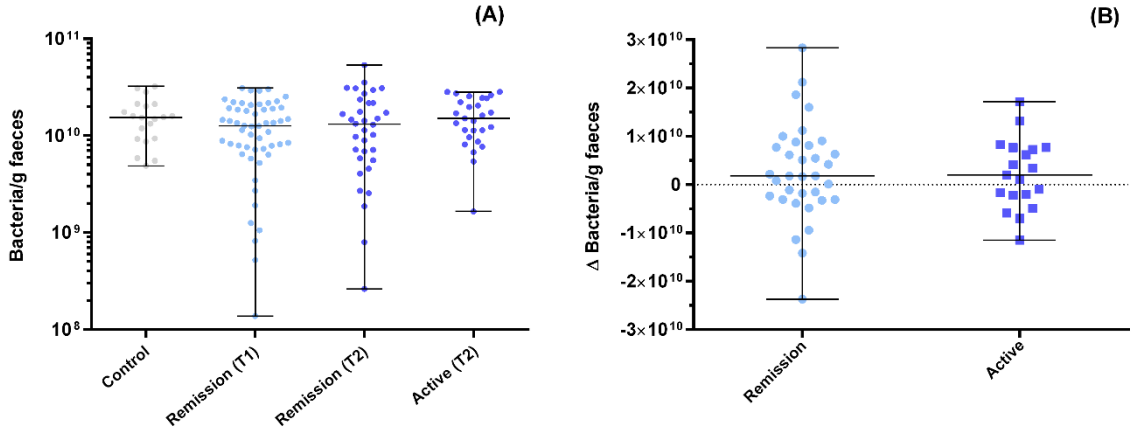

Figure 9. Bacterial density in Control and CD Patients with active disease or in remission.
(A) *The amount of bacteria/g faeces in Control (20 healthy individuals), in CD Patients in remission on the first sampling time (Remission (T1), 54 patients); in CD Patients with disease in a state of remission (Remission (T2), 34 patients) and with active disease (Active (T2), 19 patients), on the second-time point (T2).* ***(B)*** *The patient-specific difference (Δ) between the amount of bacteria/g faeces at the two visits. For each of the 53 patients, the amount of bacteria/g faeces at visit 1 was subtracted from the amount measured at visit 2. Indicated is median ± range. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

The first part of the data analysis was performed to investigate the immunologic host-microbiota interaction related to CD patients and healthy individuals. To reveal the profile of bacteria-coating by IgA, IgG and more specifically the IgG subclasses, a data analysis using the developed flow cytometry assay was performed. The results are depicted in Figures 10 and 11.

The amount of bacteria coated with IgA /g faeces tends to be decreased in Active (T2) group comparing to the Remission (T2) (Figure 10.A). The slightly increase seen for the IgA-coating (2.49×10^{10} bacteria/g faeces, Figure 10.A), between visits, for patients whose disease state switch from remission to active, is found to be significant (Wilcoxon signed-rank test, 0.01<*P-value < 0.05). The patient-specific amount of IgA-coated bacteria/g faeces appears to be decreased in patients whose disease become active comparing to the patients that remained in remission however, the decreases is not statistically significant (Figure 10.B).

The amount of bacteria coated/g faeces with IgG is slightly higher in the active group comparing to the other groups (Figure 10.C). The same is observed in the data relative to patient-specific difference over time; the patients whose disease changed from a remission state to an active one presented an increased amount of IgG-coated bacteria/g faeces comparing to patients where the disease state remained the same (Figure 10.D). The healthy individuals tend to have an increased number of IgG-coated bacteria/g faeces comparing to the patients in remission (Figure 10.C). On the other hand, healthy individuals present a lower level of IgG-coated bacteria/g faeces comparing com patients with active disease.

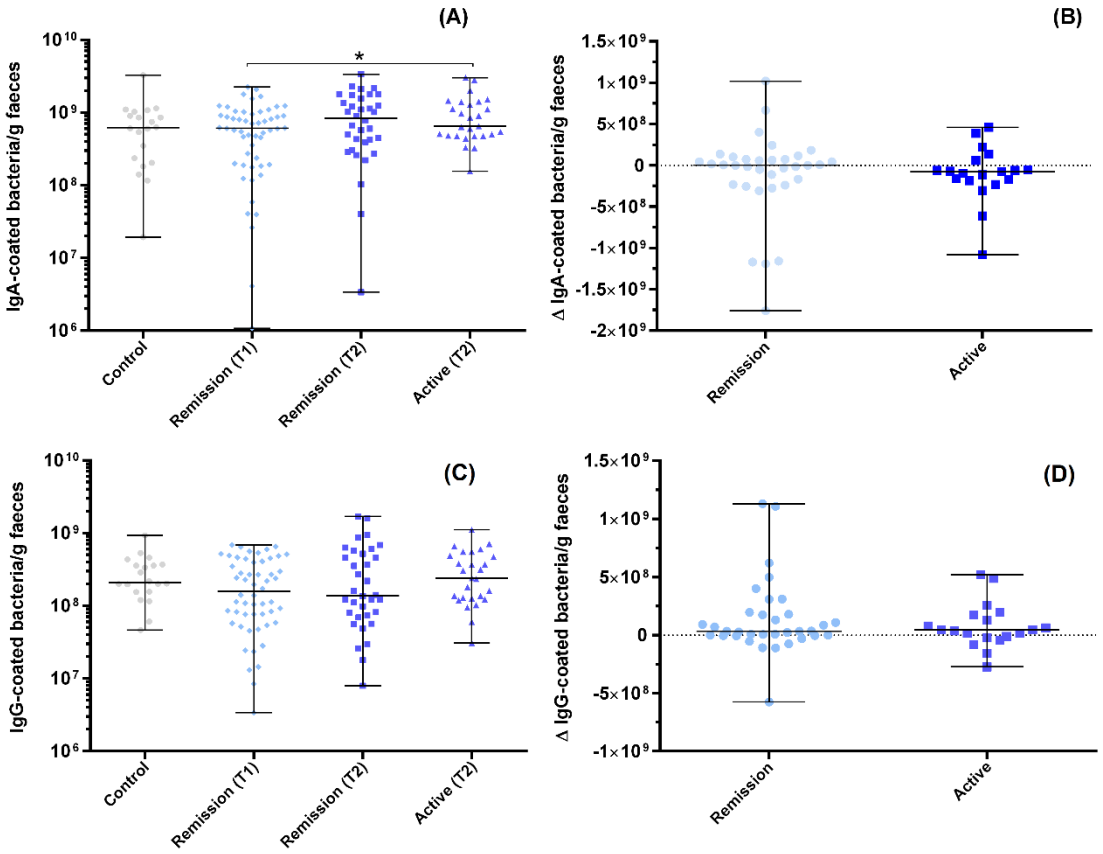

Figure 10. Comparison of the profile of bacteria-coating by IgA and IgG in CD patients with active disease or in remission.

(A) *and* ***(C)*** *the quantitative amount of IgA and IgG 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. For samples in which no bacteria are observed to be coated the data point is set to 1 to enable the presentation of the results on a logarithmic axis. 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 IgA and total IgG coated bacteria/g faeces at visit 1 and visit 2. For each patient, the amount of antibody coated bacteria 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.*

The profiles of Ig coating of the four IgG subclasses are depicted in Figure 11. The levels of bacteria coated by a specific IgG subclass are similar in healthy individuals, patients in remission and active disease in T2. Generally, the amount of bacteria coated is lower in the Remission (T1) group comparing to the other groups (Figure 11.A). Analysing the median values of the bacteria coated with each IgG subclasses, IgG2, and IgG3 tend to be higher in the patients whose disease remained in remission at T2 and IgA follows the same tendency (Figure 11.A). On the other hand, IgG1, and IgG4 levels tend to be higher in the patients with active disease comparing with patients in remission (Figure 11.A).

When comparing the patient-specific differences for all the IgG subclasses, no significant differences are observed between patients that enter a state of active disease and patients whose disease remained in remission for IgG1, IgG3 and IgG4. On the other hand, a significant difference was found for IgG2-coated bacteria/g (Mann-Whitney test. 0.01<*P-value < 0.05) where patients that remained in remission present a higher amount than patients whose disease evolved for an active state (Figure 11.B).

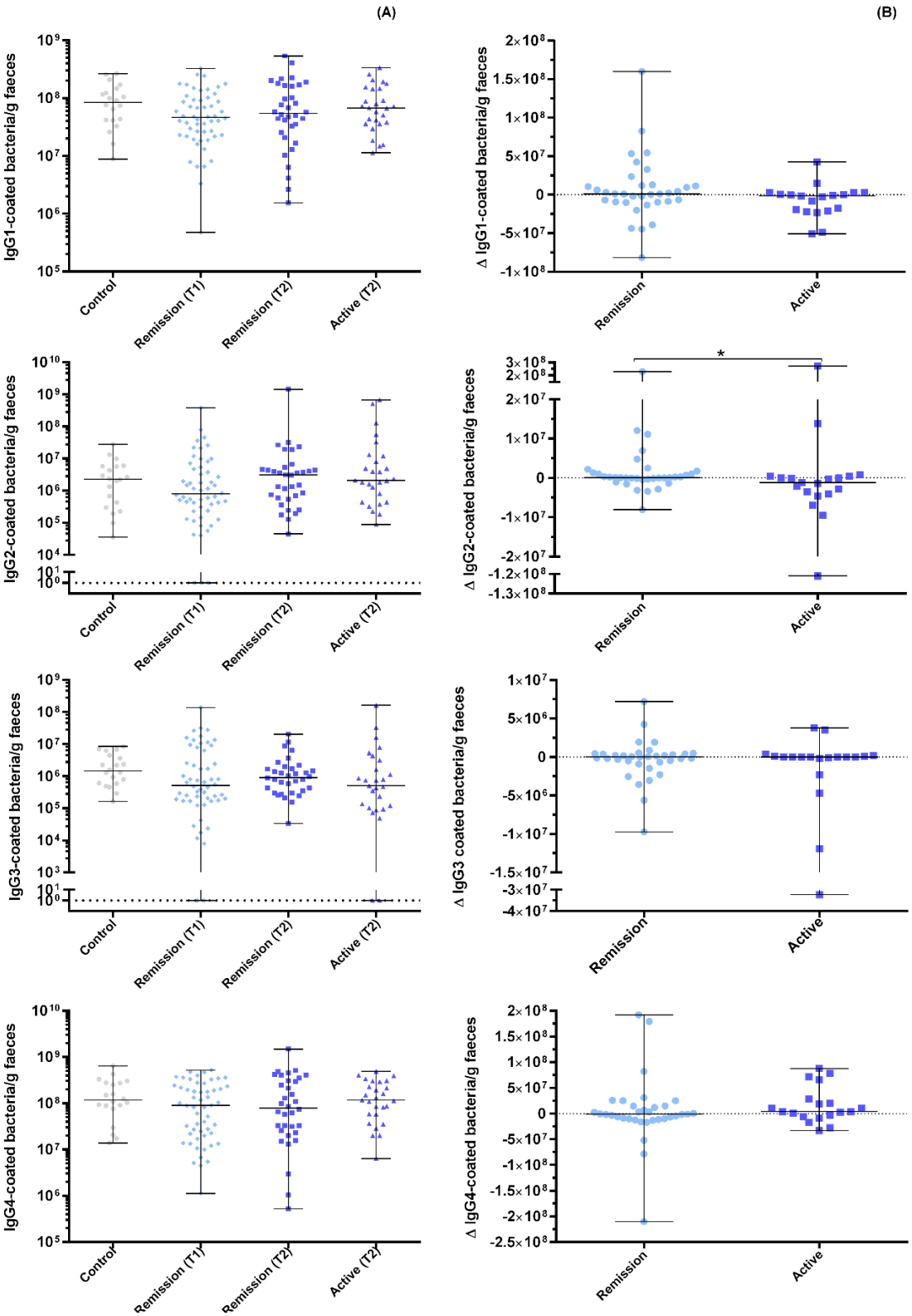

Figure 11. Comparison of the profile of bacteria-coating by IgG subclasses in CD patients with active disease or in remission.

(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.*

To enlighten if the IgG1-4 coated bacteria is patient-specific, the amount of bacteria coated by the four IgG subclasses in CD patients and controls, was analysed (Figure 12). The amount of IgA is represented in different plots due to the number of IgA-coated bacteria/g faeces being much higher comparing to the amount of IgG1-4 coated bacteria/g faeces (Figure 13). The profile of bacteria coating in each group is available in Appendix B, Figure 30.

It is observed that the profile of bacteria-coating varies a lot from one patient to another even when comparing samples within the same group which suggests that the production of IgG tend to be higher in some patients than in others. It is observed in all the groups that the most coated antibody is IgA followed by IgG4, IgG1, IgG2, and IgG3 (decreased order, Figure 12).

The IgGx-coating profile of healthy individuals is similar to the one observed for patients in remission at the first visit (Figure 12.A and 12.B). In the second visit, it seems to exists an increase of IgG2 and a decrease of IgG4, comparing to the first visit, for patients whose disease changed to an active state (Figure 12.B, 12.C).

Although it was expected that the IgGX profile remained similar, patients that remained in remission at T2 have a profile relatively different between visits (Figure 12.B, 12.D).

In the Remission T1 group is visible that the patients that will remained in remission in the second visit have a lower amount of IgG2 comparing to the patients whose disease will change to active (Figure 12.B). Even though the profile changes a lot between patients, a decrease of IgG4 and an increase of IgG1 appear to happen between visits for most of the patients.

Based on Figure 13, it is visible that the profile of IgA-coating also varies between patients, being patient-specific. All the groups show a similar distribution of the amount of IgA-coated bacteria. No significant differences were observed between T1 and T2 samples.

As seen in Figure 12 and Figure 13, some of the patients appear to have 0 % of one antibody coating. For some patients that is true but most of the patients have such a low amount that is not possible to distinguish it in the figure. The samples that presented 0 % of a particular antibody coating were not related to a patient's deficiency to produce that antibody, once it only appears in one time point for each patient (for example, M0270 does not present IgG3-coating in visit 2 but had it in visit 1). Furthermore, it is also observed that some patients have an amount of Ig-coating much higher than the median (outliers). Patients that represent outliers were analysed concerning their age at diagnosis, disease location and phenotype and smoking history but common characteristic to all of them were not found. Patient M0185 stood out because in visit 2 presented an elevated amount of IgG4- and a low amount of IgG2-coated bacteria which may be an indication of an inverse correlation between these two antibodies. In Appendix C, Table 2, is shown a table that summarizes the patients that lack the coating of a specific antibody and also the patient's characteristics of all the outliers.

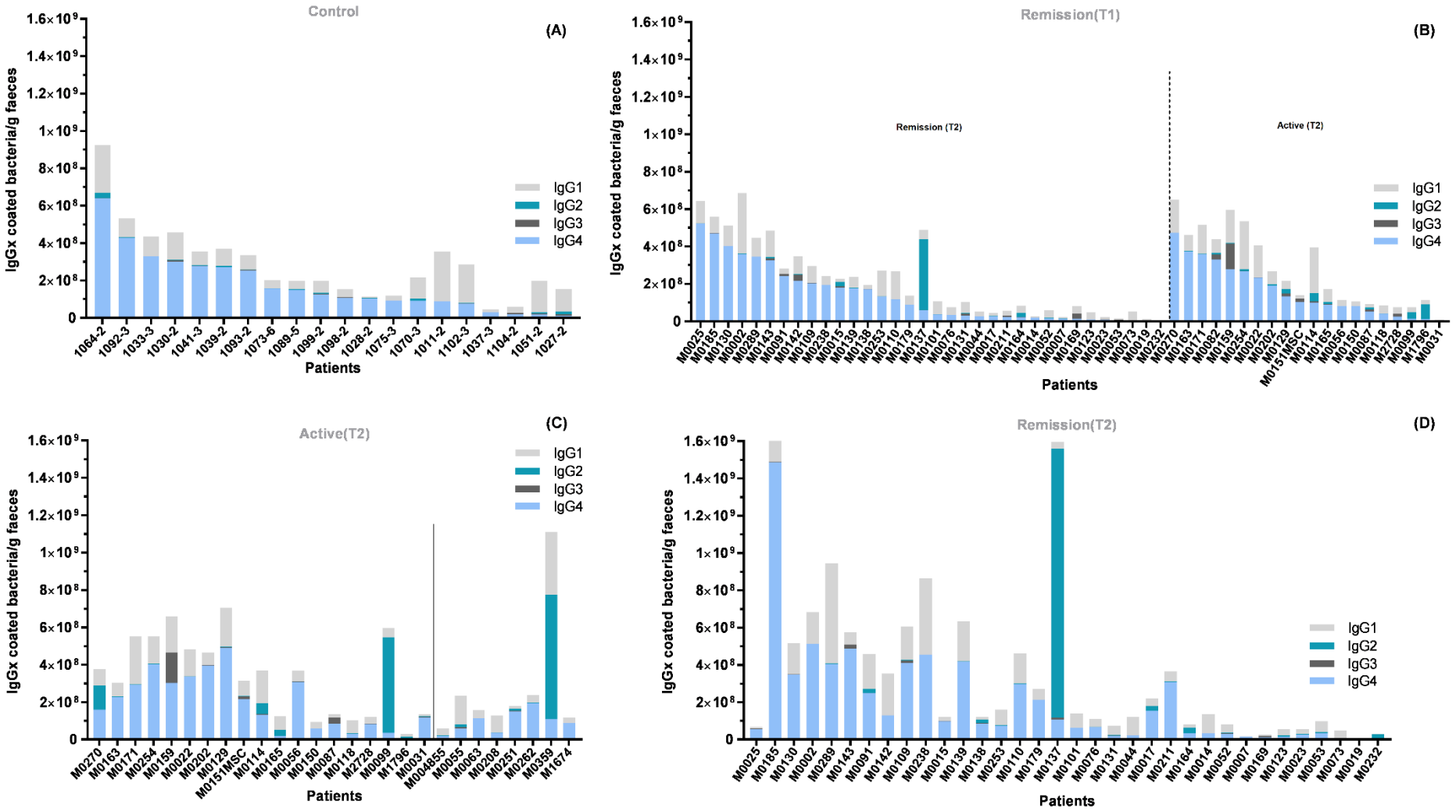

Figure 12. Comparison of the profile of bacteria-coating by IgG subclasses in CD patients in the different groups.

*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.*

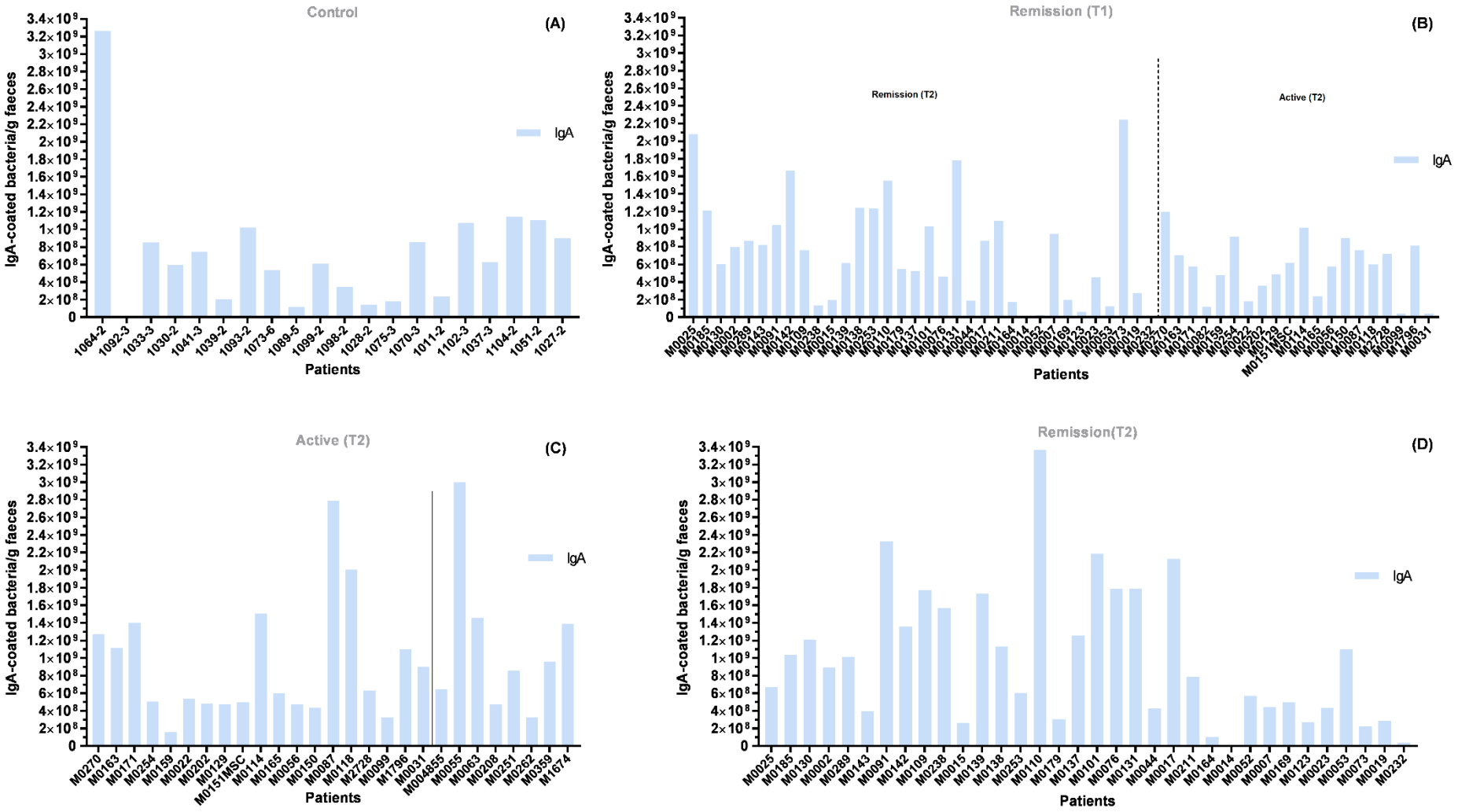

Figure 13. Comparison of the profile of bacteria-coating by IgA in CD patients in the different groups.

*The quantitative amount of IgA-coated bacteria/g faeces in samples from **(A):** healthy individuals; **(B)** 54 CD patients at visit 1 (Remission (T1)), where all patients were 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 at the second visit (Remission (T2)). (- - -) divides the patients that remained in remission at the second visit from the patients that presented active disease. (——) divides the patients that only had one sample from the others. The patients appear in a descending order of IgG4, according to Figure 6, 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.*

To investigate how an antibody-specific coating is correlated with the others, a Spearman rank test or a Pearson correlation test was performed between the quantitative number of bacteria coated by IgA and the four IgG subclasses (Figure 14). A significantly positive correlation is found between IgA- and IgG1-, and IgG1- and IgG4- coated bacteria in patients that were initially in remission and in patients that remained in remission in T2. Hence, it is expected that more bacteria coated with IgA leads to more bacteria coated with IgG1 that consequently, is correlated with a higher level of IgG4-coated bacteria. Furthermore, a significantly positive correlation occurs in Remission (T1) between IgG3- and IgG4-coated bacteria and between IgA- and IgG4-coated bacteria for patients in Remission (T2) group.

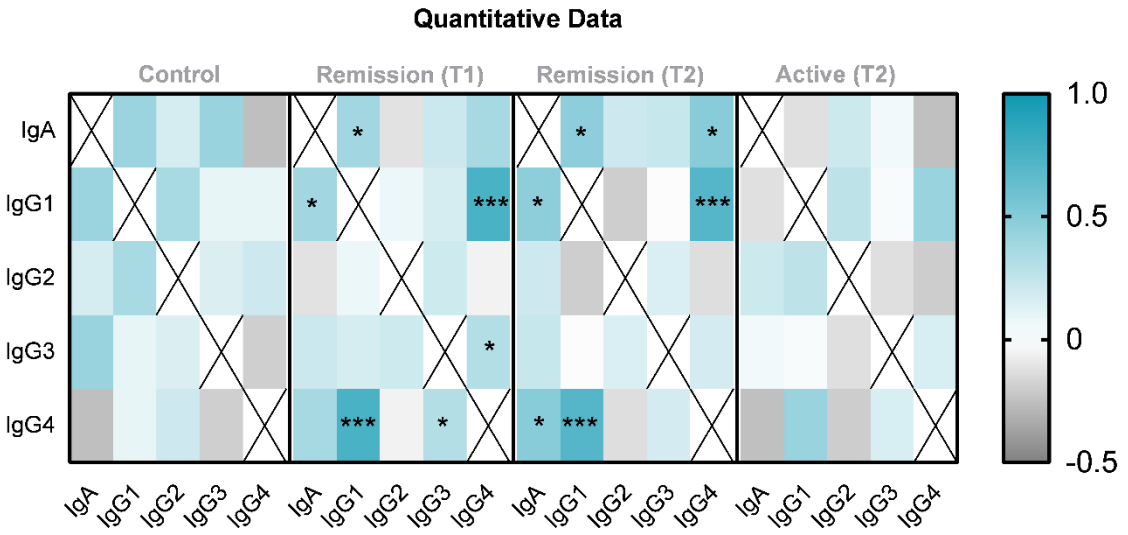

Figure 14. Correlations between the quantitative amounts of bacteria coated by specific IgG subclasses.
*Heat map presenting correlation coefficients between the level of bacteria coated by specific IgG subclasses. Negative correlation coefficients (grey) represent invers associations between the parameters, whereas positive correlation coefficients (blue) represent positive associations. Significant correlations are marked by asterisks. * P-value < 0.05, ** P-value < 0.01, 0.0001<*** P-value < 0.001. A Spearman Rank test was performed for all the correlations in Control (except for IgG1-IgG3), Remission (T1) and Remission (T2) groups. A Pearson correlation was used in the other cases. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

The IgA is the most abundant antibody coated by bacteria (0.64 – 35.84 %) hence, the percentage of bacteria coated with IgG is relatively low. The relative amount of the total bacteria coated with IgG1, IgG2, IgG3 and IgG4 is, respectively, 0.06 - 3.86 %, 0 - 30.81%, 0 – 0.82 % and 0.02 – 4.95 % (Appendix C, Table 3). For the relative data shown in Figure 15, the percentage of coated bacteria by each antibody shows an increased level tendency of IgG1-, IgG2- and IgG4 coated bacteria for patients with active disease. However the difference between the patients that remained in remission and the patients with active disease is not significant. As observed in the quantitative data, the most abundant IgG subclass is IgG4 followed by IgG1, IgG2, and IgG3 (decreased order) (Appendix C, Table 4). For the relative data, it was used the same correlation analyses used in the quantitative data. It was observed a significantly positive correlation between IgG1 and IgG4 percentage of coated bacteria on Remission (T1) group; between IgA and IgG2 for patients in Remission (T2) group and between IgA and IgG2 and IgG1 and IgG2 in patients that are part of Active (T2) group (Figure 15). No significant correlation is observed for the Control group.

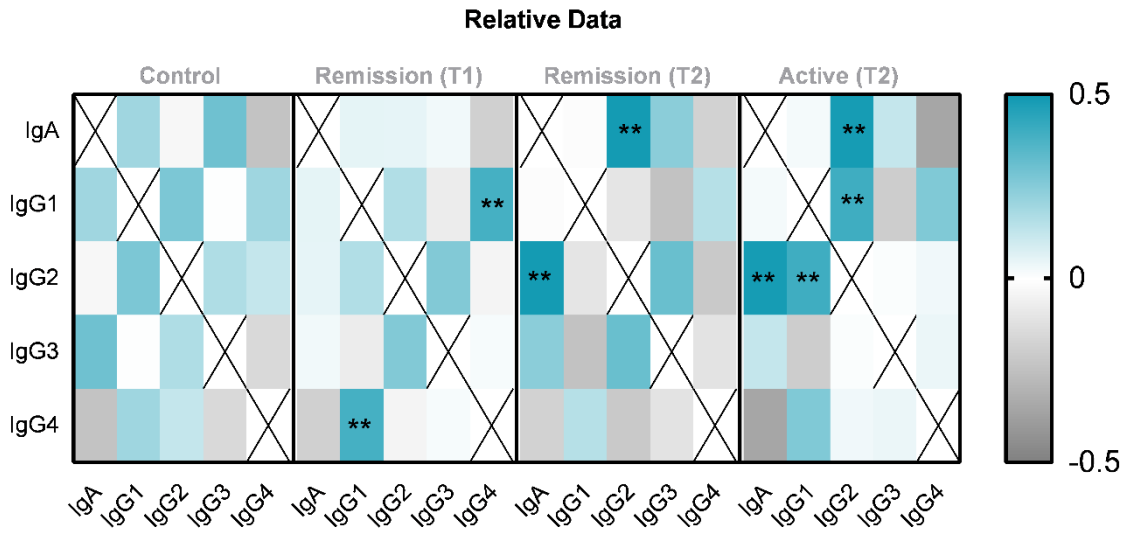

Figure 15. Correlations between the percentage of bacteria coated by specific IgG subclasses.

*Heat map presenting correlation coefficients between the level of bacteria coated by specific IgG subclasses. Negative correlation coefficients (grey) represent invers associations between the parameters, whereas positive correlation coefficients (blue) represent positive associations. Significant correlations are marked by asterisks. * P-value < 0.05, ** P-value < 0.01, 0.0001<*** P-value < 0.001. A Pearson test was performed for the Control group (to log transformed values) and a Spearman Rank test was performed for the other groups. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

As previously mentioned, Figure 12 enlighten that the overall coating by IgG subclasses varies between patients. Taking this into account and using the relative data, the ratio of each subclass relatively to the percentage of bacteria coated by IgG was calculated. The relative ratio of bacteria coated by each antibody is 2.21 – 98.90 %, 0 – 90.47%, 0 – 33.83 % and 1.07 – 89.21% for IgG1, IgG2, IgG3, and IgG4, respectively (Appendix C, Table 5). As observed in the quantitative and relative data, the most abundant IgG subclass is IgG4 followed by IgG1, IgG2, and IgG3 (decreased order). When analysing the percentage of bacteria coated with IgG4 comparing to the percentage of total IgG bacteria coated, it is observed an increased amount of IgG4 in patients with active CD. For IgG1 and IgG2 it is observed an increase in patients that remained in remission in T2. The control group shows an increased ratio of IgG2, IgG3 and IgG4 comparing to the other groups.

For the relative ratio data, was used the same correlation analyses used in the quantitative and relative data (Figure 16). It is observed a significantly negative correlation between IgG1 and IgG4 in all groups and between IgG2 and IgG4 in the groups with CD patients. Furthermore, it is expected that the more IgG1 or IgG2 is present in the total IgG coating, the less IgG4-coating is found. However, in the quantitative data analysis, IgG1 and IgG4 were positively correlated which differ from the correlation found for the relative ratio data.

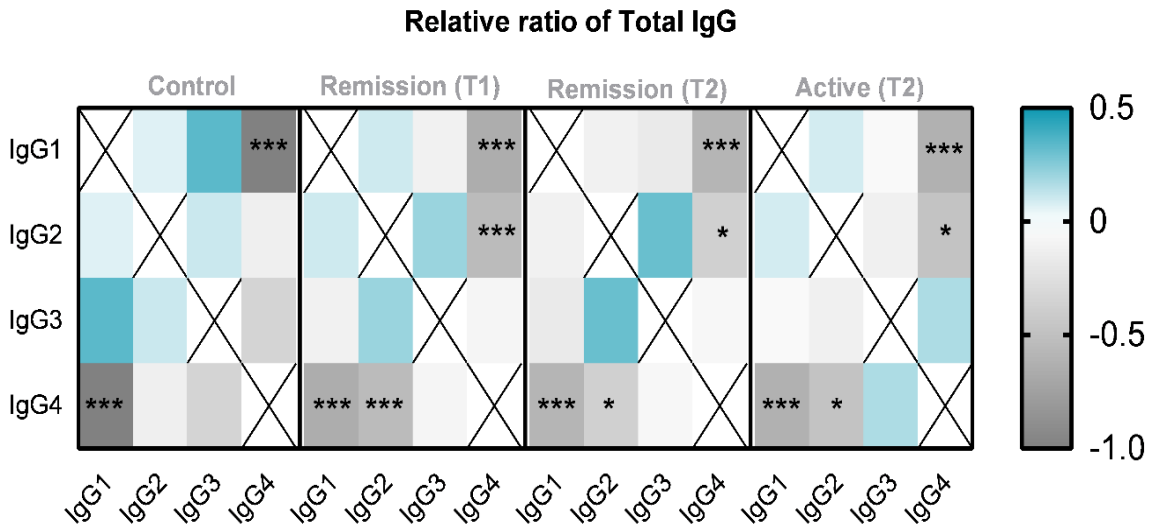

Figure 16. Correlations between the relative ratios of bacteria coated by specific IgG subclasses compared to the total IgG coating.

*Heat map presenting correlation coefficients between the level of bacteria coated by specific IgG subclasses. Negative correlation coefficients (grey) represent invers associations between the parameters, whereas positive correlation coefficients (blue) represent positive associations. Significant correlations are marked by asterisks. * P-value < 0.05, ** P-value < 0.01, 0.0001<*** P-value < 0.001. A Spearman Rank test was performed for all the correlations except between IgG1 and IgG4 where a Pearson test was performed instead. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

For most of the data sets, a significant difference between the two groups of patients in remission is found, but this could be related to few outliers. The outliers were not removed once the Ig-profiling is patient-specific and all the patients are considered valuable.

In summary, the different groups analysed do not present significant differences. Particularly, patients presenting active disease and the ones remaining in remission in the second visit, appear to have almost the same profile of Ig-coated bacteria. The profile of Ig-coating appears to be patient-specific and not group specific.

IV.2.Profile of Bacteria Double-Coating by IgA and IgG Subclasses in Controls and CD Patients

To investigate the amount of bacteria coated by more than one antibody at the same time, the profile of bacteria double coated by IgA and IgG subclasses were analysed using the flow cytometry assay. The profile of bacteria double coated was analysed for all the possible pairs, namely, bacteria coating simultaneously by IgA and IgG1 (IgA_IgG1); IgA and IgG2 (IgA_IgG2); IgA and IgG3 (IgA_IgG3); IgA and IgG4 (IgA_IgG4); IgG1 and IgG2 (IgG1_IgG2); IgG1 and IgG3 (IgG1_IgG3); IgG1 and IgG4 (IgG1_IgG4); IgG2 and IgG3 (IgG2_IgG3); IgG2 and IgG4 (IgG2_IgG4); and IgG3 and IgG4 (IgG3_IgG4).

The profile of bacteria double coated in each group of study, namely Control; Remission (T1); Remission (T2) and Active (T2) are illustrated in Figure 17. For all the groups it is observed that the pair that has higher level of coated bacteria/g faeces is IgA and IgG1 followed by IgA and IgG4; IgG1 and IgG4; IgA and IgG2; IgG1 and IgG2; IgG3 and IgG4; IgG2 and IgG4; IgA and IgG3; IgG1 and IgG3; and IgG2 and IgG3 (decreased order).

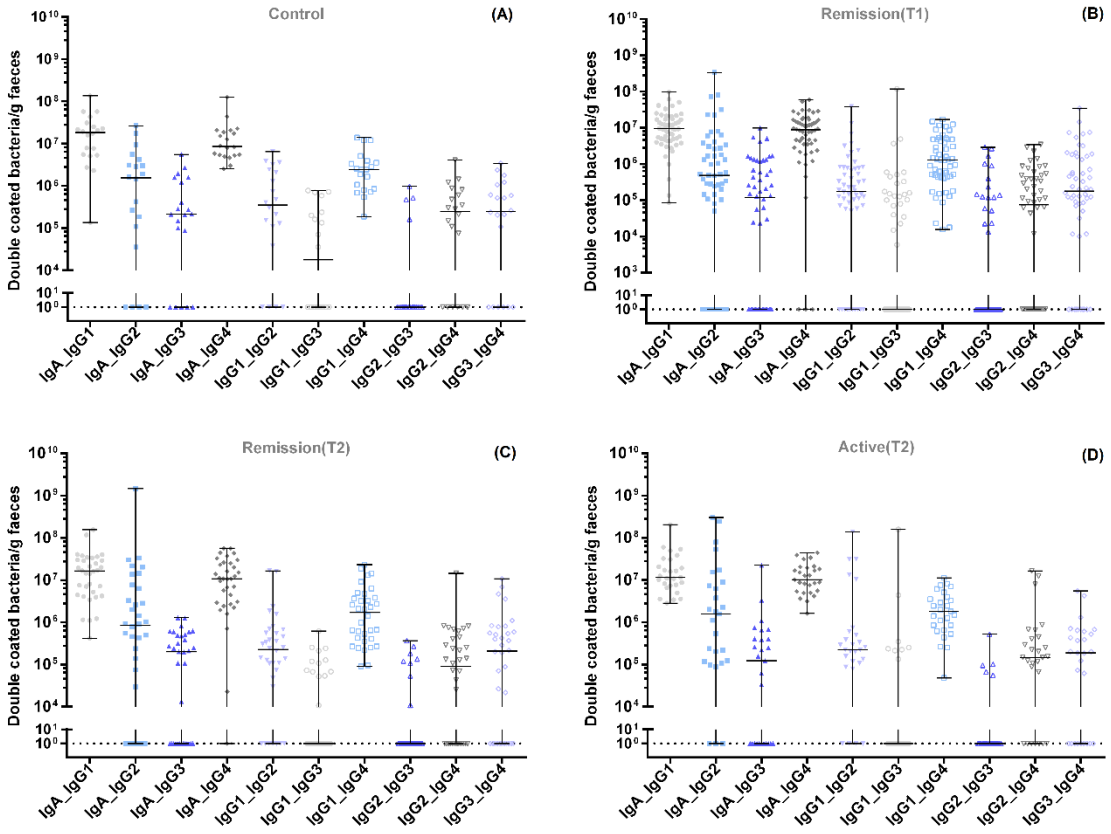

Figure 17. Comparison of the profile of bacteria-coating by pairs of antibodies in CD patients in the different groups studied.

*The quantitative amount of bacteria/ g faeces coated simultaneously by IgA and IgG1 (IgA_IgG1); IgA and IgG2 (IgA_IgG2); IgA and IgG3 (IgA_IgG3); IgA and IgG4 (IgA_IgG4); IgG1 and IgG2 (IgG1_IgG2); IgG1 and IgG3 (IgG1_IgG3); IgG1 and IgG4 (IgG1_IgG4); IgG2 and IgG3 (IgG2_IgG3); IgG2 and IgG4 (IgG2_IgG4); and IgG3 and IgG4 (IgG3_IgG4) in samples from **(A):** healthy individuals; **(B)** 54 CD patients at visit 1 (Remission (T1)), where all patients were in remission; **(C)** 34 patients whose disease remained in remission at the second visit (Remission (T2)) and **(D)** 19 CD patients whose disease change from remission to active at visit 2 and 8 CD patients that disease state was active (Active (T2)). For samples in which no bacteria are observed to be coated, the data point is set to 1 to enable the presentation of the results on a logarithmic axis. Indicated are median ± range. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5.*

The profiles of double-coating for all the pairs are represented in Figure 18. The levels of bacteria coating by all pairs are similar in healthy individuals, patients in remission and active disease in T2. The amount of bacteria coated is lower in the Remission (T1) group comparing to the other groups, excepts for bacteria coated at the same time by IgA and IgG4 where the Control is the group with lower amount of IgA_IgG4-coated bacteria/g faeces.

The percentage of patients that have bacteria coated by two antibodies is very low comparing to the percentage coated by only a specific antibody. The relative levels of bacteria coated by the pair IgA and IgG1; IgA and IgG2; IgA and IgG3; IgA and IgG4; IgG1 and IgG2; IgG1 and IgG3; IgG1 and IgG4; IgG2 and IgG3; IgG2 and IgG4; and IgG3 and IgG4 corresponds to 0.001-2.340 %; 0.000-21.30 %; 0.000-0.140 %; 0.000-0.510 %; 0.000-1.890 %; 0.000-0.710 %; 0.001-0.750 %; 0.000-0.1400 %; 0.000-0.750 % and 0.000-0.180 %, respectively).

To study if the profile of double coating is patient-specific, it was analysed the amount of the bacteria coated in each patient by the ten pairs of antibodies (Figure 19). In line with previous results, it is observed that the profile of bacteria-coating varies a lot from one patient to another even when comparing samples within the same group, suggesting that the production of the same antibodies tends to be higher in same patients.

No significant differences are observed between patients that enter a state of active disease and patients whose disease remained in remission. The patients whose disease remained in remission in visit 2 appear to have a decrease in the amount of double coated by IgA and IgG1, comparing to visit 1. A few outliers were found, corresponding to patients that have high amount of bacteria coated by a specific pair. The outliers found are the same referred in the previous section (Profile of IgA and IgG1-4-coated bacteria in Control and CD patients).

To investigate how the different pairs of antibodies are correlated, a Spearman Rank test was performed between the quantitative numbers of bacteria coated by all pairs in the different groups of study. Several significant positive correlations were found between the pairs. The following significant positive correlations are common to the four groups (Control; Remission (T1); Remission (T2) and Active (T2)): IgA_IgG1 and IgA_IgG4; IgA_IgG1 and IgG1_IgG4; IgA_IgG2 and IgG1_IgG2; IgA_IgG2 and IgG2_IgG4; IgA_IgG3 and IgG1_IgG3; IgA_IgG3 and IgG3_IgG4; and IgG1_IgG2 and IgG2_IgG4. Furthermore, a very significant positive correlation was found between IgA_IgG4 and IgG1_IgG4 in the remission groups.

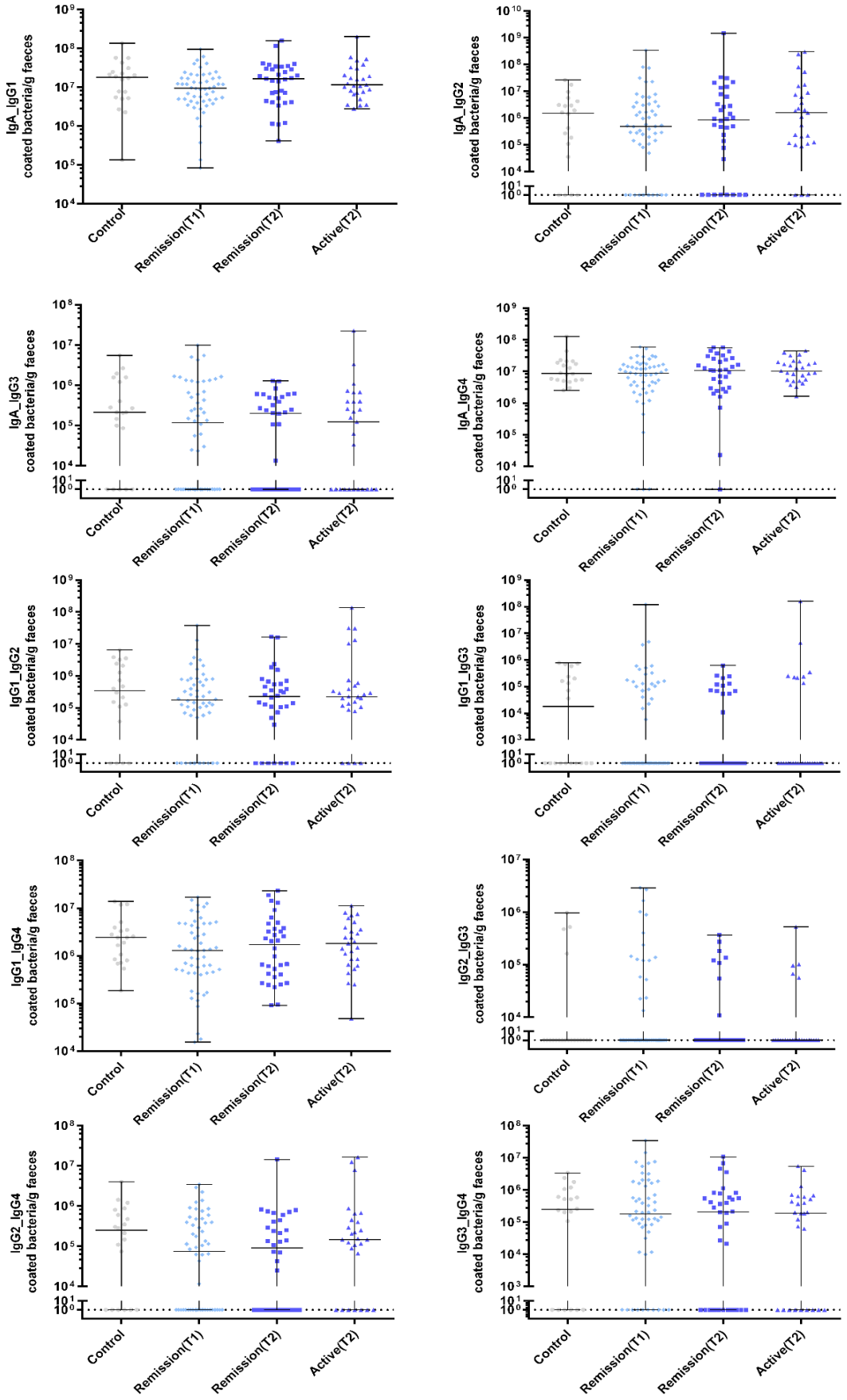

Figure 18. Comparison of the profile of bacteria-coating by all the antibodies pairs in CD patients with active disease or in remission.

The quantitative amount of bacteria /g faeces coated simultaneously by IgA and IgG1 (IgA_IgG1); IgA and IgG2 (IgA_IgG2); IgA and IgG3 (IgA_IgG3); IgA and IgG4 (IgA_IgG4); IgG1 and IgG2 (IgG1_IgG2); IgG1 and IgG3 (IgG1_IgG3); IgG1 and IgG4 (IgG1_IgG4); IgG2 and IgG3 (IgG2_IgG3); IgG2 and IgG4 (IgG2_IgG4); and IgG3 and IgG4 (IgG3_IgG4) in samples from CD patients at visit 1 (T1), where all 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, were also studied the samples of 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. For samples in which no bacteria are observed to be coated the data point is set to 1 to enable the presentation of the results on a logarithmic axis. Indicated are median ± ranges. In plots that the median is not visible the value is 1. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5.

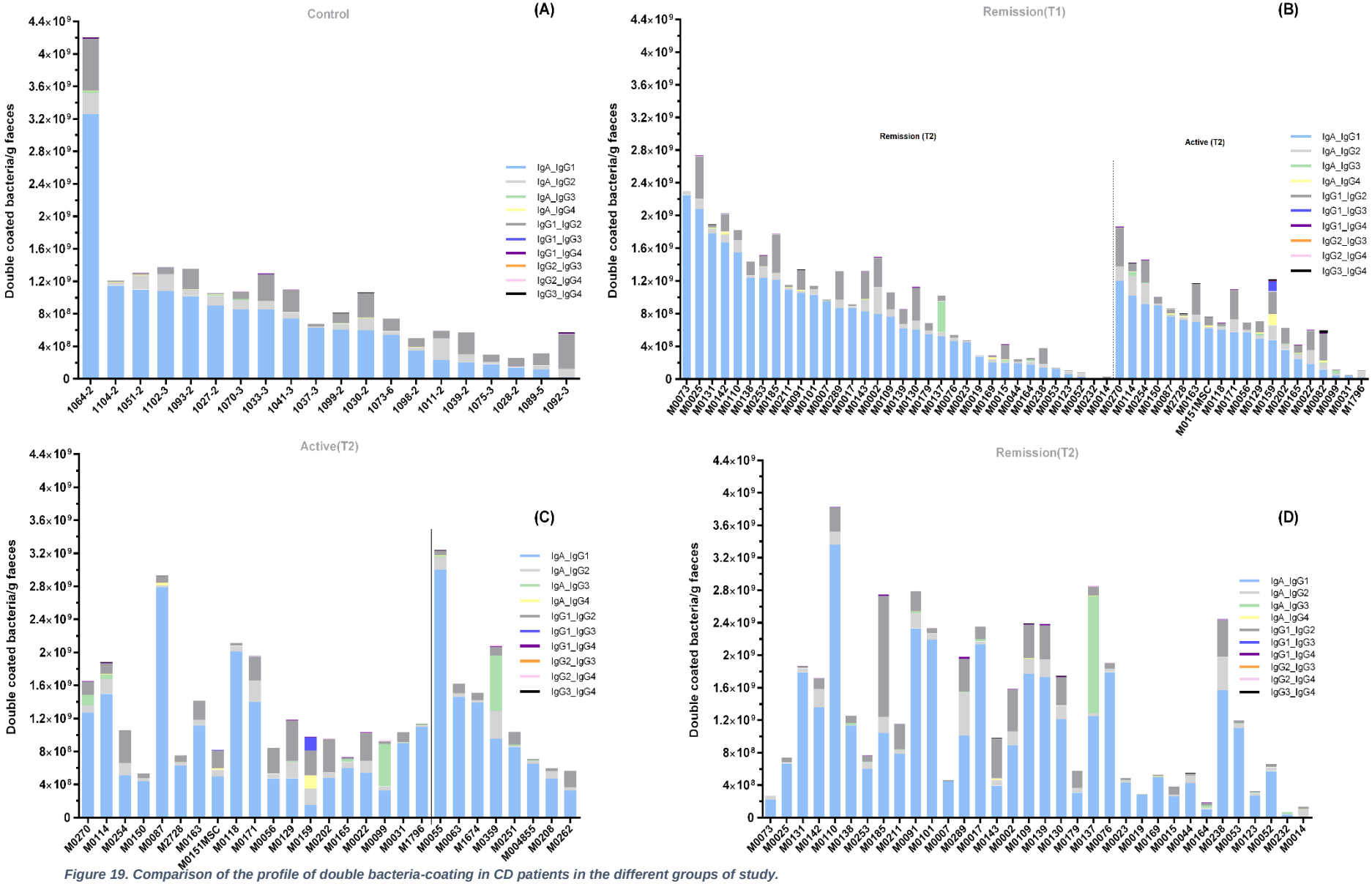

Figure 19. Comparison of the profile of double bacteria-coating in CD patients in the different groups of study.

*The quantitative amount of by IgA and IgG1 (IgA_IgG1); IgA and IgG2 (IgA_IgG2); IgA and IgG3 (IgA_IgG3); IgA and IgG4 (IgA_IgG4); IgG1 and IgG2 (IgG1_IgG2); IgG1 and IgG3 (IgG1_IgG3); IgG1 and IgG4 (IgG1_IgG4); IgG2 and IgG3 (IgG2_IgG3); IgG2 and IgG4 (IgG2_IgG4); and IgG3 and IgG4 (IgG3_IgG4) 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 IgA_IgG1 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.*

IV.3.Associations Between Currently Used Disease Markers and the Profile of Bacteria-Coating by IgA and IgG Subclasses in CD Patients

Several disease markers are used for CD, namely, the Harvey-Bradshaw Index (HBI), the level of C-reactive protein in blood samples, calprotectin in faecal samples and the Bristol Stool Scale (BS) value.

Harvey-Bradshaw Index is a disease activity index that is used for CD patients. Patients with a score higher than 4 are considered to have active disease.[166]

The level of C-reactive protein in blood is a biomarker associated with inflammation. In response to an inflammation, the levels of CRP increase, therefore CRP is classified as an acute-phase protein. The protein is produced by the liver and its levels rise as a response to pro-inflammatory cytokines. Although being used for CD, CRP is not a specific marker for intestines since it can be produced due to extraintestinal inflammations as well. Healthy individuals normally present less than 1 mg/L of CRP in blood whereas in periods of acute inflammation the levels cans rise 100-fold (200-400 mg/L)[167].

Calprotectin is a calcium and zinc-binding protein that is used as a biomarker for disease activity. An increase level of calprotectin in faeces indicates the presence of mucosal inflammation. The protein is produced by granulocytes at the site of mucosal inflammation leading to an increase of the level of calprotectin in faeces[168]. Concerning the level of faecal calprotectin (FC), active disease is associated to a value higher than 250 µg/g, healthy individuals usually present a value less than 50 µg/g. However, these cut-off levels are not consensual.

The Bristol Stool Scale (BS) is used to differentiate the faecal samples according to their texture. The Scale has seven categories, 1 represents the most solid consistence and 7 the most liquid. A Bristol Stool Scale classification of 3-4 is considered within the normal range.

To determine the possible associations of the disease markers (above-mentioned) to the amount of bacteria/g faeces and to the quantitative amount of IgA and IgG1-4 coated bacteria/g faeces, a correlation analysis was performed. Furthermore, it was also analysed the existence of correlations between the parameters and the relative amount of IgA and IgG1-4 bacteria coated and the IgG1-4 relative ratio of total IgG bacteria coated. The resulting correlation coefficients and significant findings are shown in Figure 20. A Spearman Rank test was performed for all the pairs of data.

A significantly negative correlation was found between **HBI** and the amount of bacteria coated/g faeces and the amount of IgG4 coated bacteria/g faeces among the Remission T1 and T2 groups. In addition, a significantly positive correlation was observed in those groups between the parameter mentioned and the percentage of bacteria coated by IgA. Furthermore, it is also spotted a significantly negative correlation between HBI and the amount of IgG1 bacteria coated/ g faeces in Remission (T1) and Active (T2) groups (Figure 20).

For **C-Reactive protein**, Remission T1 group is the only group where significant correlations were found. A significantly negative correlation was found between CRP and the amount of IgA

coated bacteria/g faeces; IgG1 coated bacteria/g faeces and the IgG1 relative ratio of bacteria coated by total IgG. Moreover, a positively significant correlation was found between CRP and the IgG4 relative ratio of bacteria coated by total IgG.

In relation to **Faecal Calprotectin**, a single significantly positive correlation was observed in Active (T2) group between FC and the amount of IgA-coated bacteria/g faeces.

The **Bristol Stool Scale** is the disease marker showing more correlations. The IgG1 relative ratio of total IgG bacteria coated seems to be significantly positively correlated to the BS in samples from the first time point (T1). In all the groups, a significantly negative correlation is detected between BS and the amount of IgG4 bacteria coated/g faeces. BS in Remission T2 and Active T2 groups appear to have a significantly negative correlation to the total number of bacteria/g faeces; the amount IgG4 bacteria coated/g faeces and the IgG4 relative ratio of total IgG bacteria coated. A significantly negative correlation was established in Remission T2 group, between BS and the amount of IgA bacteria coated/g faeces; the amount of IgG1 bacteria coated/g faeces and the relative amount of IgG4 bacteria coated. In Active T2 group several significantly positive correlations are described namely, correlations to: relative amount of bacteria coated by IgA; relative amount of IgG1 bacteria coated; amount of IgG2 bacteria coated/g faeces; relative amount of bacteria coated by IgG2 and IgG2 relative ratio of total IgG bacteria coated.

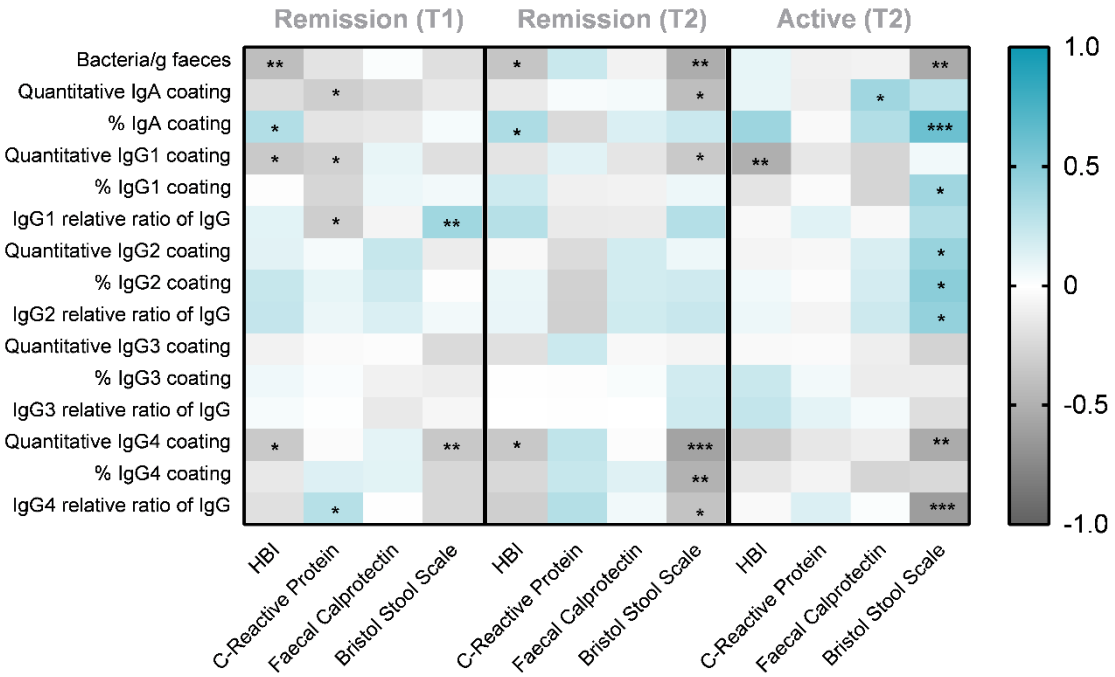

Figure 20. Correlations between bacteria-coating by IgA and IgG subclasses and currently used disease markers in CD patients.

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.*

Given that Bristol Scale is the biomarker that presents a higher amount of significant correlations was considered relevant to further study the importance of faecal texture as a biomarker to categorize the state of disease in CD patients. To study the influence of the faecal samples texture, the amount of bacteria (Figure 21) and the profile of Ig-coating bacteria (Figure 22) were studied for the two time points (Time 1 and Time 2). The patients were divided in two groups, Group 1 (G1) where all patient's faecal sample were classified as 3, 4 or 5 in the Bristol Scale and Group 2 (G2) where the samples were classified as 6 or 7. The comparison between visit 1 and visit 2 was performed considering that between visits the patient's faecal remained in the same Bristol Scale category and that the ones that did change the category, remained in the same group.

It is observed that within the same visit, independently of which visit, the number of bacteria is higher in G1 comparing to G2 (Figure 21). Group 2 includes samples that were classified as 6 or 7 in the Bristol Scale which means that they have higher quantity of water and less amount of faecal matter. Hence, it makes sense that a lower amount of faecal matter is related with a lower amount of coated bacteria. In the second visit, it is visible an increase in the number of bacteria coated, both in Group 1 and 2, comparing to the first visit. Of note is the significant findings of the opposite association with bacteria at visit 2 between the groups 1 and 2 (Mann-Whitney test. 0.0001<***P-value < 0.001).

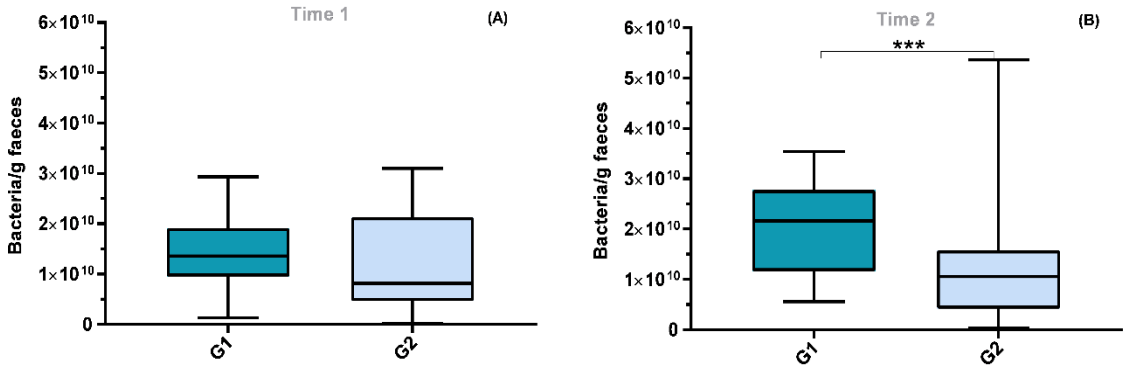

Figure 21. Comparison between the Bristol Scale and the amount of bacteria-coating in CD patients in remission in T1 and with active disease or in remission in T2.

(A) *The amount of bacteria/g faeces in samples from CD patients at visit 1 (Time 1), where all patients were in in remission.* ***(B)*** *The amount of coated bacteria/g faeces in samples from CD patients at visit 2 (Time 2), where 19 patient presented active disease and 34 remained in remission. The samples were divided in two groups, according to the category on the Bristol Scale. Group 1 (G1) is composed by patients that presented 3, 4 or 5 on the Bristol Scale and Group 2 (G2) of patients that had 6 or 7. The significant finding is based on a Mann-Whitney test. 0.0001<***P-value < 0.001. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

For all the Ig-coating bacteria it is observed an increase of the level of bacteria coated within the Group 1 between the first and second visit. The median level of the Group 2 remains almost the same between visits excepts for IgG2-coated bacteria that is relatively higher on visit 2 comparing to visit 1 (Figure 22). Of note is the significant finding of the opposite association with IgG4-coating at visit 1 and visit 2 between the Groups 1 and 2.

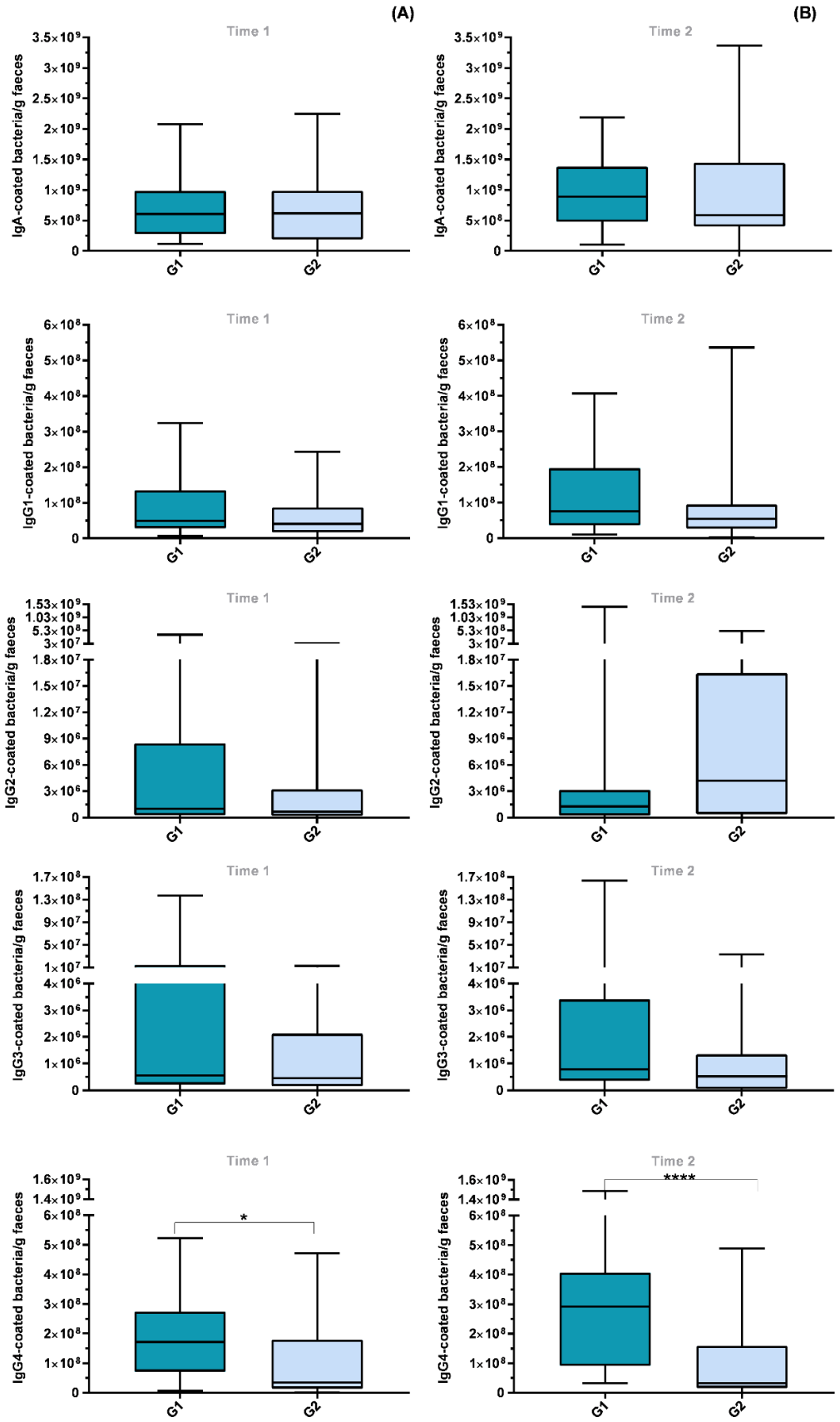

Figure 22. Comparison between the Bristol Scale and the profile of bacteria-coating by IgA and IgG subclasses in CD patients in remission in T1 and with active disease or in remission in T2.

(A) *The quantitative amount of IgA and IgG1-4 coated bacteria/g faeces in samples from CD patients at visit 1 (Time 1), where all patients were in in remission.* ***(B)*** *The quantitative amount of IgA and IgG1-4 coated bacteria/g faeces in samples from CD patients at visit 2 (Time 2), where 19 patient presented active disease and 34 remained in remission. The samples were divided in two groups, according to the category on the Bristol Scale. Group 1 (G1) is composed by patients that presented 3, 4 or 5 on the Bristol Scale and Group 2 (G2) for patients that had 6 and 7. The significant finding is based on an Unpaired T-test. 0.01<*P-value<0.005; ****P-value < 0.0001. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

IV.4. Relation of the Profile of Bacteria-Coating by IgA and IgG Subclasses in CD Patients to Disease Phenotype, Disease Location, Age at Diagnosis; Smoking History and Gender

Crohn's Disease onset and outcome can be influenced by several factors. Hence, it was considered relevant to study the information's concerning the patient-specific characteristics (Appendix B). To investigate some of the factors that can be related to CD, a statistical analysis was performed to identify the relation between the bacteria-coating by IgA and IgG1-4 and the smoking history; disease phenotype; disease location; age at diagnosis; and patient's gender. The follow data refers to samples in the same study groups presented in the previous sections (Remission T1; Remission T2 and Active T2).

IV.4.1. *Phenotype*

Disease phenotype is related with the behaviour of CD which can change due to disease's progression. Throughout life, patients can have complications such as strictures, fistulas and abscesses. As previously mentioned, CD behaviour is classified in three categories according to the existent complication. B1 represents a disease with no complication; B2 is related to a stricturing behaviour and B3 to a penetrating behaviour where fistulas, perianal ulcers, and/or abscesses are included[169].

A statistical analysis was performed to identify the relation between the disease behaviour and the bacteria-coating by IgA and IgG subclasses. Furthermore, it was examined if the number of bacteria/g faeces was affected by the disease phenotype. Figure 23 summarizes the results found. Appendix E.1. shows the scatter plots and median levels regarding the profile of IgA and IgG1-4 when patients were grouped accordingly to their disease phenotype.

Overall, the amount of bacteria coated decreases with the increase of disease complications (B1 to B3) in all the groups; no significant difference were observed between the same phenotype in the three groups.

Patients belonging to remission groups appear to have decrease amount of bacteria/g faeces; IgA- and IgG1-coated bacteria/g faeces with the increased of the phenotype, *i.e.*, a penetrating behaviour has less amount of IgA coating than a structuring behaviour. A structuring behaviour in Active T2 group presents higher amount of IgA coating comparing to the others phenotypes.

For IgG1-coated bacteria it seems that in remission groups the higher the disease complications the lower the amount of bacteria coated.

The amount of bacteria/coated by IgG2 is higher in patients with B3 that present active disease. In the three phenotypes it is observed an increase of IgG2-coated bacteria with the complications severity (B1 to B3).

Regarding IgG3-coating, the amount of IgG3-coated bacteria in Active T2 group decreases with the increase of phenotype.

The amount of IgG4-coated bacteria is higher in the active group that in the remission groups. The decrease with the increase of the severity of complications (B1 to B3) seen for the IgG4-

coating within the Active T2 group is found to be significant (Kruskal-Wallis test, 0.01<*P-value < 0.05).

In general, phenotype B2 is related to higher amount of bacteria/g faeces; IgA and IgG1 coating in active disease and IgG3 in Remission T1 group. Furthermore, in all groups it is observed that a structuring phenotype is associated with a higher value of IgG4-coated bacteria comparing to phenotypes B1 and B3.

Phenotype B1 is related with higher amount of bacteria in all the groups, IgA and IgG1 in Remission (T1) and (T2). Lastly, phenotype B3 is responsible for higher amount of IgG2 in all the groups. Results found for the relative data are consistent to the ones seen for quantitative data.

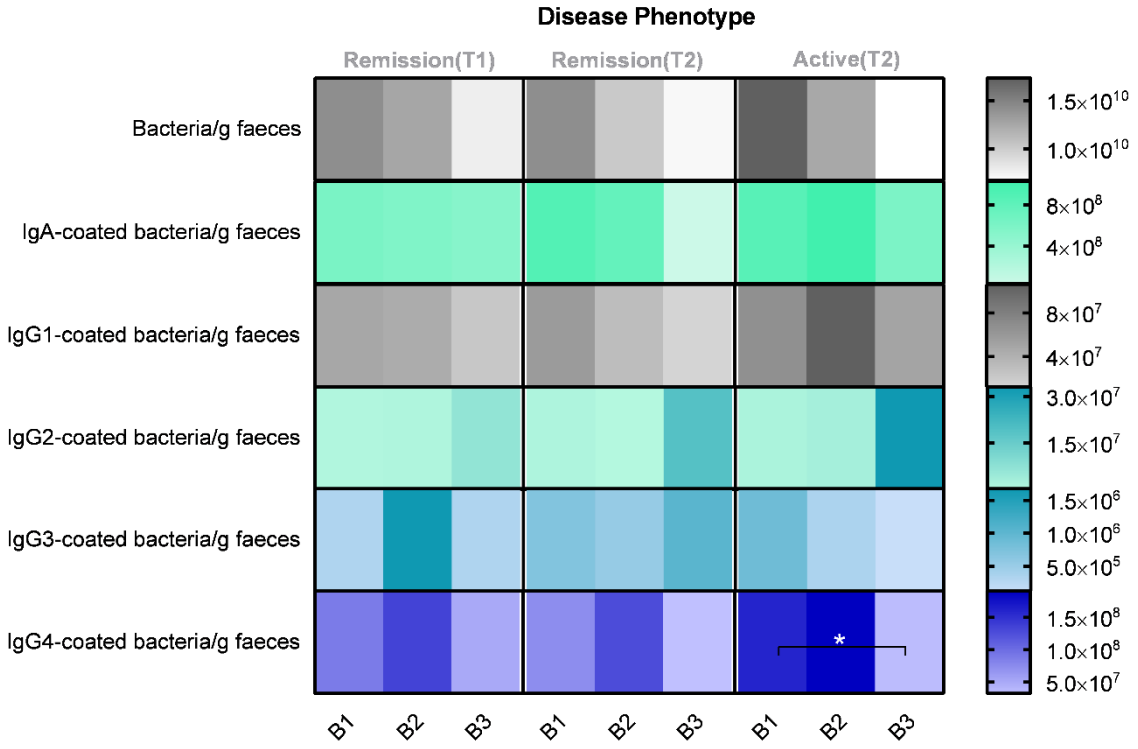

Figure 23. The profile of bacteria-coating by IgA and IgG subclasses in patients grouped according to their disease phenotype.

The values represented in the heat map are the median levels of IgA and IgG subclass-coating and/or amount of bacteria/g faeces in every category of disease phenotype. B1 category represents a non-stricturing, non-penetrating phenotype, B2 represents a stricturing phenotype, and B3 a penetrating phenotype where fistulas, perianal ulcers and/or abscesses occur. The heat map is divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. The significant finding is based on a Kruskal-Wallis test. 0.01< P-value < 0.05. Data represented by scatter plots can be found in Appendix E.1. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

IV.4.2.*Location*

CD can affect different locations within the gastrointestinal tract which, once again, can change according to disease's progression. As previously mentioned, the Montreal Classification divides disease location into four categories, L1; L2; L3; and L4, representing ileum, colon, ileum and colon and the upper gastrointestinal tract, respectively[169]. To study the influence of disease location, a statistical analysis was performed to found out associations between location and the bacteria coating by IgA and IgG1-4, as well as the number of bacteria/g faeces. Figure 24,

summarizes the results observed. Scatter plots and median levels related with the profile of bacteria coated when patients are grouped accordingly to their disease location can be found in Appendix E.2.

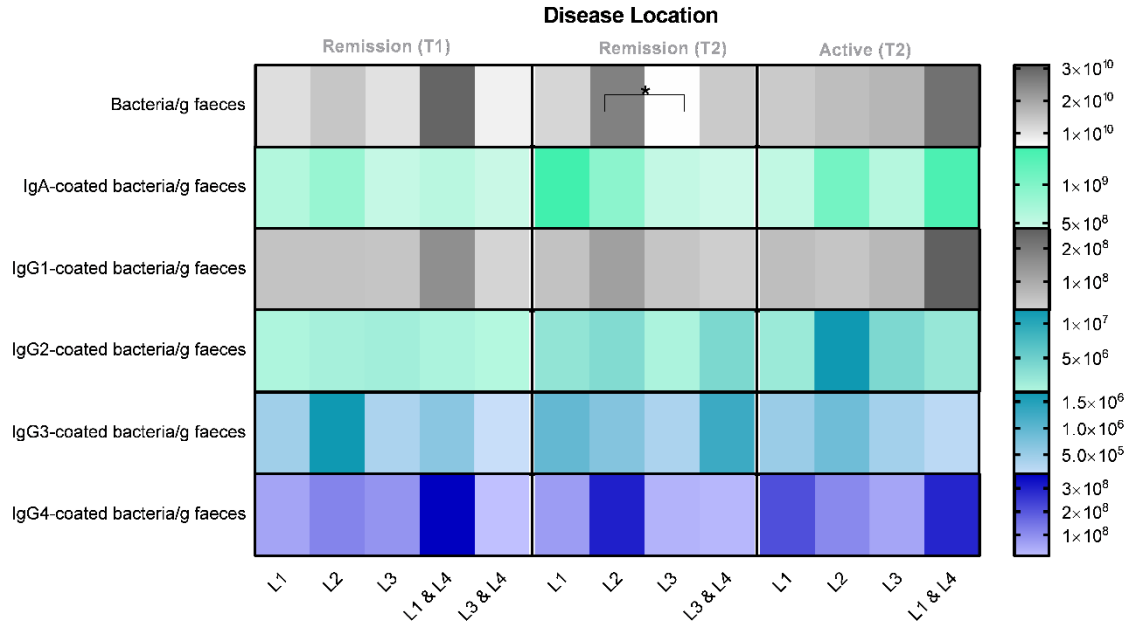

Figure 24. The profile of bacteria-coating by IgA and IgG subclasses in patients grouped according to their disease location.

The values represented in the heat map are the median levels of IgA and IgG subclass-coating and/or amount of bacteria/g faeces in every category of disease location. Disease location was divided in four categories that include ileum (L1), colon (L2), ileum and colon (L3), and the upper gastrointestinal tract (L4) which covers disease from the mouth to ileum. Patients presenting disease in ileum and gastrointestinal tract and in ileum, colon and gastrointestinal tract, were allocated to L1 & L4 and L3 and L4, respectively. The heat map is divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. The significant finding is based on a one-way ANOVA test. 0.01< P-value < 0.05. Data represented by scatter plots can be found in Appendix E.2. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

Overall, disease in colon (L2) and both ileum and the upper gastrointestinal tract (L1 & L4) are the categories possessing the highest amount of bacteria as well as the highest amount of bacteria coated by the different antibodies.

Disease in colon region (L2) appears to be associated with higher amount of IgG3 in Remission (T1) group, IgA in Remission (T2) and IgG2 in Active (T2) group.

The amount of bacteria coated by IgA in Remission T2 group seems to be higher in disease located in ileum (L1), colon (L2) and ileum and colon (L3)-ascending order.

Disease in ileum and gastrointestinal tract (L1 & L4) seems to be related with high amount of bacteria coated by all the antibodies and also with higher amount of bacteria coated. Only one patient presented the disease in this location hence, no conclusions about this data should be extrapolated. The same is true for disease located in colon, ileum and gastrointestinal tract (L3 & L4) which was present in one patient only.

In the Remission group at visit 2, patients having disease in both ileum and colon (L3) compared to disease restricted only to colon (L2) have significantly higher amount of bacteria/g faeces (one-way ANOVA, P-value < 0.05). Results found for the relative data are consistent to the ones seen for the quantitative data.

IV.4.3.*Age at diagnosis*

The patient's age when diagnosed with CD can influence the disease outcome. The earlier the disease is recognised, the sooner the best treatment can be found. To investigate whether the age at diagnosis has an association with bacteria-coating by IgA and IgG subclasses, the profile of bacteria coated was studied when patients were grouped accordingly to their age at diagnosis. The scatter plots and median levels concerning the bacteria coating profile in relation to age at diagnosis can be seen in Appendix E.3. Figure 25 summarizes the results found. Patients were considered being part of group A1 when diagnosis occurred before 16 years old (included); A2 when disease was recognised after 16 and before 40 years old (included) and A3 when disease diagnosis happened after 40 years old, accordingly to Montreal Classification[169].

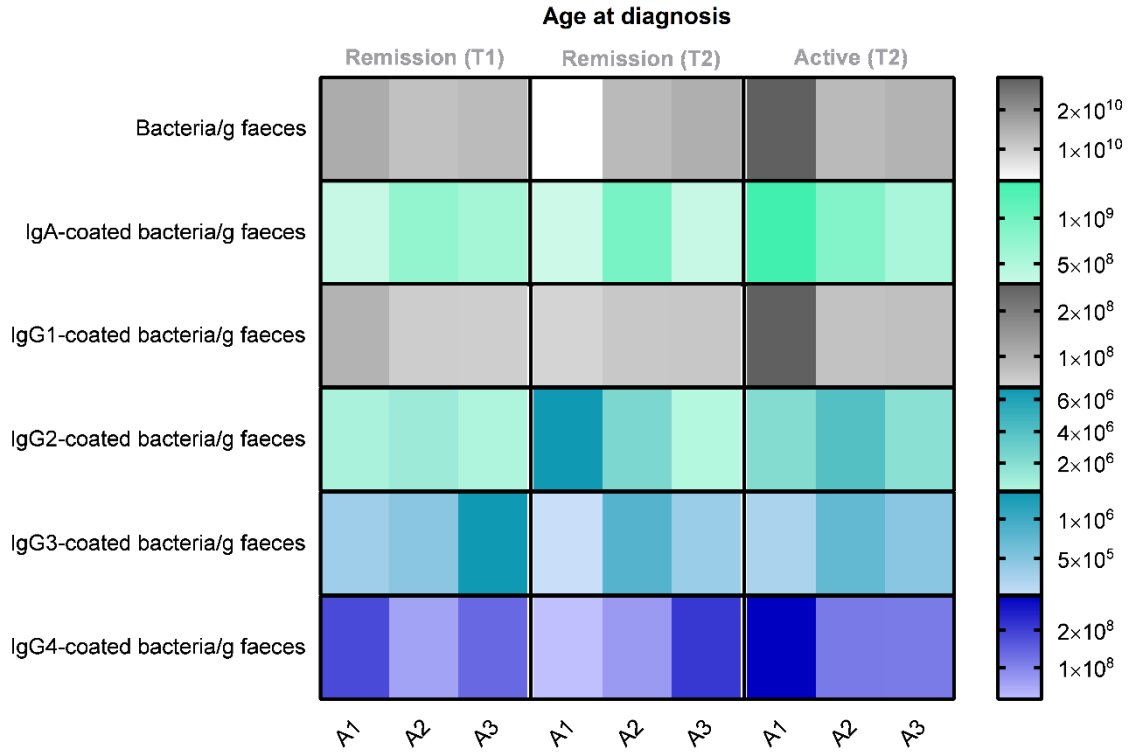

Figure 25. The profile of bacteria-coating by IgA and IgG subclasses when patients were grouped accordingly to their age at disease diagnosis.

The values represented in the heat map are the median levels of IgA and IgG subclass-coating and/or amount of bacteria/g faeces in every category of age at diagnosis. A1 represent patient that were diagnosed before the age of 16 years, A2 to refers to patients diagnosed in the age of 17-40 years, and A3 to patients diagnosed after the age of 40 years. The heat map is divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. Data represented by scatter plots can be found in Appendix E.3. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.

Patients belonging to Remission T1 and Active T2 groups, that were diagnosed before the age of 16 years old (A1) seem to be related with a higher amount of bacteria coating by IgG1 and IgG4 as well as a higher amount of bacteria/g faeces comparing to patients diagnosed in other ages. On Remission T2, the contrary is observed and in addition, diagnosis before 16 years old seem to be related to higher level of IgG2-coated bacteria/g faeces. It is noteworthy, that only two patients were diagnosed before 16 years old.

Diagnosed at the age of 17-40 years old (A2) appear to be related with an elevated amount of bacteria coating by IgA in the remission groups. Furthermore, in those groups, A2 patients appear to be related to an elevated amount of IgG4 bacteria coated/g faeces.

Regarding to relative data (available on Appendix E.3.), it was observed a decrease of the bacteria coated by IgA and IgG1 with the increased age at diagnosis. Besides that, in Remission T1 group, it seems that being diagnosed after 40 years old (A2) comparing to the age of 17-40 (A3) decreases significantly the bacteria coated by IgG4 (one-way ANOVA. 0.01<* P-value < 0.05).

IV.4.4.*Smoking*

One of the environmental factors described in the Theoretical Background section was smoking. Smoking can represent a risk in disease development and/or outcome. Therefore it was considered relevant to investigate the relation between the level of bacteria-coating by the different antibodies and patient's smoking history (summarised in Figure 26). The scatter plots and median levels that lead to Figure 26 are available in Appendix E.4. The samples were divided in three categories where "Never" represents patients that never smoked; "Ex" represents patients that used to smoke; and "Currently" the patients that were smokers at the time of samples collection.

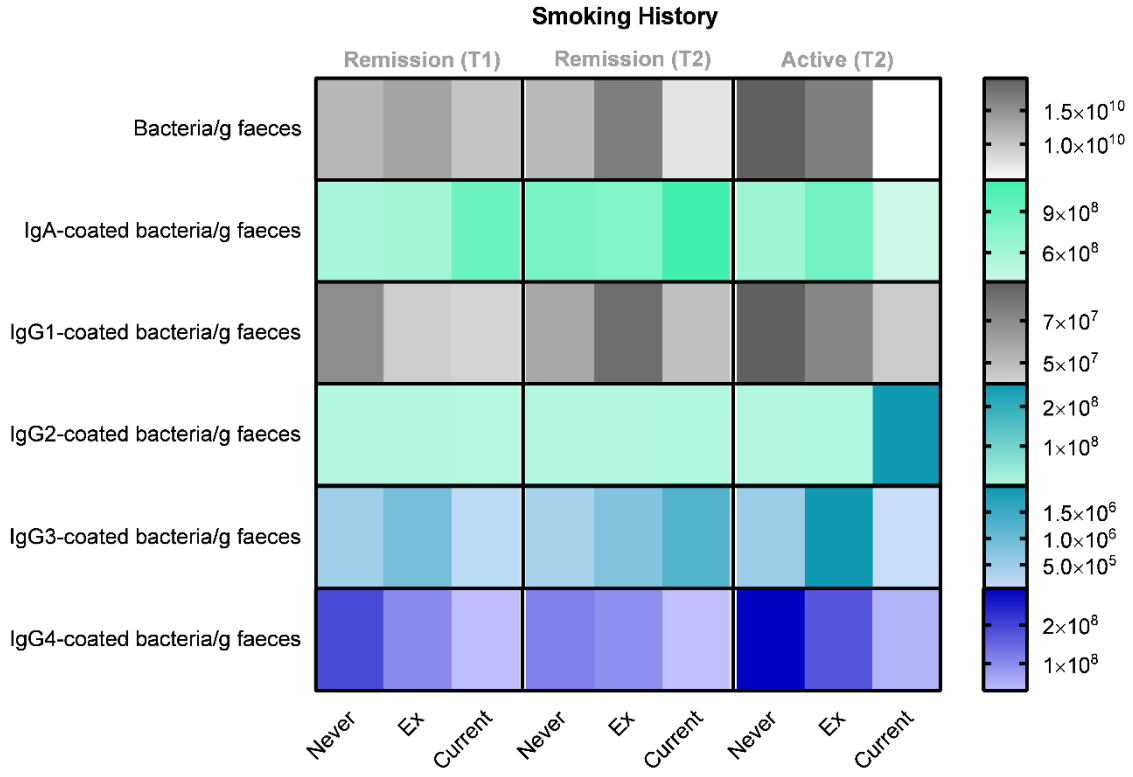

Figure 26. The profile of bacteria-coating by IgA and IgG subclasses when patients were grouped accordingly to their smoking history.

The values represented in the heat map are the median levels of IgA and IgG subclass-coating and/or amount of bacteria/g faeces in every category of smoking history i.e. never been a smoker (Never), ex-smoker (Ex) and current smoker (Currently-at the time of sample collection). The heat map is divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. Data represented by scatter plots can be found in Appendix E.4. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.

Patients with active disease that never smoked (Never) show a tendency of increase IgG2- and IgG4-coating along with an increase in the amount of bacteria/g faeces comparing to those that were smokers or ex-smokers. The tendency observed for increased IgG2-and IgG4-coating was also present in patients whose disease was in remission at the time of the first visit. It is important to note that patients that simultaneously smoke and have active disease have a much higher level of IgG2 bacteria coated/g faeces comparing to all the other patients. This observation can be an indicative of a correlation between smoking and active disease and the role of IgG2 in the development of the disease.

The amount of IgG1 coating appears to decrease with the increase of smoking history in Remission (T1) and Active (T2), *i.e.* patients that never smoked presented higher amount comparing to ex-smokers and smokers. Hence, smoking could be related to a decrease of the amount of IgG1 in CD patients.

For the three study groups (Remission (T1); Remission (T2) and Active (T2)), it is observed a decrease of IgG4 coating when patients never smoked; were ex-smokers and currently smokers, *i.e.*, it seems that smoking is related with a decrease in the level of bacteria coating by IgG4.

Patients that were previously smokers belonging to Remission (T1) and Active (T2) appear to have higher level of IgG3-coated bacteria/g faeces comparing to currently smokers or patients that never smoked. The same results are found for relative data (Appendix E.4.).

Summing up, smoking appears to be related to: a decrease of IgG1- and IgG4- coated bacteria/g faeces; a decrease of IgA-coated bacteria/g faces in patients in remission and to an increased level of bacteria coating by IgG2 in patients with active disease.

IV.4.5.*Gender*

Although, CD seems to be no sex-specific it was considered relevant to study if, in the present work, the profile of bacteria coating was influenced by the patient's gender. To investigate whether the gender has an association with bacteria-coating by IgA and IgG subclasses, the profile of bacteria coated was studied when patients were grouped as Male or Female.

Figure 27 summarizes the results that were extrapolated from the data present in the scatter plots and tables available in Appendix E.5.

Males appear seem have higher level of bacteria/g faces and also IgG1-coated bacteria/g faeces comparing to female in the remission groups. Contrastingly, it was observed that female present higher amount of IgG2- coated bacteria/g faeces in all the groups. Furthermore, female patients with active disease have higher level of IgG1-; IgG2; IgG3- coating in addition to higher amount of bacteria/g faeces comparing to male patients.

In addition, it is observed that the level of IgA coating in all groups is higher in Male comparing to Female patients.

Female patients belonging to Remission (T1) and Active (T2) groups have higher level of IgG4 coating comparing with Male.

It should be noted that the decrease seen for bacteria/g faeces between Male and Female patients, at the first visit, is found to be significant (Unpaired T-test, 0.01<*P-value < 0.05).

Comparing these findings to the relative data found in Appendix E.5, it is observed that the relative level of IgA; IgG1- and IgG4-coating has the opposite distribution seen for the quantitative data. Quantitative data considers the amount of bacteria/g faeces which adds a further dimension of information to the data and can be the reason for the different results observed between the quantitative and relative data.

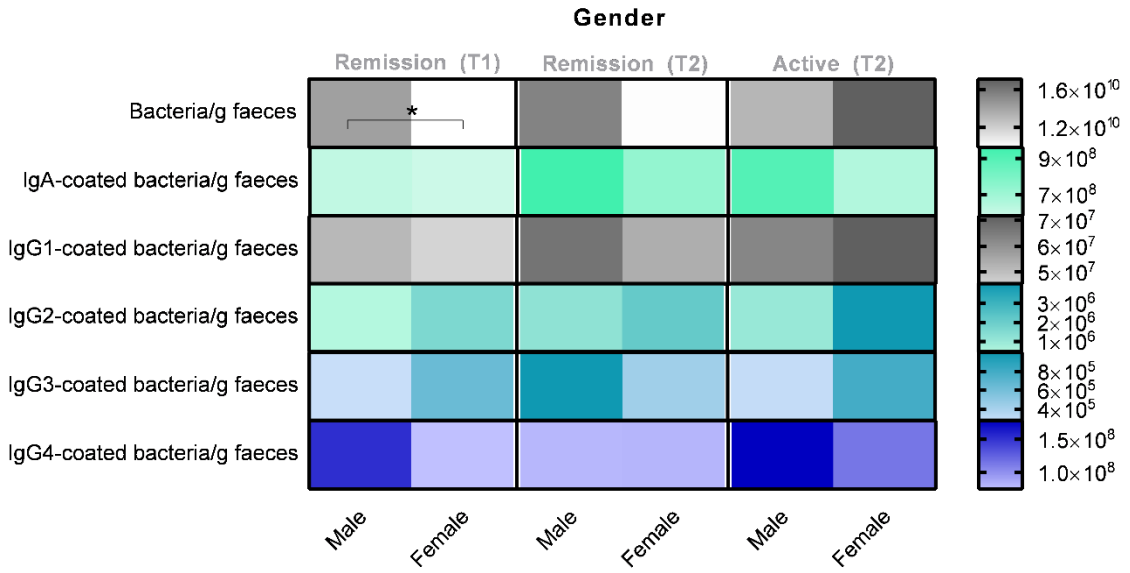

Figure 27. The profile of bacteria-coating by IgA and IgG subclasses when patients were grouped accordingly to their gender.

The values represented in the heat map are the median level of IgA and IgG subclass-coating and/or amount of bacteria/g faeces in male and female patients. The heat map is divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. The significant finding is based on an Unpaired T-test. 0.01< P-value < 0.05. Data represented by scatter plots can be found in Appendix E.5. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.*

Lastly, it is important to note that in all the previous parameters, several differences were found between the two remission groups. These differences might be related to the lower amount of patients that are considered in remission in the second visit (34) comparing to the first visit (53).

V. Discussion

In the present work, the profile of bacteria coated by IgA and IgG subclass was investigate in faecal samples from Crohn's Disease patients. The aim was to identify whether the profile could be used to properly distinguish healthy individuals from CD patients in remission and with active disease. Additionally, it was investigated if the currently used disease markers where being effective in this distinction and if patient's characteristics, namely, disease phenotype, disease location, smoking history, age at diagnosis, and gender, may influence the profile of bacteria coating.

The present study estimated the relative profile of bacteria coating as well as the quantitative profile. The quantitative data provides a more faithful inside of the profile of bacteria coating once it is determined the actual amount of bacteria coating by IgA and IgG subclass and not only a relative amount. Furthermore, once it is known that specific antibodies/subclass are responsible for coating specific microorganisms, knowing the precise amount of bacteria coated by those antibodies may hence provide a more comprehensive view of what are the predominant *taxa* in CD. Moreover, some of the used disease markers are thought to be correlated with an increased/decreased amount of bacteria in faeces thus it is important to determine the actual amount of bacteria to found whether a correlation exists or not. Nevertheless, the relative data is also displayed since most of the previous studies only measured the relative amount of bacteria and also because these data can give some insights about the disease activity as well. Furthermore, the comparison between the results obtained from both quantitative and relative data leads to more conclusive results.

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.*[170] 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.*

The distribution of the bacteria coating was found to be IgA>IgG4>IgG1>IgG2>IgG3 in all the groups, *i.e.,* healthy individuals, patients in remission and patients presenting active disease. Regarding the difference between IgA and IgG coating, 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[171,172]. In previous studies[173,174], 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. The level of Ig producing cells in the mucosa and serum may not be correlated to the profile of bacteria coating of faecal bacteria since bacteria coating depends on transepithelial transport and bacterial antigen affinity. Since Philipsen *et al.* reported low amounts of IgG4 in the blood[175] 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. Furthermore, as mentioned in the Theoretical Background section, only a

fraction of the intestinal induced IgG-producing cells returns to lamina propria in the gut, after recirculation, and the different IgG subclasses-producing cells may act through different mechanisms which may lead to differences in the intestinal homing of each subclass. On the other hand, Kett *et al.* reported that only a small amount of IgG4-producing plasma cells is present in the mucosa[174] but, once more, Kett *et al.* study was not based in faecal 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[141].

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 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[175]. However, no significant differences were found between healthy individuals and CD patients which contrasts with Palm *et al*.[155] and Peterson *et al.*[176] who reported that the proportion of IgA-coated bacteria was increased in CD patients comparing to healthy individuals. In terms of relative data, it was observed that the percentage of IgA-coated bacteria in control (3.746 %, median) was lower comparing to CD patients (5.168-6.891 %). Similarly, Waaij *et al.* reported that IBD patients present higher levels of IgA bacteria coating comparing to controls. More specifically, they concluded that controls present 36±12 % whereas IBD patients with active disease contained 69±19% IgA-coated bacteria[177]. Furthermore, they found that patients in remission for more than 2 years also presented higher percentage of IgA-coated bacteria (50±16%) comparing to the controls. The differences concerning the relative data are due to the use of means while in the present study, were analysed the median levels. In addition, their study was based in a mix of 18 CD and UC patients that is a small number of patients and can lead to biased results once the sample of a single patient can drastically change the results.

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, reporting that healthy patients expressed 11±4 % of IgG-coated bacteria in contrast with CD patients that showed 56±32 %[177]. In addition, Harmsen *et al.* found that CD patients had more IgG binding gut bacteria than healthy individuals[157] 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[178]. Moreover, IgG level was found to be normal in patients with at least 2 years of clinical remission[177]*.* In the present work, it was found that the patient's time in remission is unavailable, so it is not possible to know if our results are similar to the ones from Waaij *et al.* However, the difference between the controls and the remission's patients is so minor that it could be hypothesized that patients have been in remission for quite a while. It is thus suggested, that the time of remission could influence the profile of IgG bacteria coating and that in further studies should be included in patient's information.

No significant differences were found for IgG1 and IgG3 between controls and CD patients which is in agreement to Philipsen *et al.* who noted that in CD patients serum, no significant differences were find between these subclasses between active and remission disease[175]. In line with Wang *et al.*[179]*,* 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 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[174,175,180]. 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[173]. Similarly, Rai *et al.* noted that IBD patients display low levels of Ig in serum[181]. Again, the level of Ig in blood may not express the level of Ig in faecal samples.

Since CD is thought to be related with an increased level of IgG coating in active disease, it was expected that the same occurred to IgG subclasses. However, as previously mentioned, an increase of IgG was not observed, thus the results found for the subclasses are consistent.

Patients in remission were expected to present the same profile of antibody-coated bacteria, thus patients that remained in remission should have displayed the same profile between visits. However, the profile between groups was different in all the antibodies coating which may be due with the lower number of patients that maintained remission. In addition, it can be suggested that although disease remained in remission, the time of remission may influence the profile of antibodies thus, in future work it should be consider the time of remission and should be done a study with patients whose disease do not became active between visits.

In the Theoretical Background section, it was hypothesized that CD was related to an IgG2- and IgG3-mediated immune response, coupled with an IgA immune response as well. Thus it was expected that patients with active disease would present increased levels of IgG2, IgG3 and IgA. However, the results of the present study suggest that an increase of IgG4 and a decrease of IgG2 are related to CD immunopathogenesis. Therefore, the results do not support the idea of

CD being associated to a Th1, Th17 and Treg responses. However, the association between Th17 and IgG3 production remains to be validated. No studies concerning the profile of IgA and IgG subclass in CD were found which did not enable a direct comparison of the data displayed in the present work.

Noteworthy, the studies previously mentioned were performed in the 70-90's where the immunological knowledge and technologies were limited. Therefore, it is important that new studies concerning the antibodies levels in different sites will be conducted.

In summary, active disease in CD patients seem to be related to a decreased level of IgG2, an increased level of IgG4 comparing to healthy individuals and IgA production in comparison to patients in remission.

The antibody production showed to be patient specific 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[155]. Even though the profile changes a lot 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. However, it is not possible to predict disease relapse using the Ig profile of coated bacteria since no pattern was found within the groups to enable the distinction between patients whose disease will turn active and patients that will remain in remission.

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.

A correlation analysis showed several correlations between the antibodies however, the correlations were ambiguous because the ones observed for quantitative data differ from the relative data. When taking into account the relative ratio of the total IgG coating, an inverse correlation is seen between both IgG1- and IgG4-coating, and IgG2- and IgG4-coating in CD patients. The negative correlation between IgG1 and IgG4 is supported by the fact that Treg cells, associated to IgG4 production, are capable to inhibit Th2 responses, linked to IgG1. In addition, Treg cells can also inhibit Th1 responses which are linked to IgG2 production and may explain the inverse correlation found between IgG4 and IgG2.

The differences between the results collect by quantitative data and relative data enhance the fact that knowing the actual amount of bacteria coating by a specific antibody may change completely the conclusion taken from the results. The relative data may give a general idea of the coating profile but do not take into account the amount of microorganism present in the sample that is known to be different in healthy individuals and CD patients[182]. For this reason, it should always be considered the amount of bacteria in the faecal samples.

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.

The profile of double bacteria coating revealed that the levels of bacteria coating by all pairs are similar in healthy individuals in CD patients and it was observed that the percentage of patients that have bacteria coated by two antibodies is very low comparing to the percentage coated by only a specific antibody. In line to what was found for the profile of single antibodies coating, the double coating is patient-specific. The results showed that the combined use of two antibodies do not allow the differentiation between healthy individuals and CD patients.

Currently, several disease markers are used to distinguish healthy individuals from CD patients and specifically, distinguishing remission and active disease patients. Since it is suggested that CD is related with a decrease level of IgG2 and an increase level of IgA and IgG4, it was expected that these antibodies show correlations with the used disease markers. The results obtained from quantitative data, relative data and their ratio are, once more, not consistent.

The higher the value of HBI, the strongest is the disease thus, it was expected that HBI was positively correlated with IgG4 and IgA and negatively correlated with IgG2, especially in the patients with active disease. Regarding IgG2, no correlation was found. In general, in remission patients, HBI is observed to be negatively correlated with the amount of bacteria/g faces and the amount of IgG4 coating and positively correlated with the percentage of IgA coating. In patients with exacerbating disease, HBI and IgG1-coated bacteria are significantly negatively correlated. Besides, it appears that HBI, in active disease, is positively correlated with IgA coating and negatively correlated with IgG4-coated bacteria/g faeces. Overall, the positive correlations found between HBI and IgA meet the expectations whereas the correlations found for IgG4 are the opposite of what was expected. Although the negative correlation found was not in agreement with the previous results showed in this study it actually makes sense since lower levels of IgG4 are indicative of a lower regulatory response which occurs when disease is more severe. Overall, HBI do not appear to be a good marker to distinguish the state of CD patients since it showed ambiguous correlations. Similarly, Falvey *et al.* reported that HBI was not able to distinguish active from inactive Crohn's disease[183]. It is important to notice that HBI is a subjective measure since takes into account the patient's reports.

For C-reactive protein, are only found correlations for the first visit, where all the patients were in remission. CRP is negatively correlated with the amount of IgA and IgG1 coating, and the IgG1 relative ratio of total IgG. On the other hand, it was found a positive correlation between CRP and the IgG4 relative ratio of total IgG. Considering that higher CRP values correlate with worse disease activity, the correlations found for IgG4 is in accordance to what was expected. No explanation was found for the other correlations but it should be noted that CRP is not specific for intestinal inflammation thus, the correlation found in remission patients could be related to an

extraintestinal inflammations that are measured by this biomarker. In addition, Falvey *et al.* reported that C-reactive proteins was good to predict active disease but not to characterise remission disease, although in the present study no correlation was found for active disease[183]. Furthermore, Vermeire *et al.* found that one third of CD patients that had normal CRP were actually with active disease and one third had raised CRP but clinically inactive disease, which explains the low specificity of CRP for CD.

Faecal Calprotectin showed to be positively correlated with the amount of IgA coating in CD patients. The positive correlation is in accordance with what was expected since it was observed that IgA was increased in active disease and higher values of faecal calprotectin are associated with increased severity disease. Although no significant correlations were found between FC and IgG2, it was observed that IgG2 positively correlates with FC, both in remission and active disease which is in agreement with the studies that correlated CD with an increase of the level of IgG2[174,175,180]. In addition, the low number of correlations found between FC and the Ig amounts are in accordance with Harmsen *et al.,* who reported that the amount of IgG coating was not correlated with calprotectin levels and that could mean that patients had a gut with an impaired barrier function[157]. Furthermore, although FC is a better marker for mucosal inflammation than, for example CRP, it shows limit sensitivity for the proximal colon and small bowel[184].

Correlations for the last disease marker, Bristol Stool Scale, illustrate 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, as previously mentioned, 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, once again, is in agreement with the reports that associate CD to a IgG2 increase[174,175,180]. 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.

A further detailed study of the Ig profile when patients are divided in two groups, accordingly with BS values (G1: patients classified with 3, 4 and 4 in BS and G2: classification of 6 or 7), shows that the bacteria number is higher in G1 comparing to G2. Once again, this result is expected since G2 is comprised of samples with higher content of liquid thus less faecal content and less bacteria. In this study the profile of Ig coating was studied separately in samples from visit 1 and visit 2. It was observed that in visit 1, the profile of Ig coating in the two groups is relatively equal which was expected since at that time all the patients were in remission. An exception is found for IgG4 coating where a significant decrease is observed between the median level of G1 and G2. The decrease is observed both in visit 1 and visit 2 thus, it is reasonable to assume that CD is associated with a decreased IgG4 level since both patients with active disease and in remission display this decrease. By contrast, in visit 2, where 34 patients remained in remission and 19 had active disease, only IgG1 and IgG3 level were similar in the two groups. In

addition, the amount of IgG2 coating was increased in G2 comparing to G1 which, again, may reveal the possible relation between the level of IgG2 and Crohn's Disease.

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.

Regarding disease phenotype, it was found that a penetrating behaviour display increased levels of IgG2. On the other hand, a stricturing behaviour is associated to an increased level of IgG4-coated bacteria. Stricturing involves excessive scar formation and may result in higher transit time, since IgG4 is thought to mediate, to a certain level, transepithelial transport, it can be hypothesised that the strictures do need higher amount of IgG4 to help in the food passage. Finally, it is observed that more aggressive disease, when it comes to complications, implies lower amount of bacteria which once more shows that CD patients are characterized by a reduced amount of microorganisms.

The colon appears to be the most distinctive location since it is observed an increased level of IgA-, IgG2-, IgG3 and IgG4-coated bacteria. It is known that the mucosal immune system interacts more with the small intestine which could mean that patients that had disease in the ileum or in both in ileum and colon would present a higher amount of IgG2 than patients whose disease only affect the colon, which is not the case. However, it was found that the colon is the region showing higher level of bacteria which can be related to the fact that, as well known, colon is the intestine's region with higher population of microorganism. Although, the results are not consistent between remission and active patients, the results show, once more, that CD may be related to an increase of IgA, IgG2 and IgG4. It must be noted that only one patient presented disease in colon, ileum and upper gastrointestinal tract and the same happen for ileum and upper gastrointestinal tract.

A risk factor associated to Crohn's disease is the age at diagnosis however, no association was found to the antibodies profile. The profile of Ig coating does not significantly change with the different classes of age. It must be noted that only two patients were diagnosed before 16 years old.

For smoking history, it is observed an increased in both IgA- and IgG2-coating and a decreased in IgG1, IgG4-coating, and in the number of bacteria/g faeces, although no significant associations were found. Based on the knowledge that smoking is a risk factor for CD and considered that previously it was shown that increased IgA and IgG2 as well a decrease level of IgG4 were related to the disease, it may confirm the importance of smoking history for CD patients. However, it is important to note that the increased level of IgG2 is based in only one patient.

Finally, it was examined whether an association could be found to patient's gender although CD is considered to be no sex-specific. It was observed that Females and Males display different profiles of Ig coating although that is contrary to studies that reported that IgA and IgG profiles was the same[185]. Therefore, it is shown that Females are associated with higher amounts of IgG2 whereas Males have higher levels of IgA- and IgG4-coated bacteria. In line with that, French *et*

al. reported that males presented higher concentration of IgG4 in serum samples than woman[127]. Studies comparing the antibodies concentrations in faecal samples of men and women were not found.

Finally, it should be noted that the Ig profiles were rather similar in healthy individuals and CD patients not only when patients were divided accordingly to their disease state, but also when were divided into phenotypes, location, age at diagnosis, smoking history and gender. The similar profiles must be related to the use of medicines since all the patients were taking drugs at the time of sample collection or at least had taken previously to that thus the CD patients were showing the same profile than the controls. Although it is known the specific medicines that each patients was taking, it was not possible to analyse the implication of those drugs in the Ig profiles once it is not possible to determine what drug affects what, particularly because most of the patients were taking more than one at the time. In agreement to that, several studies[156,182,186] reported that antibiotics, thiopurines and anti-TNF, some of the medicines used in CD patients, affect the gut microbial composition and diversity which has implication on the Ig profile. In future work, is crucial to use samples of CD patients that were not in treatment and, in addition, to do a longitudinal study of those patients *i.e.,* study the alterations that are found in the Ig Profile when patients start treatment.

Furthermore, it must be noted that in the present work, there was a lack of patients that displayed the same characteristics which led to biased results. The following topics are a summary of the limitations of the previous study as well as suggestion that should be used in future studies, so that a more comprehensive view of the profile of bacteria coating by Immunoglobulins in Crohn's disease could be obtained:

- Have samples of patients that were never treated, samples of before and after the treatment; samples of patients that were taking only one type of medicine instead of a mix of medicines to understand the role of each medicine;
- Have a higher number of controls; equal numbers of female and male patients; equal numbers of patients diagnosed: at A1; A2 and A3; with B1; B2 and B3; and with disease in L1; L2; L3; L3&L4 and L1&L4; same number of patients that never smoked; were smokers or are smokers; and equal number of patients that remained in remission and patients whose disease change to active state and follow the time that patients remain in remission.

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.

VI. References

1. Tarabova, L., Makova, Z., Piesova, E., Szaboova, R. & Faixova, Z. Intestinal Mucus Layer and Mucins (A Review). *Folia Vet.* **60,** 21–25 (2016).
2. Purchiaroni, F. *et al.* The role of intestinal microbiota and the immune system. *Eur. Rev. Med. Pharmacol. Sci.* **17,** 323–333 (2013).
3. Martinez-Guryn, K. *et al.* Small Intestine Microbiota Regulate Host Digestive and Absorptive Adaptive Responses to Dietary Lipids. *Cell Host Microbe* **23,** 458–469.e5 (2018).
4. Marchiando, K. C. . Function of Epithelial Barriers. in *Encyclopedia of Cell Biology* (ed. Stahl, R. A. and B. P. D.) 687–694 (2016).
5. Mowat, A. M. & Agace, W. W. Regional specialization within the intestinal immune system. *Nat. Rev. Immunol.* **14,** 667–685 (2014).
6. Okumura, R. & Takeda, K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. *Exp. Mol. Med.* **49,** e338 (2017).
7. McGhee, J. R. & Fujihashi, K. Inside the Mucosal Immune System. *PLoS Biol.* **10,** 1–5 (2012).
8. Ramanan, D. & Cadwell, K. Intrinsic Defense Mechanisms of the Intestinal Epithelium. *Cell Host Microbe* **19,** 434–441 (2016).
9. Peterson, L. W. & Artis, D. Intestinal epithelial cells: Regulators of barrier function and immune homeostasis. *Nat. Rev. Immunol.* **14,** 141–153 (2014).
10. Sartor, R. B. The intestinal microbiota in inflammatory bowel diseases. *Nestle Nutr Inst Work. Ser* **79,** 29–39 (2014).
11. Torres, J., Mehandru, S., Colombel, J. F. & Peyrin-Biroulet, L. Crohn's disease. *Lancet* (2017). doi:10.1016/S0140-6736(16)31711-1
12. Molodecky, N. A. *et al.* Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* **142,** 46–54.e42 (2012).
13. Ng, S. C. *et al.* Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* **390,** 2769–2778 (2017).
14. Baumgart, D. C. & Sandborn, W. J. Crohn's disease. *Lancet* **380,** 1590–1605 (2012).
15. Strober, W., Fuss, I. and Mannon, P. The fundamental basis of inflammatory bowel disease. *J Clin Invest* **117,** 514–521 (2007).
16. Louis, E. *et al.* Behaviour of Crohn's disease according to the Vienna classification: changing pattern over the course of the disease. *Gut* **49,** 777–782 (2001).
17. Neurath, M. F. Crohn's Disease Chapter Outline. (2015). doi:10.1016/B978-0-12-415847-4.00082-3
18. Solberg, I. C. *et al.* Clinical Course in Crohn's Disease: Results of a Norwegian Population-Based Ten-Year Follow-Up Study. *Clin. Gastroenterol. Hepatol.* **5,** 1430–1438 (2007).
19. Tontini, G. E., Vecchi, M., Pastorelli, L., Neurath, M. F. & Neumann, H. Differential diagnosis in inflammatory bowel disease colitis: State of the art and future perspectives. *World J. Gastroenterol.* (2015). doi:10.3748/wjg.v21.i1.21
20. Strong, S. A. *A service of the National Library of Medicine, National Institutes of Health. Holzheimer RG, Mannick JA, editors. Surgical Treatment: Evidence-Based and Problem-Oriented*. (2001).
21. Pizarro, T. T., Arseneau, K. O., Bamias, G. & Cominelli, F. Mouse models for the study of Crohn's disease. *Trends in Molecular Medicine* (2003). doi:10.1016/S1471-4914(03)00052-2
22. Manichanh, C., Borruel, N., Casellas, F. & Guarner, F. The gut microbiota in IBD. *Nat. Rev. Gastroenterol. Hepatol.* **9,** 599–608 (2012).
23. Annese, V. *et al.* Clinical features in familial cases of Crohn's disease and ulcerative colitis in Italy: a GISC study. Italian Study Group for the Disease of Colon and Rectum. *Am. J. Gastroenterol.* **96,** 2939–45 (2001).
24. Brant, S. Update on the heritability of inflammatory bowel disease: the importance of twin studies. *Inflamm Bowel Dis.* **17,** 1–5 (2011).
25. Liu, J. Z. & Anderson, C. A. Genetic studies of Crohn's disease: Past, present and future. *Best Pract. Res. Clin. Gastroenterol.* (2014). doi:10.1016/j.bpg.2014.04.009
26. Limbergen, J. Van, Wilson, D. C. & Satsangi, J. The Genetics of Crohn's Disease. (2009). doi:10.1146/annurev-genom-082908-150013
27. Hugot, J. *et al.* Mapping of a susceptibility locus for Crohn's disease on chromosome 16.

Nature **379,** 821–823 (1996).
28. Hugot, J. P. *et al.* Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411,** 599–603 (2001).
29. Ogura, Y. *et al.* Expression of NOD2 in Paneth cells: A possible link to Crohn's ileitis. *Gut* **52,** 1591–1597 (2003).
30. Inohara, N. *et al.* Host recognition of bacterial muramyl dipeptide mediated through NOD2: Implications for Crohn's disease. *J. Biol. Chem.* **278,** 5509–5512 (2003).
31. Magalhaes, J. G. Nod2-dependent Th2 polarization of antigen-specific. *J Immunol* **181,** 7925–35. (2008).
32. Schreiber, S., Nikolaus, S. & Hampe, J. Activation of nuclear factor kappa B inflammatory bowel disease. *Gut* **42,** 477–84 (1998).
33. Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A. & Karin, M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science (80-.).* **270,** 286–90 (1995).
34. Watanabe, T., Kitani, A., Murray, P. J. & Strober, W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat. Immunol.* **5,** 800–808 (2004).
35. O'Neill, L. A. J. M. How NOD-ing off leads to Crohn disease. **5,** 775–776 (2004).
36. Lala, S. *et al.* Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology* **125,** 47–57 (2003).
37. Gasche, C. & Grundtner, P. Genotypes and phenotypes in Crohn's disease: Do they help in clinical management? *Gut* **54,** 162–167 (2005).
38. Simms, L. A. *et al.* Reduced α-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. *Gut* **57,** 903–910 (2008).
39. Reference, G. H. Genetics Home Reference. *National Library of Medicine (US).* (2017). Available at: https://ghr.nlm.nih.gov/condition/noonan-syndrome.
40. Levine, B., Mizushima, N. & Virgin, H. W. Autophagy in immunity and inflammation. *Nature* **469,** 323–335 (2011).
41. Cadwell, K. *et al.* A unique role for autophagy and Atg16L1 in Paneth cells in murine and human intestine. *Nature* **456,** 259–263 (2009).
42. Wallace, K. L., Zheng, L. B., Kanazawa, Y. & Shih, D. Q. Immunopathology of inflammatory bowel disease. *World J. Gastroenterol.* **20,** 6–21 (2014).
43. Sidiq, T., Yoshihama, S., Downs, I. & Kobayashi, K. S. Nod2: A critical regulator of ileal microbiota and Crohn's disease. *Front. Immunol.* **7,** 18–20 (2016).
44. Cooney, R. *et al.* NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat. Med.* **16,** 90–97 (2010).
45. Danese, S., Sans, M. & Fiocchi, C. Inflammatory bowel disease: The role of environmental factors. *Autoimmunity Reviews* (2004). doi:10.1016/j.autrev.2004.03.002
46. Lakatos, P. L., Szamosi, T. & Lakatos, L. Smoking in inflammatory bowel diseases: Good, bad or ugly? *World J. Gastroenterol.* **13,** 6134–6139 (2007).
47. Rodríguez, L. A. G., Ruigómez, A. & Panés, J. Acute Gastroenteritis Is Followed by an Increased Risk of Inflammatory Bowel Disease. *Gastroenterology* **130,** 1588–1594 (2006).
48. Kiesslich, R. *et al.* Local barrier dysfunction identified by confocal laser endomicroscopy predicts relapse in inflammatory bowel disease. *Gut* **61,** 1146–1153 (2012).
49. Moussata, D. *et al.* Confocal laser endomicroscopy is a new imaging modality for recognition of intramucosal bacteria in inflammatory bowel disease in vivo. *Gut* **60,** 26–33 (2011).
50. Swidsinski, A. *et al.* Mucosal flora in inflammatory bowel disease. *Gastroenterology* **122,** 44–54 (2002).
51. Nishida, A. *et al.* Gut microbiota in the pathogenesis of inflammatory bowel disease. *Clin. J. Gastroenterol.* **11,** 1–10 (2018).
52. Eckburg, P. B. *et al.* Diversity of the Human Intestinal Microbial Flora. *Science (80-.).* **308,** 1635–1638 (2005).
53. Sartor, R. B. Microbial influences in inflammatory bowel diseases. *Gastroenterology* **134,** 577–594 (2008).
54. Rajilić-Stojanović, M. Function of the microbiota. *Best Pr. Res Clin Gastroenterol.* **27,** 5–16 (2013).
55. Ramakrishna, B. S. Role of the gut microbiota in human nutrition and metabolism. *J. Gastroenterol. Hepatol.* **28,** 9–17 (2013).
56. Sommer, F. & Bäckhed, F. The gut microbiota--masters of host development and physiology. *Nat. Rev. Microbiol.* **11,** 227–238 (2013).

57. Mazmanian, S. K., Cui, H. L., Tzianabos, A. O. & Kasper, D. L. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* **122,** 107–118 (2005).
58. Ewald, D. R. & Sumner, S. C. J. Human microbiota, blood group antigens, and disease. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **10,** 1–44 (2018).
59. Lepage, P. *et al.* Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. *Gastroenterology* **141,** 227–236 (2011).
60. Kostic, A. D., Xavier, R. J. & Gevers, D. The Microbiome in Iflammatory Bowel Diseases: Current Status and the Future Ahead. *Gastroenterology* **146,** 1489–1499 (2015).
61. Atarashi, K. *et al.* Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* **500,** 232–236 (2013).
62. Machiels, K. *et al.* A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. *Gut* **63,** 1204–1205 (2013).
63. Sokol, H. *et al.* Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc. Natl. Acad. Sci.* **105,** 16731–16736 (2008).
64. Darfeuille-Michaud, A. *et al.* High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* **127,** 412–421 (2004).
65. Testa, A., Castiglione, F., Nardone, O. M. & Colombo, G. L. Adherence in ulcerative colitis: An overview. *Patient Prefer. Adherence* **11,** 297–303 (2017).
66. Kirkwood, C. *et al.* Mycobacterium avium subspecies paratuberculosis in children with early-onset Crohn's disease. *Inflamm Bowel Dis.* **15,** 1643–55 (2009).
67. Friswell, M., Campbell, B. & Rhodes, J. The role of bacteria in the pathogenesis of inflammatory bowel disease. *Gut Liver* **4,** 295–306 (2010).
68. Ferwerda, G. *et al.* Mycobacterium paratuberculosis is recognized by Toll-like receptors and NOD2. *J. Leukoc. Biol.* **82,** 1011–1018 (2007).
69. Gutierrez, M. G. *et al.* Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. *Cell* **119,** 753–766 (2004).
70. Elguezabal, N. *et al.* Lactase persistence, NOD2 status and Mycobacterium avium subsp. paratuberculosis infection associations to Inflammatory Bowel Disease. *Gut Pathog.* **4,** 1–9 (2012).
71. Juste, C. *et al.* Bacterial protein signals are associated with Crohn's disease. *Gut* **63,** 1566–1577 (2014).
72. Prantera, C. *et al.* Antibiotic treatment of Crohn's disease: Results of a multicentre, double blind, randomized, placebo-controlled trial with rifaximin. *Aliment. Pharmacol. Ther.* **23,** 1117–1125 (2006).
73. Butterworth, A., Thomas, A. & Akobeng, A. Probiotics for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* **16,** (2008).
74. Eiseman, B., Silen, W., Bascom, G. & Kauvar, A. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. **44,** 854–9 (1958).
75. Quraishi, M. N. *et al.* Systematic review with meta-analysis: the efficacy of faecal microbiota transplantation for the treatment of recurrent and refractory *Clostridium difficile* infection. *Aliment. Pharmacol. Ther.* **46,** 479–493 (2017).
76. van Nood, E. *et al.* Duodenal Infusion of Donor Feces for Recurrent *Clostridium difficile*. *N. Engl. J. Med.* **368,** 407–415 (2013).
77. Suskind, D. L. *et al.* Fecal mircobial translplant effect on clinical outcomes and fecal microbiome in active Crohn's disease. *Inflamm Bowel Dis.* **21,** 556–563 (2015).
78. Colman, R. J. & Rubin, D. T. Fecal Microbiota Transplantation as Therapy for Inflammatory Bowel Disease: A Systematic Review and Meta-Analysis. *J Crohns Colitis* **8,** 1569–1581 (2014).
79. Smith, A. M. *et al.* Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J. Exp. Med.* **206,** 1883–1897 (2009).
80. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Genetics* **8,** 958–969 (2009).
81. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol.* **3,** 133–46 (2003).
82. Li, N. & Shi, R. Update review on immune factors in pathogenesis of Croh's disease. *World J. Gastroenterol.* **1,** 15–22 (2018).

83. Park, J. H., Peyrin-Biroulet, L., Eisenhut, M. & Shin, J. Il. IBD immunopathogenesis: A comprehensive review of inflammatory molecules. *Autoimmun. Rev.* **16,** 416–426 (2017).
84. Omenetti, S. & Pizarro, T. T. The Treg/Th17 axis: A dynamic balance regulated by the gut microbiome. *Front. Immunol.* **6,** 1–8 (2015).
85. Oh, S. A. & Li, M. O. TGF- : Guardian of T Cell Function. *J. Immunol.* **191,** 3973–3979 (2013).
86. Gaffen, S. L., Jain, R., Garg, A. V. & Cua, D. J. IL-23-IL-17 immune axis: Discovery, Mechanistic Understanding, and Clinical Testing. **14,** 585–600 (2015).
87. McGeachy, M. J. *et al.* TGF-β and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell–mediated pathology. *Nat Immunol.* **8,** pages 1390–1397 (2007).
88. Schmidt, C. *et al.* Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin- 27p28 in Crohn's disease but not in ulcerative colitis. *Inflamm. Bowel Dis.* **11,** 16–23 (2005).
89. Sakuraba, A. *et al.* Th1/Th17 Immune Response Is Induced by Mesenteric Lymph Node Dendritic Cells in Crohn's Disease. *Gastroenterology* **137,** 1736–1745 (2009).
90. Panaccione, R. *et al.* Briakinumab for treatment of Crohn's disease: Results of a randomized trial. *Inflamm. Bowel Dis.* **21,** 1329–1340 (2015).
91. Sandborn, W. J., Gasink, C., Blank, M., Lang, Y. & Johanns, J. O-001 a multicenter, double-blind, placebo-controlled phase3 study of ustekinumab, a human IL-12/23P40 mAB, in moderate-service Crohn's disease refractory to anti-TFN alpha: UNITI-1. *Inflamm. Bowel Dis.* **22,** (2016).
92. Kinugasa, T., Sakaguchi, T., Gu, X. & Reinecker, H. C. Claudins regulate the intestinal barrier in response to immune mediators. *Gastroenterology* **118,** 1001–1011 (2000).
93. Roeleveld, D. M. *et al.* Higher efficacy of anti-IL-6/IL-21 combination therapy compared to monotherapy in the induction phase of Th17-driven experimental arthritis. *PLoS One* **12,** 1–16 (2017).
94. Raza, A., Yousaf, W., Giannella, R. & Shata, M. T. Th17 cells: interactions with predisposing factors in the immunopathogenesis of inflammatory bowel disease. *Expert Rev Clin Immunol* **6,** 161–168 (2013).
95. Mitsuyama, K. *et al.* IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease. *Clin. Exp. Immunol.* **96,** 432–6 (1994).
96. Huh, J. R. & Littman, D. R. Small molecule inhibitors of RORγt: Targeting Th17 cells and other applications. *Eur. J. Immunol.* **42,** 2232–2237 (2012).
97. Carrier, Y., Yuan, J., Kuchroo, V. K. & Weiner, H. L. Th3 Cells in Peripheral Tolerance. II. TGF- -Transgenic Th3 Cells Rescue IL-2-Deficient Mice from Autoimmunity. *J. Immunol.* **178,** 172–178 (2007).
98. Azizi, G., Yazdani, R. & Mirshafiey, A. Th22 cells in autoimmunity: a review of current knowledge. *Eur Ann Allergy Clin Immunol.* **47,** 108–17 (2015).
99. Guo, X. *et al.* Induction of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection against intestinal infection. *Immunity* **40,** 25–39 (2014).
100. Bernink, J. H., Peters, C. P., Munneke, M. & Spits, H. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat. Immunol.* **14,** 221–229 (2013).
101. Hutnick, N. A., Kunisawa, J., Shibata, N. & Grunberg, S. Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria. *Science (80-.).* **336,** 1321–1325 (2013).
102. Hepworth, M. R. *et al.* Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4+ T cells. **348,** 1031–1035 (2015).
103. Bernink, J. H. *et al.* Interleukin-12 and -23 Control Plasticity Of Cd127+Group 1 And Group 3 Innate Lymphoid Cells In The Intestinal Lamina Propria. *Immunity* **43,** 146–160 (2015).
104. Janeway CA, J., Travers, P. & Walport, M. The Humoral Immune Response. in *Immunobiology: The Immune System in Health and Disease* (Garland Science, 2001).
105. Rispens, T. & Vidarsson, G. Human IgG Subclasses. in *Antibody Fc: Linking Adaptive and Innate Immunity* (eds. Ackerman, M. E. & Nimmerjahn, F.) 159–177 (Academic Press, 2014). doi:10.1016/B978-0-12-394802-1.00009-1
106. Lu, L. L., Suscovich, T. J., Fortune, S. M. & Alter, G. Beyond binding: Antibody effector functions in infectious diseases. *Nat. Rev. Immunol.* **18,** 46–61 (2018).
107. Bunker, J. J. *et al.* Natural polyreactive IgA antibodies coat the intestinal microbiota. *Science (80-.).* **358,** (2017).
108. Lu, L. L., Suscovich, T. J., Fortune, S. M. & Alter, G. Beyond binding: Antibody effector functions in infectious diseases. *Nat. Rev. Immunol.* **18,** 46–61 (2018).

109. Schroeder, H. W. J. & Cavacini, L. Structure and Function of Immunoglobulins. *J. Allergy Clin. Immunol.* **125,** S41–S52 (2010).
110. Berczi, I. & Szentivanyi, A. Immunoglobulins. in *The Immune-Neuroendocrine Circuitry* (ed. Szentivanyi, I. B. and A.) 117–127 (2003).
111. Pabst, O. New concepts in the generation and functions of IgA. *Nat. Rev. Immunol.* **12,** 821–832 (2012).
112. Wijburg, O. L. C. *et al.* Innate secretory antibodies protect against natural *Salmonella typhimurium* infection. *J. Exp. Med.* **203,** 21–26 (2006).
113. Macpherson, A. J., Köller, Y. & McCoy, K. D. The bilateral responsiveness between intestinal microbes and IgA. *Trends Immunol.* **36,** 460–470 (2015).
114. He, B. *et al.* Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. *Immunity* **26,** 812–826 (2007).
115. Fagarasan, S., Kinoshita, K., Muramatsu, M., Ikuta, K. & Honjo, T. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* **413,** 639–643 (2001).
116. Boursier, L., Gordon, J. N., Thiagamoorthy, S., Edgeworth, J. D. & Spencer, J. Human intestinal IgA response is generated in the organized gut-associated lymphoid tissue but not in the lamina propria. *Gastroenterology* **128,** 1879–1889 (2005).
117. Niro, R. Di *et al.* Abundance and unique repertoire of plasma cells secreting IgA autoantibodies to transglutaminase 2 in the intestinal lesion of celiac disease. **18,** 441–445 (2015).
118. Avery, D. T., Bryant, V. L., Ma, C. S., de Waal Malefyt, R. & Tangye, S. G. IL-21-Induced Isotype Switching to IgG and IgA by Human Naive B Cells Is Differentially Regulated by IL-4. *J. Immunol.* **181,** 1767–1779 (2008).
119. Tangye, S. G., Ferguson, A., Avery, D. T., Ma, C. S. & Hodgkin, P. D. Isotype Switching by Human B Cells Is Division-Associated and Regulated by Cytokines. *J. Immunol.* **169,** 4298–4306 (2002).
120. Cerutti, A. The regulation of IgA class switching. *Nat. Rev. Immunol.* **8,** 421–434 (2008).
121. Cerutti, A. *et al.* CD40 ligand and appropriate cytokines induce switching to IgG, IgA, and IgE and coordinated germinal center and plasmacytoid phenotypic differentiation in a human monoclonal IgM+IgD+ B cell line. *J. Immunol.* **160,** 2145–57 (1998).
122. Rousset, F. *et al.* Interleukin-10 induces immunoglobulin G isotype switch recombination in human CD40-activated naive B lymphocytes. *Proc Natl Acad Sci U S A* **89,** 1890–1893 (1992).
123. Fayette, J. *et al.* Human Dendritic Cells Skew Isotype Switching of CD40-activated Naive B Cells towards IgA $_1$ and IgA $_2$. *J. Exp. Med.* **185,** 1909–1918 (1997).
124. Li, M. O. & Flavell, R. A. Contextual Regulation of Inflammation: A Duet by Transforming Growth Factor-β and Interleukin-10. *Immunity* **28,** 468–476 (2008).
125. Vidarsson, G., Dekkers, G. & Rispens, T. IgG subclasses and allotypes: From structure to effector functions. *Front. Immunol.* **5,** 1–17 (2014).
126. MacDermott, R. P. & Nahm, M. H. Expression of human immunoglobulin G subclasses in inflammatory bowel disease. *Gastroenterology* **93,** 1127–1129 (1987).
127. French, M. A. & Harrison, G. Serum IgG subclass concentrations in healthy adults: a study using monoclonal antisera. *Clin. Exp. Immunol.* **56,** 473–5 (1984).
128. Jefferis, R. & Kumararatne, D. S. Selective IgG subclass deficiency: quantification and clinical relevance. *Clin. Exp. Immunol.* **81,** 357–67 (1990).
129. Bindon, B. Y. C. I., Hale, G. & Bruggemann, M. Human monoclonal igG isotypes differ in complement activating function at the level of C4 as well as C1q. **168,** (1988).
130. Kawano, Y., Noma, T. & Yata, J. Regulation of human IgG subclass production by cytokines. IFN-gamma and IL-6 act antagonistically in the induction of human IgG1 but additively in the induction of IgG2. *J. Immunol.* **153,** 4948–58 (1994).
131. Pene, J. *et al.* Cutting Edge: IL-21 Is a Switch Factor for the Production of IgG1 and IgG3 by Human B Cells. *J. Immunol.* **172,** 5154–5157 (2004).
132. Boumendjel, A. *et al.* IL-27 induces the production of IgG1 by human B cells. *Eur. Cytokine Netw.* **17,** 281–289 (2006).
133. Berger, A. Science commentary: Th1 and Th2 responses: what are they? *Bmj* **321,** 424–424 (2000).
134. Mutapi, F. *et al.* Differential recognition patterns of Schistosoma haematobium adult worm antigens by the human antibodies IgA, IgE, IgG1 and IgG4. *Parasite Immunol.* **33,** 181–

192 (2011).
135. Siber, G. R., Schur, P. H., Aisenberg, A. C., Weitzman, S. A. & Schiffman, G. Correlation between Serum IgG-2 Concentrations and the Antibody Response to Bacterial Polysaccharide Antigens. *N Engl J Med* **4,** 178–82 (1980).
136. Hjelholt, A., Christiansen, G., Sørensen, U. S. & Birkelund, S. IgG subclass profiles in normal human sera of antibodies specific to five kinds of microbial antigens. *Pathog. Dis.* **67,** 206–213 (2013).
137. Latiff, A. H. A. & Kerr, M. A. The clinical significance of immunoglobulin A deficiency The clinical significance of immunoglobulin A deficiency. 131–139 (2007). doi:10.1258/000456307780117993
138. Zuniga, R. *et al.* Identification of IgG subclasses and C-reactive protein in lupus nephritis: The relationship between the composition of immune deposits and Fcγ receptor type IIA alleles. *Arthritis Rheum.* **48,** 460–470 (2003).
139. Al-Darmaki, S. *et al.* Delineation of the Role of Platelet-Activating Factor in the Immunoglobulin G2 Antibody Response. *Clin. Vaccine Immunol.* **11,** 720–728 (2004).
140. Hammarström, L. & Smith, C. I. IgG2 deficiency in a healthy blood donor. Concomitant lack of IgG2, IgA and IgE immunoglobulins and specific anti-carbohydrate antibodies. *Clin. Exp. Immunol.* **51,** 600–604 (1983).
141. Stapleton, N. M. *et al.* Competition for FcRn-mediated transport gives rise to short half-life of human IgG3 and offers therapeutic potential. *Nat. Commun.* **2,** 599 (2011).
142. Briere, F. Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. *J. Exp. Med.* **179,** 757–762 (1994).
143. Fujieda, S., Saxon, A. & Zhang, K. E. Direct evidence that γ1 and γ3 switching in human B cells is interleukin-10 dependent. *Mol. Immunol.* **33,** 1335–1343 (1996).
144. Rispens, T. & Vidarsson, G. Human IgG Subclasses. *Antib. Fc Link. Adapt. Innate Immun.* 159–177 (2013). doi:10.1016/B978-0-12-394802-1.00009-1
145. Tsuboi, H. *et al.* Analysis of IgG4 class switch-related molecules in IgG4-related disease. *Arthritis Res. Ther.* **14,** (2012).
146. Jeannin, P., Lecoanet, S., Delneste, Y., Gauchat, J. F. & Bonnefoy, J. Y. IgE versus IgG4 production can be differentially regulated by IL-10. *J. Immunol.* **160,** 3555–61 (1998).
147. Jutel, M. & Akdis, C. A. Immunological mechanisms of allergen-specific immunotherapy. *Allergy Eur. J. Allergy Clin. Immunol.* **66,** 725–732 (2011).
148. Aalberse, R. C., Stapel, S. O., Schuurman, J. & Rispens, T. Immunoglobulin G4: An odd antibody. *Clin. Exp. Allergy* **39,** 469–477 (2009).
149. Asano, M. & Komiyama, K. Polymeric immunoglobulin receptor. *J. Oral Sci.* **53,** 147–156 (2011).
150. Cerutti, A. & Rescigno, M. The Biology of Intestinal Immunoglobulin A Responses. *Immunity* **28,** 1199–1216 (2008).
151. Yoshida, M. *et al.* Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. *Immunity* **20,** 769–783 (2004).
152. Claypool, S. M. *et al.* Bidirectional Transepithelial IgG Transport by a Strongly Polarized Basolateral Membrane Fc□-Receptor. *Mol. Biol. Cell* **15,** 3751–3737 (2004).
153. Abdiche, Y. N. *et al.* The neonatal Fc receptor (FcRn) binds independently to both sites of the IgG homodimer with identical affinity. *MAbs* **7,** 331–343 (2015).
154. Tedjo, D. I. *et al.* The fecal microbiota as a biomarker for disease activity in Crohn's disease. *Sci. Rep.* **6,** 1–10 (2016).
155. Palm, N. W. *et al.* Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* **158,** 1000–1010 (2014).
156. Frehn, L. *et al.* Distinct patterns of IgG and IgA against food and microbial antigens in serum and feces of patients with inflammatory bowel diseases. *PLoS One* **9,** (2014).
157. Harmsen, H. J. M., Pouwels, S. D., Funke, A., Bos, N. A. & Dijkstra, G. Crohn's disease patients have more IgG-binding fecal bacteria than controls. *Clin. Vaccine Immunol.* **19,** 515–521 (2012).
158. Wu, K. C., Mahida, Y. R., Priddle, J. D. & Jewell, D. P. Immunoglobulin production by isolated intestinal mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin. Exp. Immunol.* **78,** 37–41 (1989).
159. Rüthlein, J., Ibe, M., Burghardt, W., Mössner, J. & Auer, I. O. Immunoglobulin G (IgG), IgG1, and IgG2 determinations from endoscopic biopsy specimens in control, Crohn's disease, and ulcerative colitis subjects. *Gut* **33,** 507–512 (1992).

160. Macpherson, A., Khoo, U. Y., Forgacs, I. & Bjamason, I. Mucosal antibodies in inflammatory bowel diseases are directed against intestinal bacteria. 365–375 (1996).
161. MacDermott, R. P. *et al.* Alterations in Serum Immunoglobulin G Subclasses in Patients With-Ulcerative Colitis and Crohn's Disease. *Gastroenterology* **96,** 764–768 (1989).
162. Kett, K., Rognum, T. O. & Brandtzaeg, P. Mucosal subclass distribution of immunoglobulin G-producing cells is different in ulcerative colitis and Crohn's disease of the colon. *Gastroenterology* **93,** 919–924 (1987).
163. Weeke, B. & Bendixen, G. Serum immunoglobulins and organ-specific, cellular hypersensitivity in ulcerative colitis and Crohn's Disease. *Acta med. scand.* **186,** 87–91 (1969).
164. Rowley, T. Flow Cytometry - A Survey and the Basics. *Labome* **26,** 2034–5 (2012).
165. Biosciences, B. An introduction to compensation for multicolor assays on digital flow cytometers. *BD Biosci. Tech. Bull.* 1–12 (2009).
166. Vermeire, S., Schreiber, S., Sandborn, W. J., Dubois, C. & Rutgeerts, P. Correlation Between the Crohn's Disease Activity and Harvey-Bradshaw Indices in Assessing Crohn's Disease Severity. *Clin. Gastroenterol. Hepatol.* **8,** 357–363 (2010).
167. Chang, S., Malter, L. & Hudesman, D. Disease monitoring in inflammatory bowel disease. *World J. Gastroenterol.* **21,** 11246–11259 (2015).
168. Benítez, J. M. Faecal calprotectin: Management in inflammatory bowel disease. *World J. Gastrointest. Pathophysiol.* **6,** 203 (2015).
169. Satsangi, J., Silverberg, M. S., Vermeire, S. & Colombel, J. F. The Montreal classification of inflammatory bowel disease: Controversies, consensus, and implications. *Gut* **55,** 749–753 (2006).
170. Vandeputte, D. *et al.* Quantitative microbiome profiling links gut community variation to microbial load. *Nature* **551,** 507–511 (2017).
171. Bunker, J. J. *et al.* Natural polyreactive IgA antibodies coat the intestinal microbiota. *Science (80-.).* **358,** (2017).
172. Buckner, C. M. *et al.* CXCR4/IgG-expressing plasma cells are associated with human gastrointestinal tissue inflammation. *J. Allergy Clin. Immunol.* **133,** 1676–1685.e5 (2014).
173. Helgeland, L. *et al.* IgG subclass distribution in serum and rectal mucosa of monozygotic twins with or without inflammatory bowel disease. *Gut* **33,** 1358–1364 (1992).
174. Kett, K., Rognum, T. O. & Brandtzaeg, P. Mucosal subclass distribution of immunoglobulin G-producing cells is different in ulcerative colitis and Crohn's disease of the colon. *Gastroenterology* **93,** 919–924 (1987).
175. Philipsen, E. K., Bondesen, S., Andersen, J. & Larsen, S. Serum immunoglobulin G subclasses in patients with ulcerative colitis and Crohn's disease of different disease activities. *Scand.J Gastroenterol.* **30,** 50–53 (1995).
176. Peterson, D. A., McNulty, N. P., Guruge, J. L. & Gordon, J. I. IgA Response to Symbiotic Bacteria as a Mediator of Gut Homeostasis. *Cell Host Microbe* **2,** 328–339 (2007).
177. Van Der Waaij, L. A. *et al.* Immunoglobulin coating of faecal bacteria in inflammatory. *Eur. J. Gastroenterol. Hepatol.* **16,** 669–674 (2004).
178. Baklien, K. & Brandtzaeg, P. Comparative mapping of the local distribution of immunoglobulin-containing cells in ulcerative colitis and Crohn's disease of the colon. *Clin. Exp. Immunol.* **22,** 197–209 (1975).
179. Wang, Z. *et al.* High level of IgG4 as a biomarker for a new subset of inflammatory bowel disease. *Sci. Rep.* **8,** 1–11 (2018).
180. MacDermott, R. P. *et al.* Alterations in serum immunoglobulin G subclasses in patients with ulcerative colitis and Crohn's disease. *Gastroenterology* **96,** 764–768 (1989).
181. Rai, T., Wu, X. & Shen, B. Frequency and risk factors of low immunoglobulin levels in patients with inflammatory bowel disease. *Gastroenterol. Rep.* **3,** 115–121 (2015).
182. Wills, E. S. *et al.* Fecal microbial composition of ulcerative colitis and Crohn's disease patients in remission and subsequent exacerbation. *PLoS One* **9,** 1–10 (2014).
183. Falvey, J. D. *et al.* Disease activity assessment in IBD: Clinical indices and biomarkers fail to predict endoscopic remission. *Inflamm. Bowel Dis.* **21,** 824–831 (2015).
184. D'Haens, G. *et al.* Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease. *Inflamm. Bowel Dis.* **18,** 2218–2224 (2012).
185. Buttherworth, M., McClellan, B. & Aklansmith, M. Influence of Sex on Immunoglobulin Levels. *Nature* **214,** 1224–1225 (1967).
186. Gevers, D. *et al.* The treatment-naïve microbiome in new-onset Crohn ' s disease. *Cell Host Microbe* **15,** 382–392 (2014).

VII. Appendix

A. Materials and Methods

Patient-specific gating

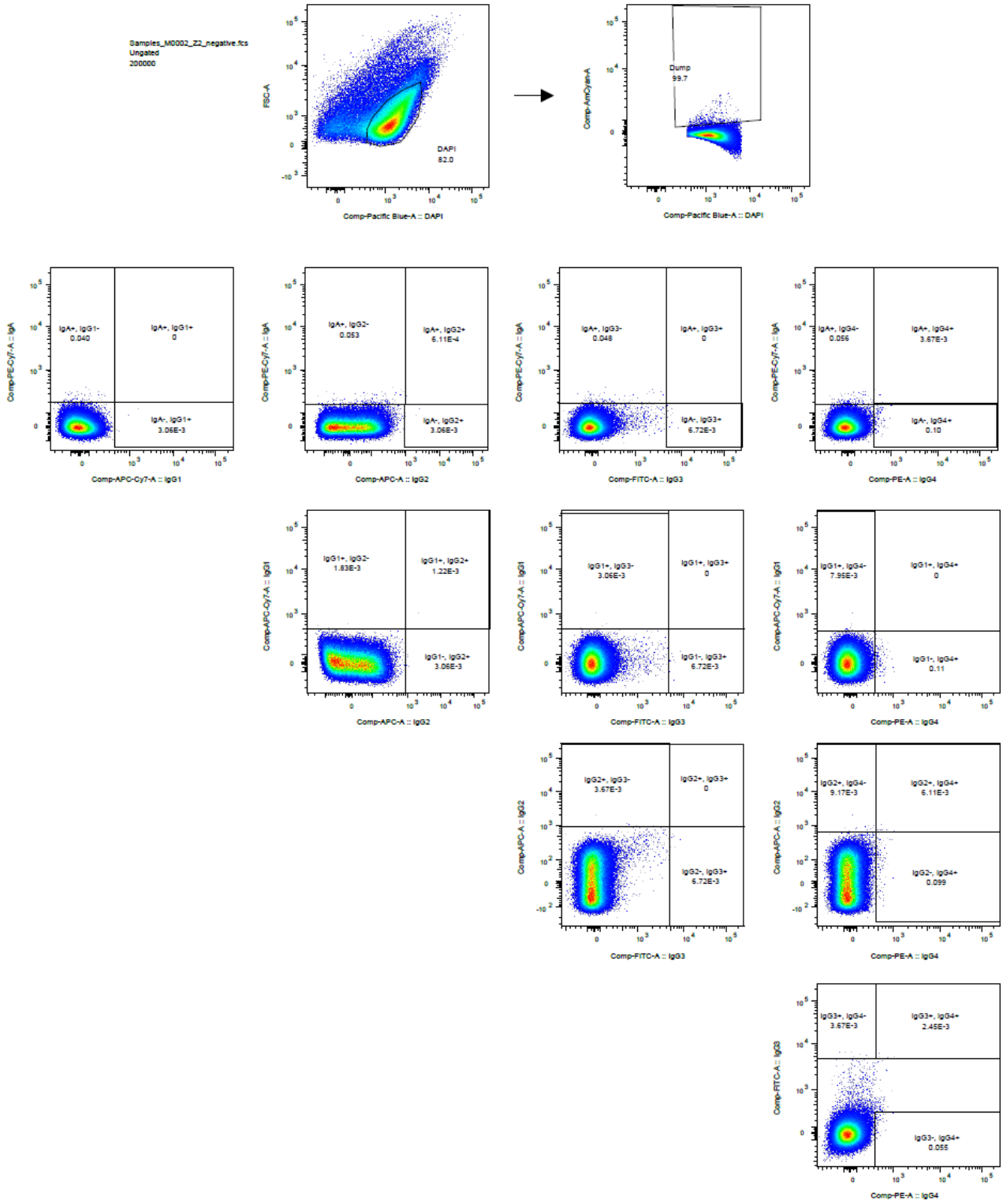

Figure 28. Applied gating strategy to all the unstained samples.

The gates were placed so that began in region not coated by bacteria. The first gate to be placed is the DAPI+ that is an indicative of viable bacteria, followed by the Dump gate that allows noise removal. Lastly, the gates considered IgA; IgG1; IgG2; IgG3 and IgG4. The gate placed in the upper corner is related to double coated data. Gating was conducted in FlowJo

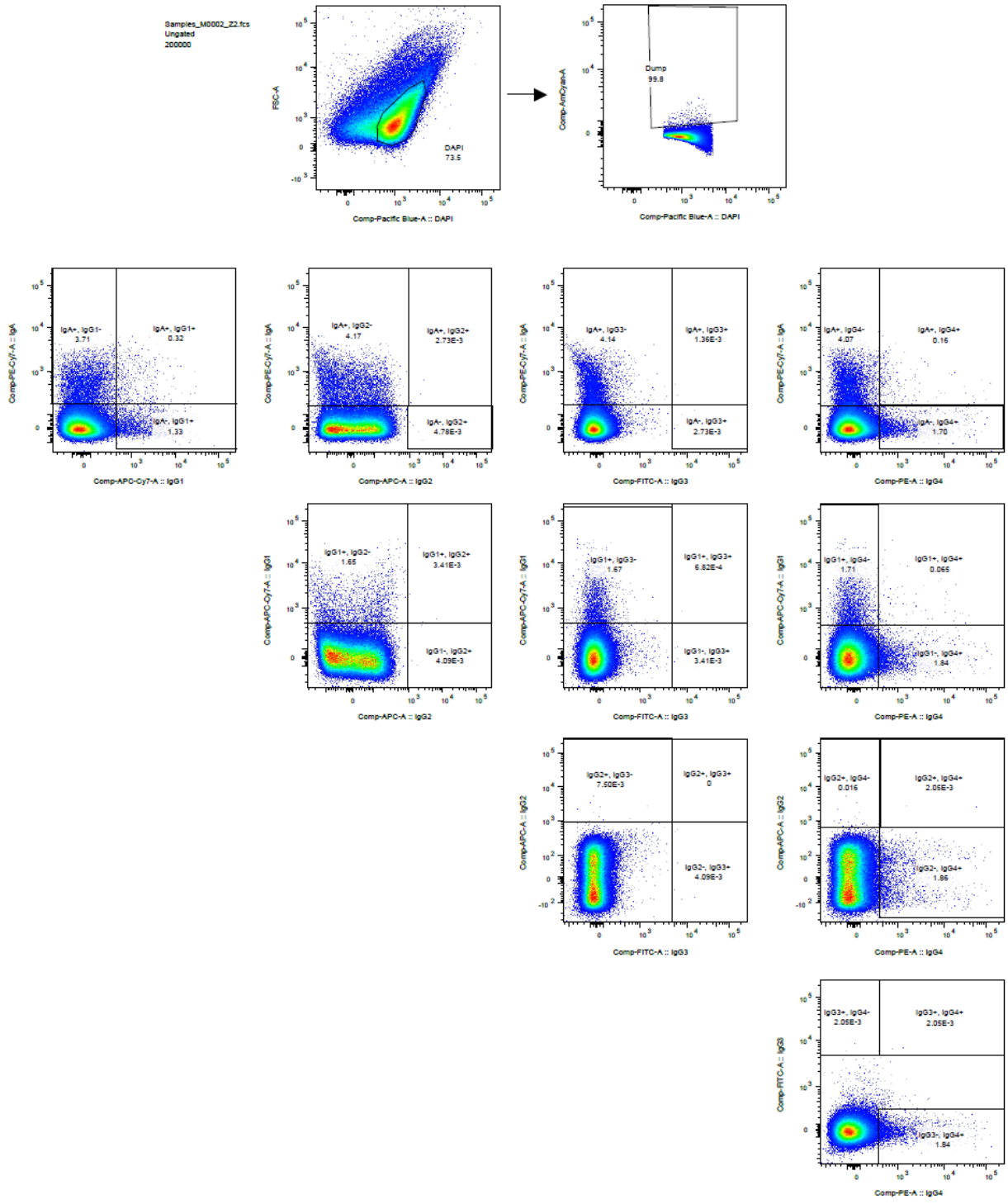

Figure 29. Applied gating strategy to all the stained samples.

The gates were placed so that began in region not coated by bacteria. The first gate to be places is the DAPI+ that is an indicative of viable bacteria, followed by the Dump gate that allows a lot of noise removal. Lastly, the gates considered IgA; IgG1; IgG2; IgG3 and IgG4 are placed. The gate placed in the upper corner is related to double coated data. Gating was conducted in FlowJo

B. Patient information

Sample ID	Visit	Status	Group	Gender	Age (visit)	Age (diagnosis)	Localization	Phenotype	HBI	CRP (mg/L)	Calprotectin (µg/g)	Smoking	BS	Surgery	Mesalazines	Thiopurines	Biologicals	Prednison	Antibiotic	PPI
M0002	1	remission_	RR	F	36	A2	L1	B1	2	2.4	15	ex	4	none	No	No	Yes	No	No	Yes
M0002	2	remission_	RR	F	36	A2	L1	B1	4	0.9	14	ex	4	none	No	No	Yes	No	No	Yes
M0007	1	remission_	RR	F	63	A2	L3	B2	1	4.6	31	ex	6	hemicolectomy	Yes	No	No	No	No	No
M0007	2	remission_	RR	F	64	A2	L3	B2	2	3.5	49	ex	6	hemicolectomy	Yes	No	No	No	No	No
M0014	1	remission_	RR	M	61	A2	L1	B3	0	4.7	14	ex	6	ileocoecaal	No	Yes	No	No	No	No
M0014	2	remission_	RR	M	61	A2	L1	B3	3	1.5	14	ex	7	ileocoecaal	No	Yes	No	No	No	No
M0015	1	remission_	RR	F	48	A3	L3	B2	1	10	34	ex	6	none	No	Yes	No	No	No	Yes
M0015	2	remission_	RR	F	48	A3	L3	B2	2	11	48	ex	5	none	No	Yes	No	No	No	Yes
M0017	1	remission_	RR	F	56	A2	L1	B2	11	0.9	27	never	6	ileocoecaal	No	No	No	No	No	No
M0017	2	remission_	RR	F	56	A2	L1	B2	3	1.4	22	never	6	ileocoecaal	No	No	Yes	No	No	No
M0019	1	remission_	RR	F	67	A2	L3	B1	9	2.7	43	ex	6	none	No	No	Yes	No	No	Yes
M0019	2	remission_	RR	F	67	A2	L3	B1	8	0.9	14	ex	7	none	Yes	No	Yes	No	No	Yes
M0022	1	remission_	RA	M	68	A3	L1	B2	3	1.4	98	never	5	ileocoecaal	Yes	No	Yes	No	No	Yes
M0022	2	active	RA	M	68	A3	L1	B2	5	4.6	315	never	6	ileocoecaal	Yes	No	Yes	No	No	Yes
M0023	1	remission_	RR	M	34	A2	L3+L4	B1	0	2.4	54	ex	7	none	No	Yes	No	No	No	Yes
M0023	2	remission_	RR	M	34	A2	L3+L4	B1	0	1.1	85	ex	6	none	No	Yes	No	No	No	Yes
M0025	1	remission_	RR	M	23	A2	L3	B1	0	1	63	never	4	none	No	Yes	Yes	No	No	No
M0025	2	remission_	RR	M	23	A2	L3	B1	0	0.9	29	never	3	none	No	Yes	Yes	No	No	No
M0031	1	remission_	RA	F	37	A2	L1	B1	8	5.4	70	never	6	none	No	Yes	No	No	No	No
M0031	2	active	RA	F	37	A2	L1	B1	13	9.8	315	never	3	none	No	Yes	Yes	No	No	No
M0044	1	remission_	RR	F	43	A2	L3	B1	4	1.3	46	never	6	rectum+sigmoid	No	No	No	Yes	No	Yes
M0044	2	remission_	RR	F	43	A2	L3	B1	10	0.9	14	never	6	rectum+sigmoid	No	No	No	No	No	Yes
M0052	1	remission_	RR	F	39	A2	L3	B1	6	4.4	71	current	6	none	No	No	Yes	No	No	Yes
M0052	2	remission_	RR	F	40	A2	L3	B1	7	1.4	51	current	6	none	No	No	No	No	No	Yes
M0053	1	remission_	RR	F	39	A2	L3	B1	7	4.6	14	never	5	none	No	No	Yes	No	No	No
M0053	2	remission_	RR	F	39	A2	L3	B1	10	3.6	14	never	6	none	No	No	Yes	No	No	No
M0056	1	remission_	RA	M	52	A3	L1	B1	2	-	44	ex	3	none	No	No	No	No	No	Yes
M0056	2	active	RA	M	52	A3	L1	B1	1	3.5	241	ex	3	none	No	No	No	No	No	Yes
M0073	1	remission_	RR	F	49	A2	L1	B1	1	0.9	14	current	6	none	No	No	Yes	No	No	No
M0073	2	remission_	RR	F	50	A2	L1	B1	4	1.5	14	current	7	none	No	No	Yes	No	No	No
M0076	1	remission_	RR	M	39	A2	L1	B1	2	1	57	ex	6	none	Yes	No	Yes	No	No	No
M0076	2	remission_	RR	M	40	A2	L1	B1	6	0.9	98	ex	5	none	No	No	Yes	No	No	No
M0082	1	remission_	RA	F	50	A2	L2	B2	3	4.3	74	ex	4	none	No	Yes	Yes	No	No	No
M0087	1	remission_	RA	M	65	A2	L2	B1	1	1.8	51	ex	6	none	Yes	No	No	No	No	No
M0087	2	active	RA	M	66	A2	L2	B1	3	1	576	ex	6	none	Yes	No	No	No	No	No
M0091	1	remission_	RR	F	53	A2	L2	B1	0	1.7	14	ex	4	colon	Yes	No	No	No	No	No
M0091	2	remission_	RR	F	54	A2	L2	B1	0	2.9	14	ex	6	colon	Yes	No	No	No	No	No
M0099	1	remission_	RA	F	33	A2	L2	B3	6	2.8	29	current	7	colon	No	Yes	Yes	No	No	No
M0099	2	active	RA	F	33	A2	L2	B3	7	3.6	236	current	7	colon	No	Yes	Yes	No	No	No
M0101	1	remission_	RR	F	22	A2	L1	B1	3	1.2	14	current	6	none	No	Yes	Yes	No	No	No
M0101	2	remission_	RR	F	22	A2	L1	B1	8	2.5	14	current	5	none	No	Yes	Yes	No	No	No
M0109	1	remission_	RR	F	45	A2	L1	B1	1	3.2	42	current	6	none	No	No	Yes	No	No	No
M0109	2	remission_	RR	F	45	A2	L1	B1	0	2.2	74	current	3	none	No	No	Yes	No	No	No
M0110	1	remission_	RR	F	40	A2	L2	B2	0	-	14	ex	5	none	No	No	Yes	No	No	No
M0110	2	remission_	RR	F	41	A2	L2	B2	0	-	14	ex	6	none	No	No	Yes	No	No	No
M0114	1	remission_	RA	F	50	A2	L2	B3	0	4.5	40	ex	6	none	No	No	Yes	No	No	No
M0114	2	active	RA	F	50	A2	L2	B3	0	11	219	ex	7	none	No	No	Yes	No	No	No
M0118	1	remission_	RA	M	29	A2	L3	B2	9	1.4	45	ex	7	none	Yes	Yes	No	No	No	Yes
M0118	2	active	RA	M	29	A2	L3	B2	8	-	1023	ex	7	none	Yes	No	No	No	No	Yes
M0123	1	remission_	RR	F	54	A1	L3	B1	0	2.5	46	current	7	hemicolectomy	Yes	No	No	No	No	No
M0123	2	remission_	RR	F	54	A1	L3	B1	3	2	37	current	7	hemicolectomy	Yes	No	No	No	No	No
M0129	1	remission_	RA	F	38	A2	L3	B2	0	-	15	ex	4	none	No	Yes	No	No	No	No
M0129	2	active	RA	F	39	A2	L3	B2	0	-	192	ex	4	none	No	No	No	No	No	No

SampleID	Visit	Status	Group	Gender	Age (visit)	Age (diagnosis)	Localization	Phenotype	HBI	CRP (mg/L)	Calprotectin (µg/g)	Smoking	BS	Surgery	Mesalazines	Thiopurines	Biologicals	Prednison	Antibiotic	PPI
M0130	1	remission_	RR	M	57	A3	L3	B1	0	3.5	14	ex	5	hemicolectomy	No	Yes	No	No	No	No
M0130	2	remission_	RR	M	58	A3	L3	B1	1	3.7	14	ex	3	hemicolectomy	No	Yes	No	No	No	No
M0131	1	remission_	RR	F	23	A2	L1	B1	4	0.9	14	current	6	none	No	No	Yes	No	No	No
M0131	2	remission_	RR	F	23	A2	L1	B1	4	0.9	14	current	6	none	No	No	Yes	No	No	No
M0137	1	remission_	RR	F	28	A2	L1	B1	1	1.7	75	never	4	none	No	Yes	No	No	No	No
M0137	2	remission_	RR	F	29	A2	L1	B1	2	0.9	38	never	4	none	No	Yes	No	No	No	No
M0138	1	remission_	RR	M	43	A2	L1	B3	3	7.2	14	current	5	none	No	No	No	No	No	No
M0138	2	remission_	RR	M	43	A2	L1	B3	3	3.2	14	current	5	none	No	No	No	No	No	No
M0139	1	remission_	RR	M	59	A2	L1	B1	0	1.7	51	ex	6	none	No	No	No	No	No	No
M0139	2	remission_	RR	M	60	A2	L1	B1	0	2.6	23	ex	4	none	No	No	No	No	No	No
M0142	1	remission_	RR	M	41	A2	L3	B1	0	1.4	44	never	4	none	No	Yes	Yes	No	No	No
M0142	2	remission_	RR	M	42	A2	L3	B1	0	1.6	14	never	3	none	No	Yes	Yes	No	No	No
M0143	1	remission_	RR	F	35	A2	L2	B1	0	3.6	14	never	6	none	No	No	Yes	No	No	No
M0143	2	remission_	RR	F	36	A2	L2	B1	0	3.8	14	never	7	none	No	No	Yes	No	No	No
M0150	1	remission_	RA	M	42	A3	L3	B3	4	7.2	31	current	5	none	No	No	Yes	No	No	No
M0150	2	active	RA	M	43	A3	L3	B3	0	-	226	current	6	none	No	No	Yes	No	No	No
M0151MSC	1	remission_	RA	F	67	A3	L1	B1	3	5	29	ex	5	none	No	No	No	No	No	Yes
M0151MSC	2	active	RA	F	67	A3	L1	B1	-	7	191	ex	5	none	No	No	No	No	No	Yes
M0159	1	remission_	RA	M	22	A2	L3	B1	3	1.3	78	never	5	none	No	Yes	Yes	No	No	Yes
M0159	2	active	RA	M	23	A2	L3	B1	2	3	281	never	4	none	No	Yes	Yes	No	No	Yes
M0163	1	remission_	RA	F	53	A3	L3	B1	4	2	91	ex	6	none	No	No	Yes	No	No	Yes
M0163	2	active	RA	F	53	A3	L3	B1	2	1.5	877	ex	7	none	No	No	Yes	No	No	Yes
M0164	1	remission_	RR	F	53	A3	L1	B1	2	3.5	15	ex	5	none	No	No	No	No	No	No
M0164	2	remission_	RR	F	54	A3	L1	B1	0	4.2	14	ex	4	none	No	No	No	No	No	No
M0165	1	remission_	RA	F	58	A2	L3	B3	2	2.8	88	ex	7	ileocoecaal	No	No	Yes	No	No	No
M0165	2	active	RA	F	59	A2	L3	B3	3	-	122	ex	7	ileocoecaal	No	No	Yes	No	No	No
M0169	1	remission_	RR	F	49	A2	L3	B2	0	-	14	ex	5	sigmoid	No	No	Yes	No	No	No
M0169	2	remission_	RR	F	49	A2	L3	B2	0	-	14	ex	7	sigmoid	No	No	Yes	No	No	No
M0171	1	remission_	RA	M	29	A1	L1+L4	B2	0	0.9	91	never	7	colon	No	Yes	Yes	No	No	No
M0171	2	active	RA	M	29	A1	L1+L4	B2	0	-	289	never	4	colon	No	Yes	Yes	No	No	No
M0179	1	remission_	RR	F	44	A3	L3	B1	8	2.6	14	never	4	none	No	No	Yes	No	No	No
M0179	2	remission_	RR	F	44	A3	L3	B1	7	-	14	never	3	none	No	No	Yes	No	No	No
M0185	1	remission_	RR	F	52	A3	L2	B2	1	5.4	33	ex	6	none	Yes	No	No	No	No	No
M0185	2	remission_	RR	F	52	A3	L2	B2	2	2.5	14	ex	5	none	Yes	No	No	No	No	No
M0202	1	remission_	RA	F	21	A2	L3	B1	0	5.1	14	never	4	none	No	No	Yes	No	No	No
M0202	2	active	RA	F	21	A2	L3	B1	0	12	126	never	3	none	No	No	Yes	No	No	No
M0211	1	remission_	RR	M	31	A2	L2	B1	1	0.9	18	ex	6	none	No	No	Yes	No	No	No
M0211	2	remission_	RR	M	32	A2	L2	B1	3	0.9	14	ex	5	none	No	No	Yes	No	No	No
M0232	1	remission_	RR	F	31	A2	L2	B3	1	0.9	14	never	6	none	No	No	No	No	No	No
M0232	2	remission_	RR	F	31	A2	L2	B3	3	0.9	24	never	7	none	No	No	No	No	No	No
M0238	1	remission_	RR	F	33	A2	L2	B1	1	3.5	26	ex	5	none	No	Yes	No	No	No	No
M0238	2	remission_	RR	F	33	A2	L2	B1	2	6.2	70	ex	3	none	No	Yes	No	No	No	No
M0253	1	remission_	RR	M	53	A2	L2	B1	0	0.9	14	ex	6	none	No	Yes	Yes	No	No	Yes
M0253	2	remission_	RR	M	53	A2	L2	B1	0	0.9	14	ex	6	none	No	Yes	Yes	No	No	Yes
M0254	1	remission_	RA	F	19	A2	L1	B1	2	0.9	39	never	5	none	No	No	Yes	No	No	No
M0254	2	active	RA	F	20	A2	L1	B1	0	1.4	219	never	3	none	No	No	Yes	No	No	No
M0270	1	remission_	RA	M	62	A2	L2	B1	0	1.9	35	never	5	none	No	No	No	No	No	No
M0270	2	active	RA	M	62	A2	L2	B1	0	0.9	449	never	7	none	No	No	No	No	No	No
M0289	1	remission_	RR	F	17	A2	L3	B1	1	1	14	never	6	none	No	No	Yes	No	No	No
M0289	2	remission_	RR	F	17	A2	L3	B1	1	0.9	43	never	6	none	No	No	No	No	No	No
M1796	1	remission_	RA	F	27	A2	L2	B3	13	3.1	76	never	5	none	No	Yes	Yes	Yes	No	Yes
M1796	2	active	RA	F	27	A2	L2	B3	4	4.6	335	never	6	none	Yes	Yes	Yes	Yes	No	Yes
M2728	1	remission_	RA	F	24	A2	L2	B1	3	-	14	never	5	none	No	Yes	No	No	No	No
M2728	2	active	RA	F	24	A2	L2	B1	2	1.9	209	never	5	none	No	Yes	Yes	No	No	No
M0109	2	remission_	RR	F	45	A2	L1	B1	0	2.2	74	current	3	none	No	No	Yes	No	No	No
M0110	1	remission_	RR	F	40	A2	L2	B2	0	-	14	ex	5	none	No	No	Yes	No	No	No
M0110	2	remission_	RR	F	41	A2	L2	B2	0	-	14	ex	6	none	No	No	Yes	No	No	No
M0114	1	remission_	RA	F	50	A2	L2	B3	0	4.5	40	ex	6	none	No	No	Yes	No	No	No
M0114	2	active	RA	F	50	A2	L2	B3	0	11	219	ex	7	none	No	No	Yes	No	No	No
M0118	1	remission_	RA	M	29	A2	L3	B2	9	1.4	45	ex	7	none	Yes	Yes	No	No	No	Yes
M0118	2	active	RA	M	29	A2	L3	B2	8	-	1023	ex	7	none	Yes	No	No	No	No	Yes
M0123	1	remission_	RR	F	54	A1	L3	B1	0	2.5	46	current	7	hemicolectomy	Yes	No	No	No	No	No
M0123	2	remission_	RR	F	54	A1	L3	B1	3	2	37	current	7	hemicolectomy	Yes	No	No	No	No	No
M0129	1	remission_	RA	F	38	A2	L3	B2	0	-	15	ex	4	none	No	Yes	No	No	No	No
M0129	2	active	RA	F	39	A2	L3	B2	0	-	192	ex	4	none	No	No	No	No	No	No

CD patients with only one sample

Sample ID	Status	Group	Gender	Age (visit)	Age (diagnosis)	Localization	Phenotype	HBI	CRP (mg/L)	Calprotectin (µg/g)	BS	Surgery	Mesalazines	Thiopurines	Biologicals	Prednison	Antibiotic	PPI
M004855	active	CD	F	58	A3	L3	B2	4	11	673	5	0	0	0	0	0	0	0
M0055	active	CD	F	68	A3	L3	B1	0	16	654	5	0	0	0	0	1	0	0
M0063	active	CD	F	40	A2	L2	B1	0	2.1	611	4	0	0	0	0	0	0	0
M0208	active	CD	F	51	A3	-	-	4	7.1	275	5	0	0	0	0	1	0	0
M0359	active	CD	F	30	A2	L2	B1	0	1.9	786	6	0	0	1	0	0	0	0
M1674	active	CD	M	27	A2	L1	B2	14	50	601	5	0	0	1	0	1	0	0
M0251	active	CD	F	19	A2	L2	B1	3	5.3	1545	4	1	0	0	0	0	0	0
M0262	active	CD	M	24	A2	L1	B1	1	113	722	5	0	0	0	0	1	0	0

Control group information

SampleID	Group	Gender	Age	BS
1011-2	Control	F	43	5
1027-2	Control	F	23	2
1028-2	Control	F	41	3
1030-2	Control	F	22	4
1033-3	Control	M	25	5
1037-3	Control	F	68	5
1039-2	Control	M	55	4
1041-3	Control	M	30	5
1051-2	Control	F	36	6
1064-2	Control	F	27	6
1070-3	Control	F	60	6
1073-6	Control	M	66	2
1075-3	Control	F	23	5
1089-5	Control	F	69	6
1092-3	Control	F	41	5
1093-2	Control	M	26	5
1098-2	Control	M	32	4
1099-2	Control	M	23	4
1102-3	Control	F	57	5
1104-2	Control	F	49	5

C. Profile of Bacteria-Coating by IgA and IgG Subclasses in Control and CD Patients

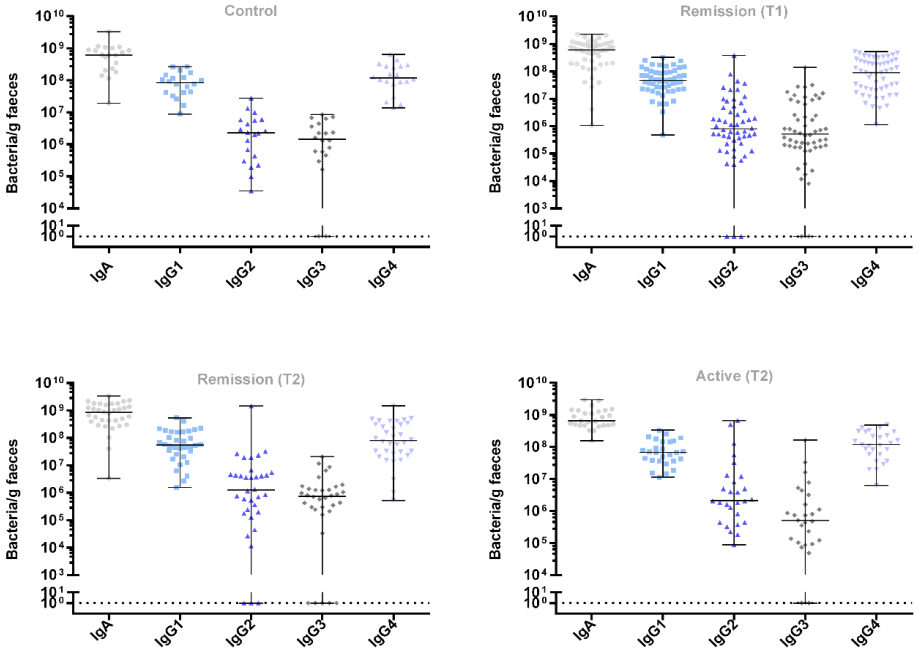

Figure 30. Profile of bacteria coating by IgA and IgG subclasses in the populations studied.

The amount of IgA; IgG1; IgG2; IgG3; and IgG4/g faeces in Control (20 healthy individuals), in CD Patients in remission on the first sampling time (Remission (T1), 54 patients); in CD Patients with disease in a state of remission (Remission (T2), 34 patients) and with active disease (Active (T2), 19 patients), on the second-time point (T2). Indicated is median ± range. Statistical analyses and graphic presentation were conducted in GraphPad Prism 5 and 7.

Table 2. Summary of the patients that present 0 % of a specific antibody coating or presented elevated values of bacteria coated for a specific antibody.

0 % of IgX-coated bacteria						
X	**Patient ID**	**Group**	**Age of Diagnosis**	**Location**	**Phenotype**	**Smoking**
IgG2	M0109	Remission (T1)	A2	L1	B1	Current
	M0130		A3	L3	B1	Ex
	M0289		A2	L3	B1	Never
IgG3	1089-5	Control	-	-	-	
	1033-3		-	-	-	
	M0052	Remission (T1)	A2	L3	B1	Current
	M0110		A2	L2	B2	Ex
	M0179	Remission (T2)	A3	L3	B1	Never
	M0185		A3	L2	B2	Ex
	M0238		A2	L2	B1	Ex
	M0017		A2	L1	B2	Never
	M0289		A2	L3	B1	Never
	M0142		A2	L3	B1	Never
	M0007		A2	L3	B2	Ex
	M0270	Active (T2)	A2	L2	B1	Never
	M0150		A3	L3	B3	Current
High % of IgX-coated bacteria						
IgG2	M0137	Remission(T1)	A2	L1	B1	Never

	M0137	Remission (T2)	A2	L1	B1	Never
	M0359	Active (T2)	A2	L2	B1	-
	M0099		A2	L2	B3	Current
IgG3	M0159	Remission (T1)	A2	L3	B1	Never
IgG4	M0185	Remission (T2)	A3	L2	B2	Ex
IgA	M0073	Remission (T1)	A2	L1	B1	Current
	M0110	Remission (T2)	A2	L2	B2	Ex
	M0087	Active (T2)	A2	L2	B1	Ex
	M0055	Active (T2)	A3	L3	B1	-

Table 3. Quantitative data regarding the median, minimum and maximum levels of bacteria coated by all the antibodies

The table is divided into four groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 (34 patients) and Active (T2) the patients that have entered an active state (19 patients). Control group is composed by 20 healthy individuals.

	Control			Remission (T1)			Remission (T2)			Active (T2)		
	Median	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**
IgA-coated bacteria/g faeces	6.17E+08	1.92E+07	3.26E+09	6.11E+08	1.08E+06	2.25E+09	8.38E+08	3.39E+06	3.36E+09	6.50E+08	1.56E+08	3.00E+09
IgG1-coated bacteria/g faeces	8.40E+07	8.82E+06	2.66E+08	4.62E+07	476112	3.24E+08	5.48E+07	1.56E+06	5.37E+08	6.76E+07	1.13E+07	3.36E+08
IgG2-coated bacteria/g faeces	2.28E+06	35563	2.80E+07	794023	0	3.79E+08	1.24E+06	0	1.44E+09	2.09E+06	88251	6.66E+08
IgG3-coated bacteria/g faeces	1.45E+06	0	8.44E+06	516118	0	1.38E+08	739844	0	2.04E+07	511014	0	1.63E+08
IgG4-coated bacteria/g faeces	1.17E+08	1.38E+07	6.40E+08	8.95E+07	1.13E+06	5.23E+08	7.92E+07	523639	1.49E+09	1.17E+08	6.37E+06	4.90E+08

Table 4.Relative data regarding the median, minimum and maximum levels of bacteria coated by all the antibodies

The table is divided into four groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 (34 patients) and Active (T2) the patients that have entered an active state (19 patients). Control group is composed by 20 healthy individuals.

	Control			Remission (T1)			Remission (T2)			Active (T2)		
	Median	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**
IgA-coated bacteria/g faeces	3.746	0.222	19.5	5.168	0.015	34.92	6.891	0.03	35.84	5.689	0.638	19.73
IgG1-coated bacteria/g faeces	0.622	0.057	2.228	0.453	0.113	5.116	0.5125	0.097	2.131	0.527	0.087	3.863
IgG2-coated bacteria/g faeces	0.012	0.001	0.118	0.0085	0	7.23	0.012	0	20.87	0.015	0.001	30.81
IgG3-coated bacteria/g faeces	0.008	0	0.116	0.0055	0	0.823	0.0055	0	0.164	0.004	0	0.669
IgG4-coated bacteria/g faeces	0.742	0.056	4.95	0.9405	0.086	3.741	0.7155	0.021	4.772	1.037	0.095	2.968

Table 5. Data concerning the median, minimum and maximum values of relative ratio of total IgG bacteria coating by the four IgG subclasses.

The table is divided into four groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 (34 patients) and Active (T2) the patients that have entered an active state (19 patients). Control group is composed by 20 healthy individuals.

	Control			Remission (T1)			Remission (T2)			Active (T2)		
	Median	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**
IgG1-coated bacteria/g faeces	28.01	7.64	85.62	31.6	8.015	89.31	34.33	2.211	98.9	29.29	8.242	71.18
IgG2-coated bacteria/g faeces	0.7125	0.05376	8.392	0.635	0	77.55	0.7402	0	90.47	0.5655	0.02771	85.59
IgG3-coated bacteria/g faeces	0.6452	0	11.23	0.394	0	33.83	0.3716	0	24.86	0.1457	0	24.79
IgG4-coated bacteria/g faeces	69.84	8.676	89.2	54.22	10.25	87.72	51.75	1.074	88.07	61.78	5.894	86.71

D. Profile of Bacteria Double-Coating by IgA and IgG Subclasses in Controls and CD Patients

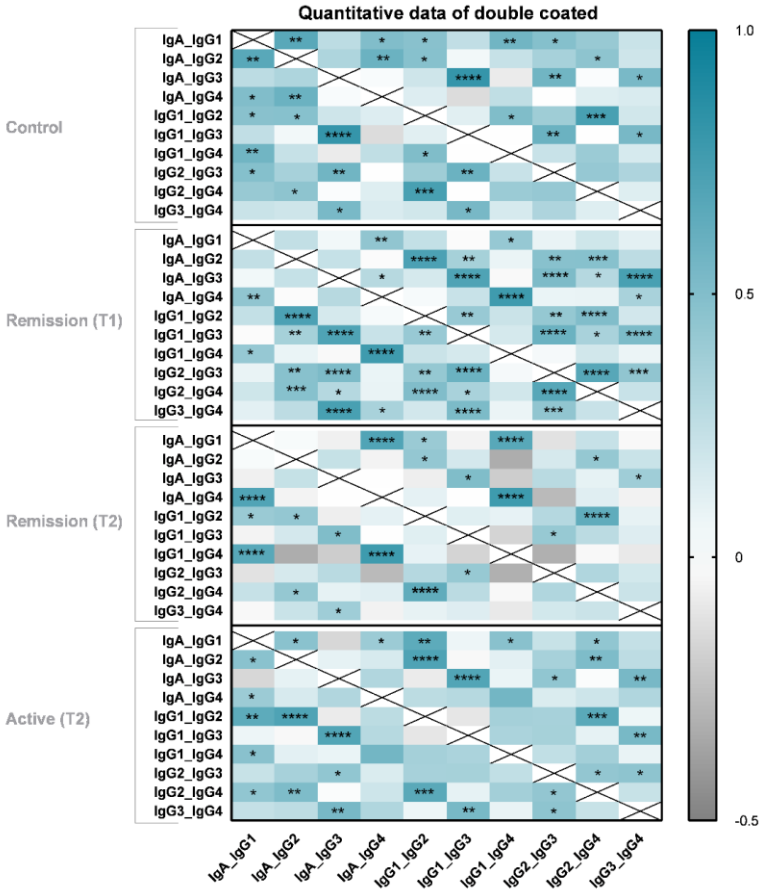

Figure 31.Correlations between the quantitative amounts of bacteria coated by the pairs of antibodies.

*Heat map presenting correlation coefficients between the level of bacteria coated by specific pair of antibodies. Negative correlation coefficients (grey) represent invers associations between the parameters, whereas positive correlation coefficients (blue) represent positive associations. Significant correlations are marked by asterisks. * P-value < 0.05, ** P-value < 0.01, 0.0001<*** P-value < 0.001; ****P-value < 0.0001. A*

E. Disease Phenotype, Disease Location, Age at Diagnosis, Smoking History and Patient Gender Relate to the Profile of Bacteria-Coating by IgA and IgG Subclasses

1. Phenotype

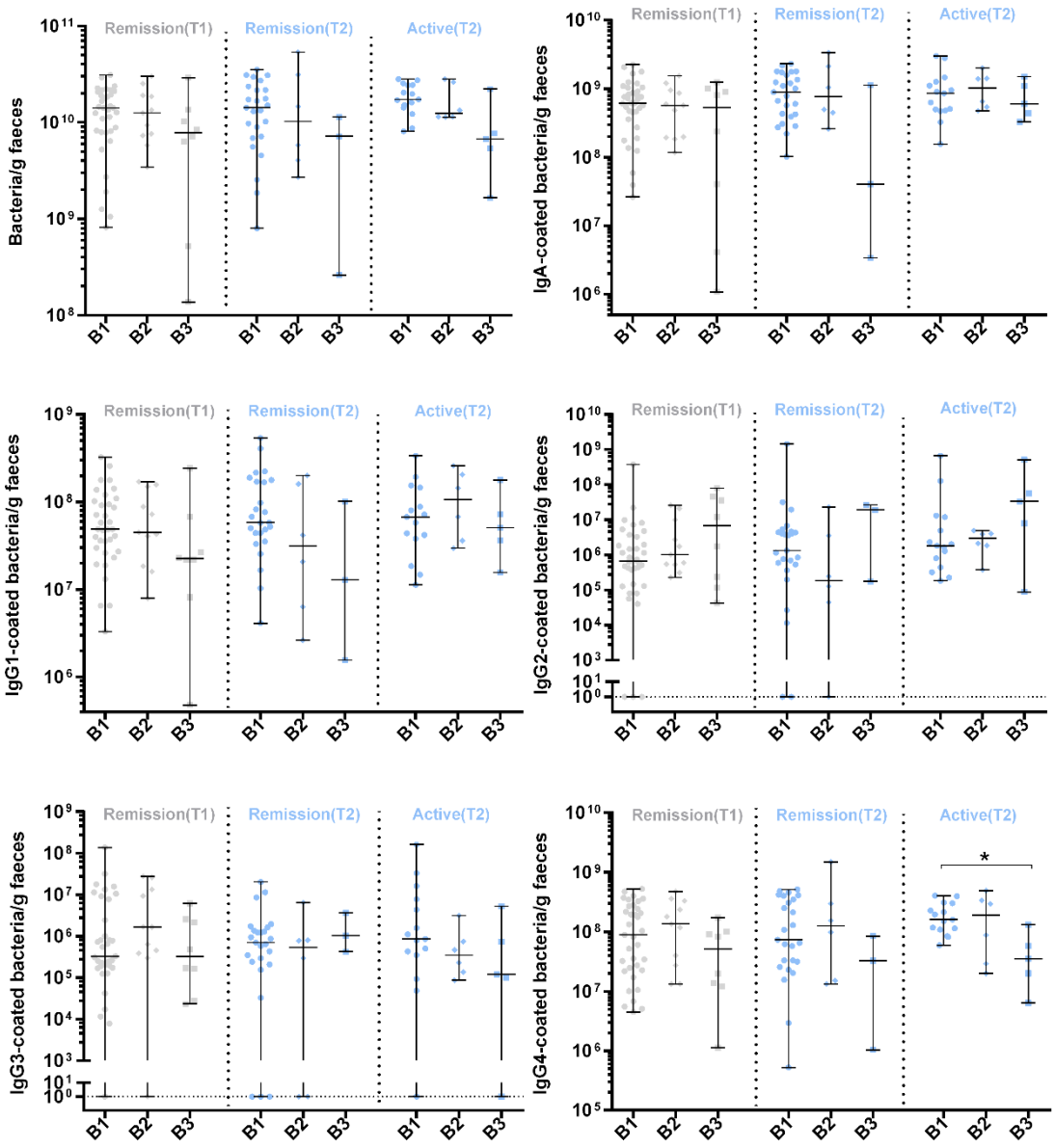

Figure 32. The profile of the quantitative amount of bacteria-coating by IgA and IgG subclasses in patients grouped according to their disease phenotype.

*Data is represented as IgA and IgG subclass-coating and/or amount of bacteria/g faeces in every category of disease phenotype. B1 category represents a non-stricturing, non-penetrating phenotype, B2 represents a stricturing phenotype, and B3 a penetrating phenotype where fistulas, perianal ulcers and/or abscesses occur. The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. 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. The significant finding is based on a Kruskal-Wallis test. * P-value < 0.05. Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.*

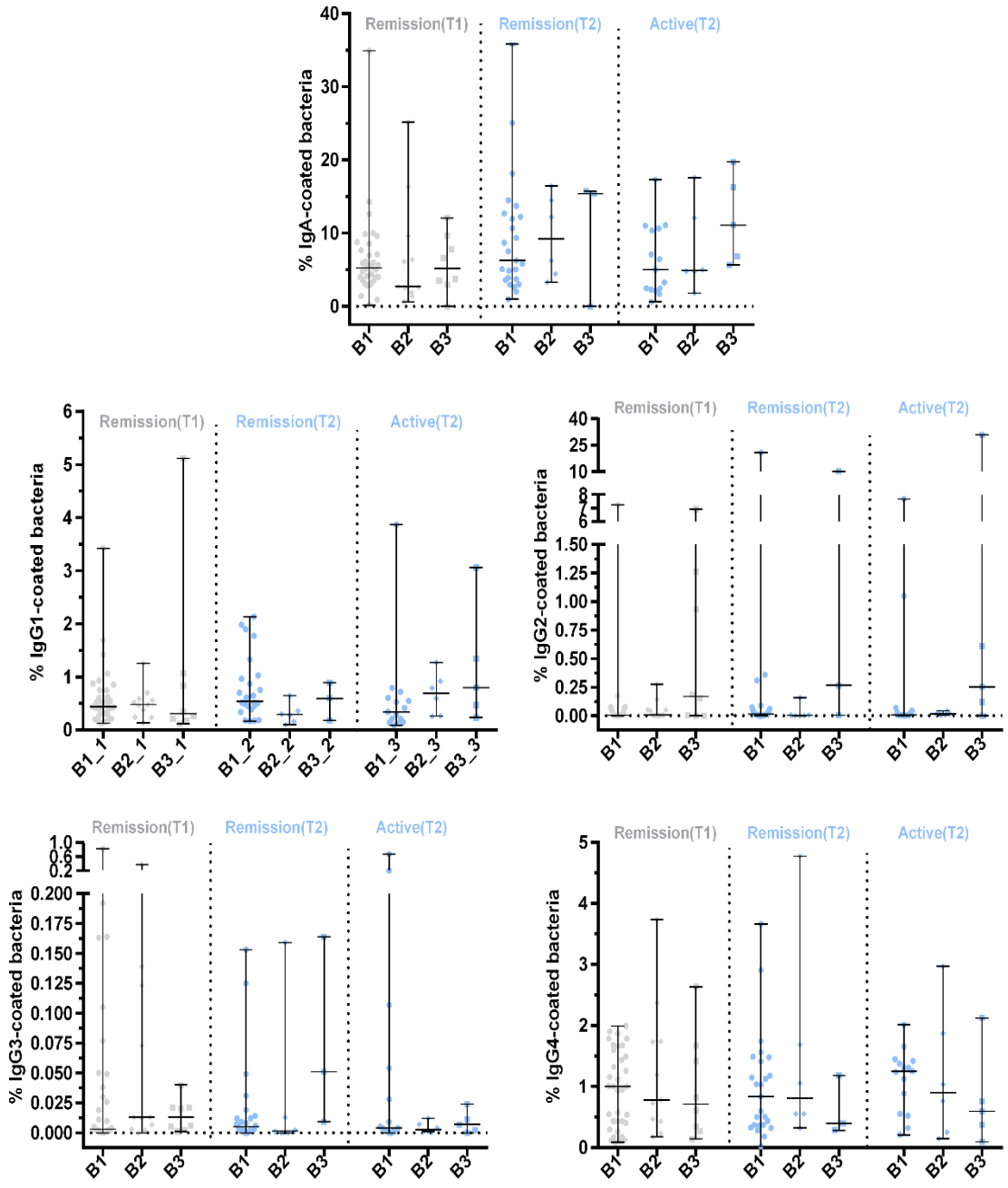

Figure 33. The profile of relative amount of bacteria-coating by IgA and IgG subclasses in patients grouped according to their disease phenotype.

Data is represented as the relative amount of bacteria-coating by each antibody in every category of disease phenotype. B1 category represents a non-stricturing, non-penetrating phenotype, B2 represents a stricturing phenotype, and B3 a penetrating phenotype where fistulas, perianal ulcers and/or abscesses occur. The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. 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. Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.

Table 6. Quantitative data regarding the median levels of bacteria coated by all the antibodies relative to disease phenotype.

B1 category represents a non-stricturing, non-penetrating phenotype, B2 represents a stricturing phenotype, and B3 a penetrating phenotype where fistulas, perianal ulcers and/or abscesses occur. The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.

	Remission (T1)			**Remission (T2)**			**Active (T2)**		
	B1	B2	B3	B1	B2	B3	B1	B2	B3
Bacteria/g faeces	1.42E+10	1.26E+10	7.81E+09	1.42E+10	1.03E+10	7.18E+09	1.73E+10	1.25E+10	6.74E+09
IgA-coated bacteria/g faeces	6.17E+08	5.74E+08	5.26E+08	8.92E+08	7.69E+08	4.03E+07	8.57E+08	1.02E+09	6.01E+08
IgG1-coated bacteria/g faeces	4.87E+07	4.48E+07	2.25E+07	5.79E+07	3.12E+07	1.28E+07	6.70E+07	1.06E+08	5.07E+07
IgG2-coated bacteria/g faeces	6.54E+05	1.02E+06	6.87E+06	1.32E+06	1.85E+05	1.90E+07	1.83E+06	2.98E+06	3.30E+07
IgG3-coated bacteria/g faeces	3.30E+05	1.67E+06	3.25E+05	7.00E+05	5.38E+05	1.04E+06	8.65E+05	3.52E+05	1.21E+05
IgG4-coated bacteria/g faeces	8.87E+07	1.35E+08	5.11E+07	7.37E+07	1.27E+08	3.26E+07	1.60E+08	1.90E+08	3.52E+07

Table 7. Relative data regarding the median levels of bacteria coated by all the antibodies relative to disease phenotype.

B1 category represents a non-stricturing, non-penetrating phenotype, B2 represents a stricturing phenotype, and B3 a penetrating phenotype where fistulas, perianal ulcers and/or abscesses occur. The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.

	Remission (T1)			**Remission (T2)**			**Active (T2)**		
	B1	B2	B3	B1	B2	B3	B1	B2	B3
% IgA coated bacteria	5.271	2.715	5.19	6.279	9.247	15.41	5.022	4.9	11.12
% IgG1 coated bacteria	0.441	0.477	0.304	0.538	0.291	0.597	0.339	0.6905	0.799
% IgG2 coated bacteria	0.006	0.007	0.171	0.015	0.0045	0.265	0.008	0.0155	0.253
% IgG3 coated bacteria	0.003	0.013	0.013	0.005	0.0015	0.051	0.004	0.0025	0.007
% IgG4 coated bacteria	1.001	0.781	0.7155	0.834	0.8075	0.398	1.251	0.9005	0.596

2. Location

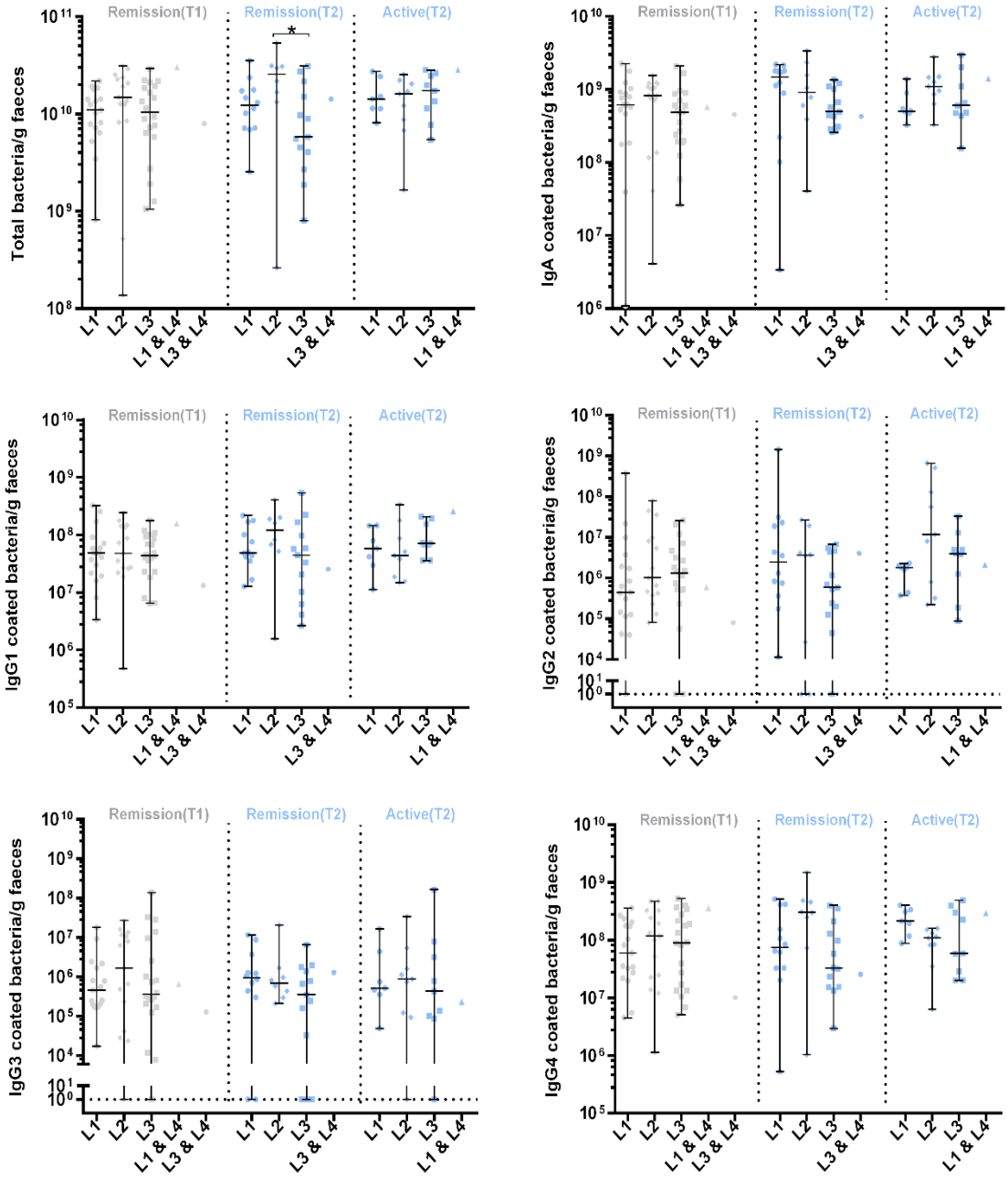

Figure 34. The profile of quantitative amount of bacteria-coating by IgA and IgG subclasses in patients grouped according to their disease location.

*Data is represented as the quantitative amount of bacteria-coating by each antibody in every category of disease location. Disease location was divided in four categories that include ileum (L1), colon (L2), ileum and colon (L3), and the upper gastrointestinal tract (L4) which covers disease from the mouth to ileum. Patients presenting disease in ileum and gastrointestinal tract and in ileum, colon and gastrointestinal tract, were allocated to L1 & L4 and L3 and L4, respectively. The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. 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. The significant finding is based on a one-way ANOVA test. * P-value < 0.05. . Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.*

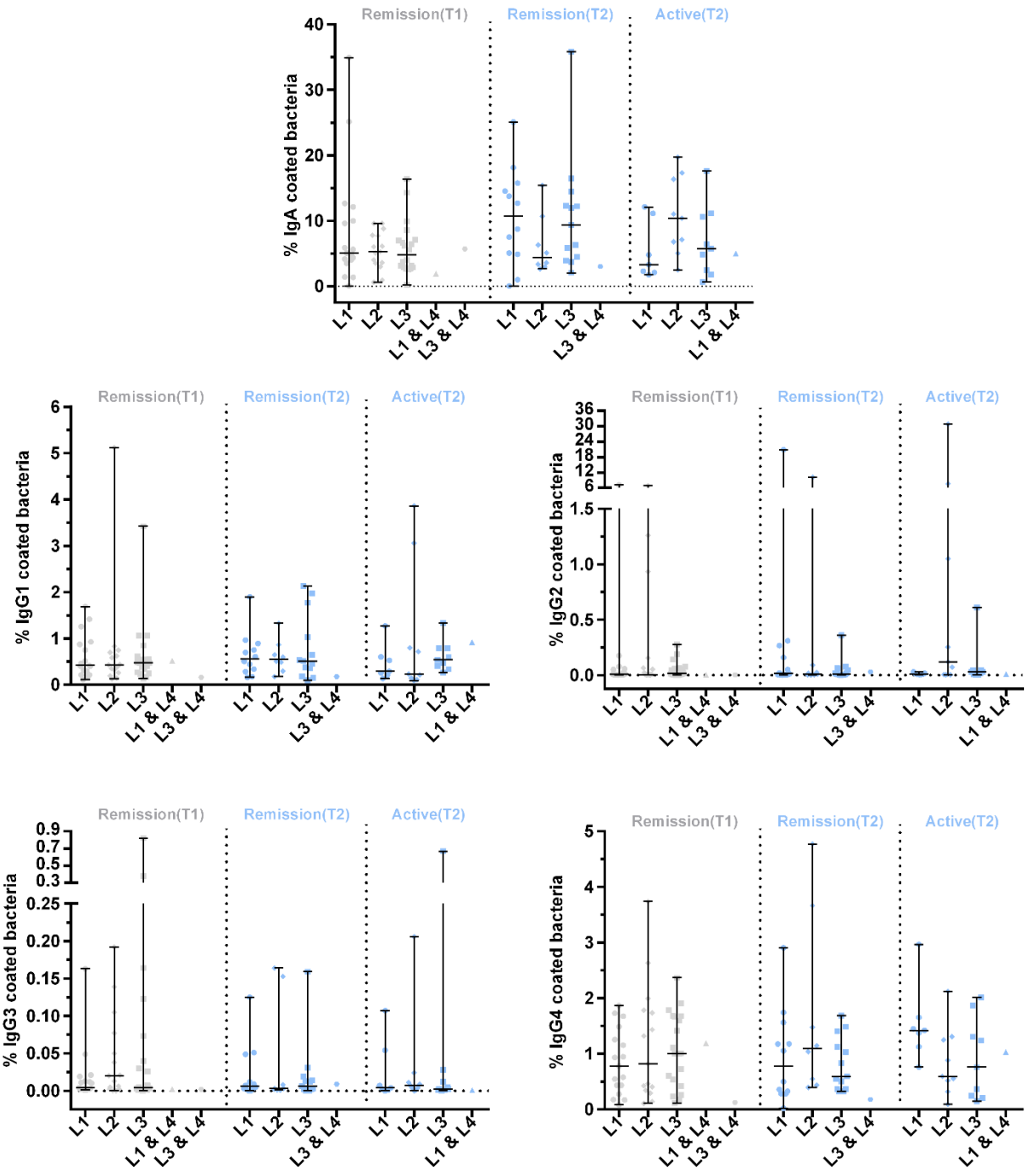

Figure 35. The profile of relative amount of bacteria-coating by IgA and IgG subclasses in patients grouped according to their disease location.

Data is represented as the relative amount of bacteria-coating by each antibody in every category of disease location. Disease location was divided in four categories that include ileum (L1), colon (L2), ileum and colon (L3), and the upper gastrointestinal tract (L4) which covers disease from the mouth to ileum. Patients presenting disease in ileum and gastrointestinal tract and in ileum, colon and gastrointestinal tract, were allocated to L1 & L4 and L3 and L4, respectively. The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. . Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.

Table 8. Quantitative data regarding the median levels of bacteria coated by all the antibodies relative to disease location.

Disease location was divided in four categories that include ileum (L1), colon (L2), ileum and colon (L3), and the upper gastrointestinal tract (L4) which covers disease from the mouth to ileum. The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.

	Remission (T1)					**Remission (T2)**				**Active (T2)**			
	L1	L2	L3	L1 & L4	L3 & L4	L1	L2	L3	L3 & L4	L1	L2	L3	L1 & L4
Total bacteria/g faeces	1.10 E+10	1.48 E+10	1.04 E+10	3.02 E+10	7.94 E+09	1.22 E+10	2.57 E+10	5.84 E+09	1.42 E+10	1.42 E+10	1.61 E+10	1.73 E+10	2.82 E+10
IgA coated bacteria/g faeces	6.17 E+08	8.23 E+08	4.83 E+08	5.74 E+08	4.52 E+08	1.49 E+09	9.12 E+08	4.98 E+08	4.30 E+08	5.07 E+08	1.10 E+09	6.01 E+08	1.40 E+09
IgG1 coated bacteria/g faeces	4.81 E+07	4.76 E+07	4.39 E+07	1.57 E+08	1.31 E+07	4.91 E+07	1.21 E+08	4.41 E+07	2.54 E+07	5.81 E+07	4.37 E+07	7.16 E+07	2.58 E+08
IgG2coated bacteria/g faeces	4.50 E+05	1.02 E+06	1.31 E+06	5.94 E+05	8.00 E+04	2.46 E+06	3.63 E+06	5.92 E+05	4.04 E+06	1.82 E+06	1.19 E+07	3.98 E+06	2.09 E+06
IgG3 coated bacteria/g faeces	4.56 E+05	1.67 E+06	3.60 E+05	6.44 E+05	1.28 E+05	9.57 E+05	6.93 E+05	3.55 E+05	1.29 E+06	5.11 E+05	8.65 E+05	4.32 E+05	2.33 E+05
IgG4 coated bacteria/g faeces	6.05 E+07	1.18 E+08	8.95 E+07	3.60 E+08	1.01 E+07	7.50 E+07	3.03 E+08	3.30 E+07	2.56 E+07	2.15 E+08	1.09 E+08	5.90 E+07	2.92 E+08

Table 9. Relative data regarding the median levels of bacteria coated by all the antibodies relative to disease location.

Disease location was divided in four categories that include ileum (L1), colon (L2), ileum and colon (L3), and the upper gastrointestinal tract (L4) which covers disease from the mouth to ileum. The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.

	Remission (T1)					**Remission (T2)**				**Active (T2)**			
	L1	L2	L3	L1 & L4	L3 & L4	L1	L2	L3	L3 & L4	L1	L2	L3	L1 & L4
% IgA coated bacteria	5.064	5.271	4.793	1.904	5.692	10.7	4.342	9.35	3.02	3.294	10.37	5.689	4.975
% IgG1 coated bacteria	0.418	0.434	0.4795	0.52	0.165	0.56	0.553	0.515	0.178	0.295	0.231	0.545	0.916
% IgG2 coated bacteria	0.006	0.004	0.018	0.002	0.001	0.014	0.0105	0.01	0.028	0.008	0.119	0.029	0.007
% IgG3 coated bacteria	0.004	0.02	0.004	0.002	0.002	0.006	0.003	0.006	0.009	0.004	0.007	0.002	0.001
% IgG4 coated bacteria	0.781	0.823	1.007	1.192	0.127	0.7795	1.093	0.597	0.18	1.424	0.596	0.759	1.037

3. Age at diagnosis

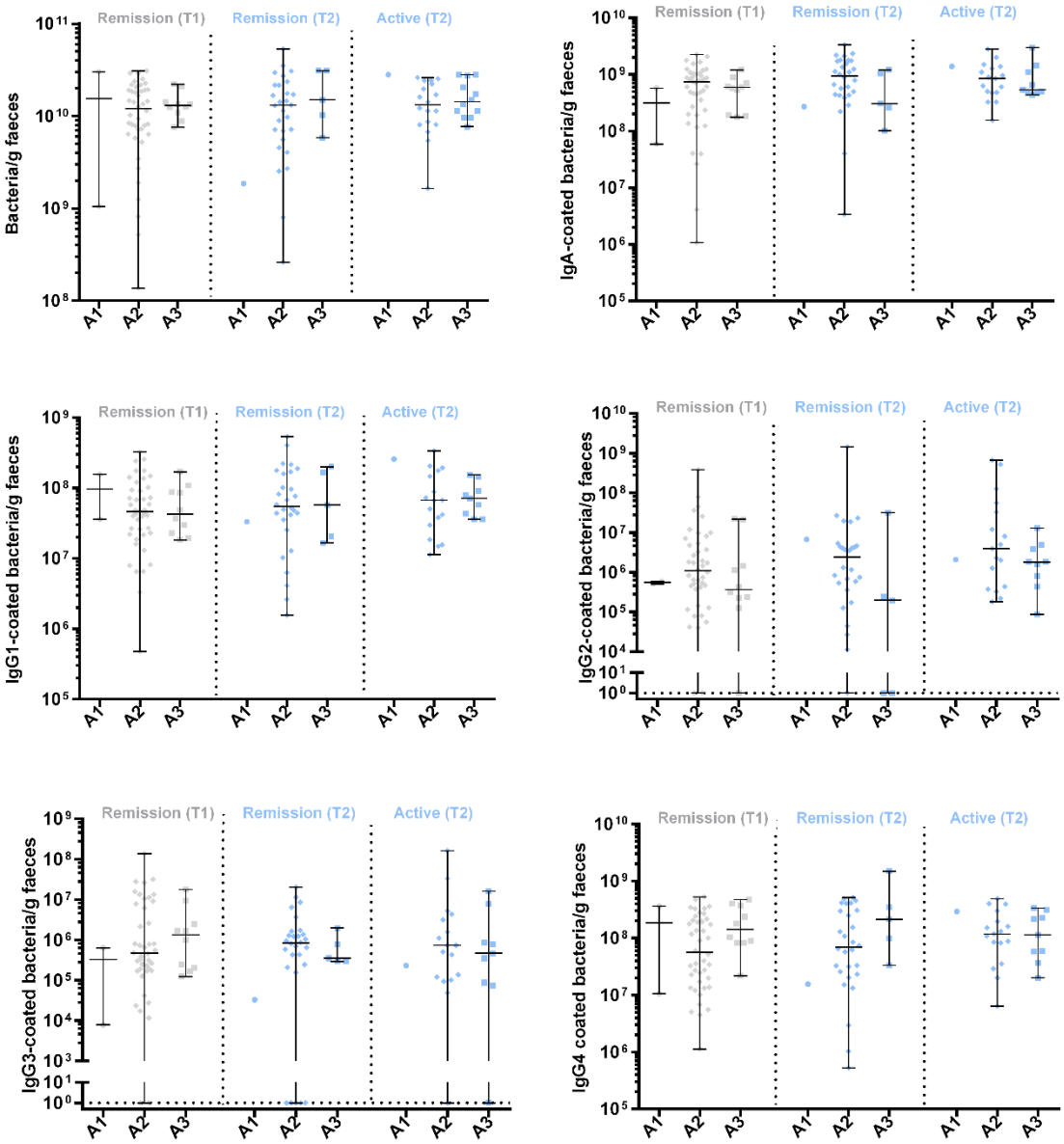

Figure 36. The profile of quantitative amount of bacteria-coating by IgA and IgG subclasses when patients are groups accordingly to their age at diagnosis.

Data is represented as IgA and IgG subclass-coating and/or amount of bacteria/g faeces accordingly to patients age at diagnosis. A1 represent patient that were diagnosed before the age of 16 years, A2 to refers to patients diagnosed in the age of 17-40 years, and A3 to patients diagnosed after the age of 40 years. The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. 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. Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.

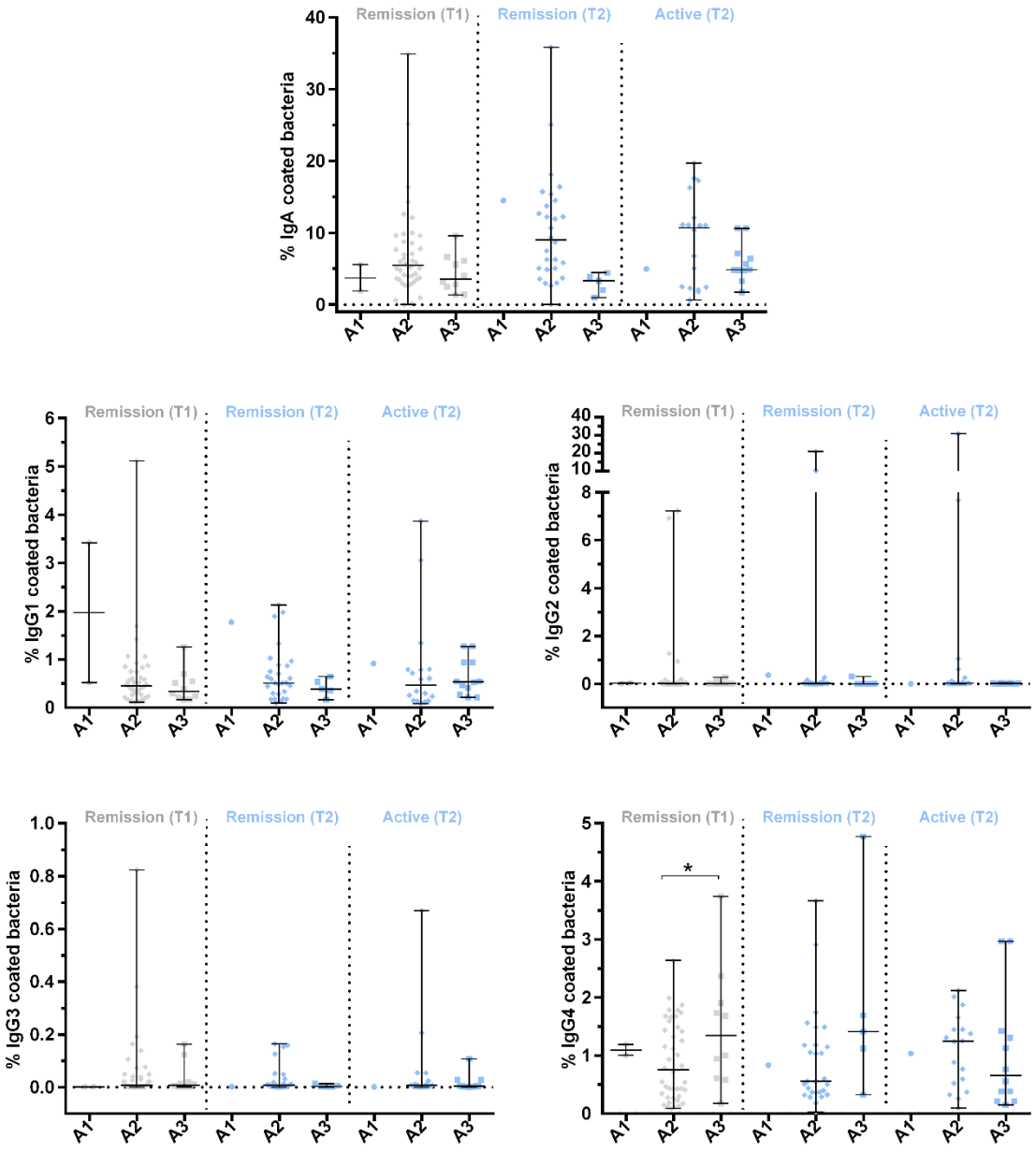

Figure 37. The profile of relative amount of bacteria-coating by IgA and IgG subclasses when patients are groups accordingly to their age at diagnosis.

*Data is represented as the relative amount of bacteria-coating by each antibody according to patients age at diagnosis. A1 represent patient that were diagnosed before the age of 16 years, A2 to refers to patients diagnosed in the age of 17-40 years, and A3 to patients diagnosed after the age of 40 years. The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. The significant find is based in an Unpaired T-teste. 0.01<*P-value<0.05. Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.*

Table 10. Quantitative data regarding the median levels of bacteria coated by all the antibodies relative to patient age at diagnosis.

A1 represent patient that were diagnosed before the age of 16 years, A2 to refers to patients diagnosed in the age of 17-40 years, and A3 to patients diagnosed after the age of 40 years. The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.

	Remission (T1)			**Remission (T2)**			**Active (T2)**		
	A1	A2	A3	A1	A2	A3	A1	A2	A3
Total bacteria/g faeces	1.56E+10	1.21E+10	1.31E+10	1.87E+09	1.32E+10	1.50E+10	2.82E+10	1.32E+10	1.43E+10
IgA coated bacteria/g faeces	3.17E+08	7.42E+08	5.90E+08	2.71E+08	9.51E+08	3.06E+08	1.40E+09	8.57E+08	5.40E+08
IgG1 coated bacteria/g faeces	9.66E+07	4.62E+07	4.30E+07	3.31E+07	5.50E+07	5.75E+07	2.58E+08	6.70E+07	7.16E+07
IgG2coated bacteria/g faeces	5.52E+05	1.09E+06	3.70E+05	6.71E+06	2.42E+06	1.98E+05	2.09E+06	3.98E+06	1.82E+06
IgG3 coated bacteria/g faeces	3.26E+05	4.69E+05	1.34E+06	3.32E+04	8.39E+05	3.55E+05	2.33E+05	7.40E+05	4.70E+05
IgG4 coated bacteria/g faeces	1.85E+08	5.61E+07	1.42E+08	1.56E+07	6.95E+07	2.12E+08	2.92E+08	1.17E+08	1.14E+08

Table 11. Relative data regarding the median levels of bacteria coated by all the antibodies relative to patient age at diagnosis.

A1 represent patient that were diagnosed before the age of 16 years, A2 to refers to patients diagnosed in the age of 17-40 years, and A3 to patients diagnosed after the age of 40 years. The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.

	Remission (T1)			**Remission (T2)**			**Active (T2)**		
	A1	A2	A3	A1	A2	A3	A1	A2	A3
% IgA coated bacteria	3.74	5.48	3.60	14.50	9.03	3.32	4.98	10.69	4.89
% IgG1 coated bacteria	1.97	0.45	0.34	1.77	0.51	0.38	0.92	0.47	0.54
% IgG2 coated bacteria	0.03	0.01	0.00	0.36	0.01	0.00	0.01	0.03	0.01
% IgG3 coated bacteria	0.00	0.01	0.01	0.00	0.01	0.00	0.00	0.01	0.00
% IgG4 coated bacteria	1.10	0.75	1.34	0.83	0.56	1.41	1.04	1.25	0.66

4. Smoking History

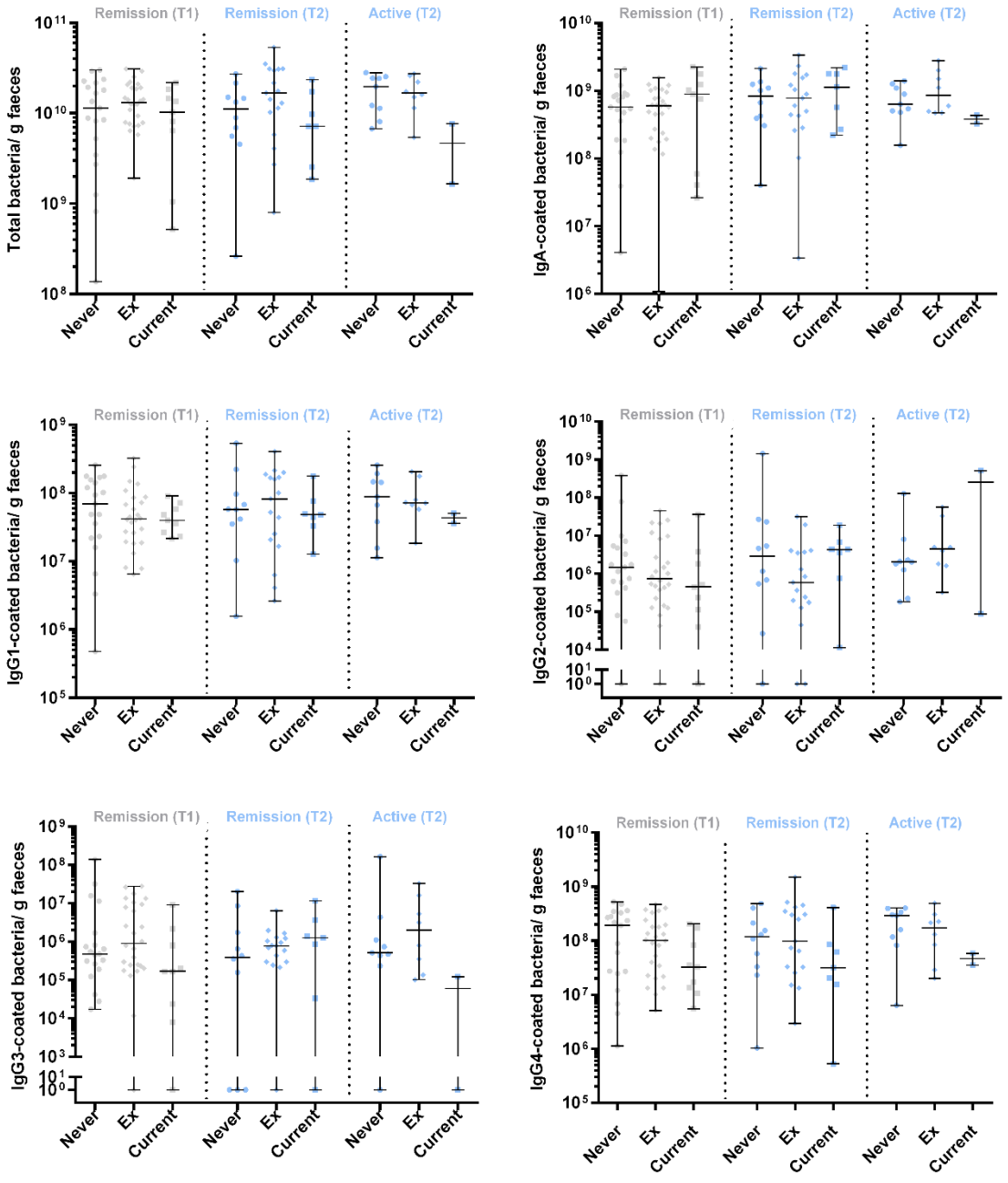

Figure 38. The profile of quantitative amount of bacteria-coating by IgA and IgG subclasses when patients are grouped accordingly to their smoking history.

Data is represented as IgA and IgG subclass-coating and/or amount of bacteria/g faeces accordingly to smoking history, i.e. never been a smoker (Never), ex-smoker (Ex) and current smoker (Currently-at the time of sample collection). The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. 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. Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.

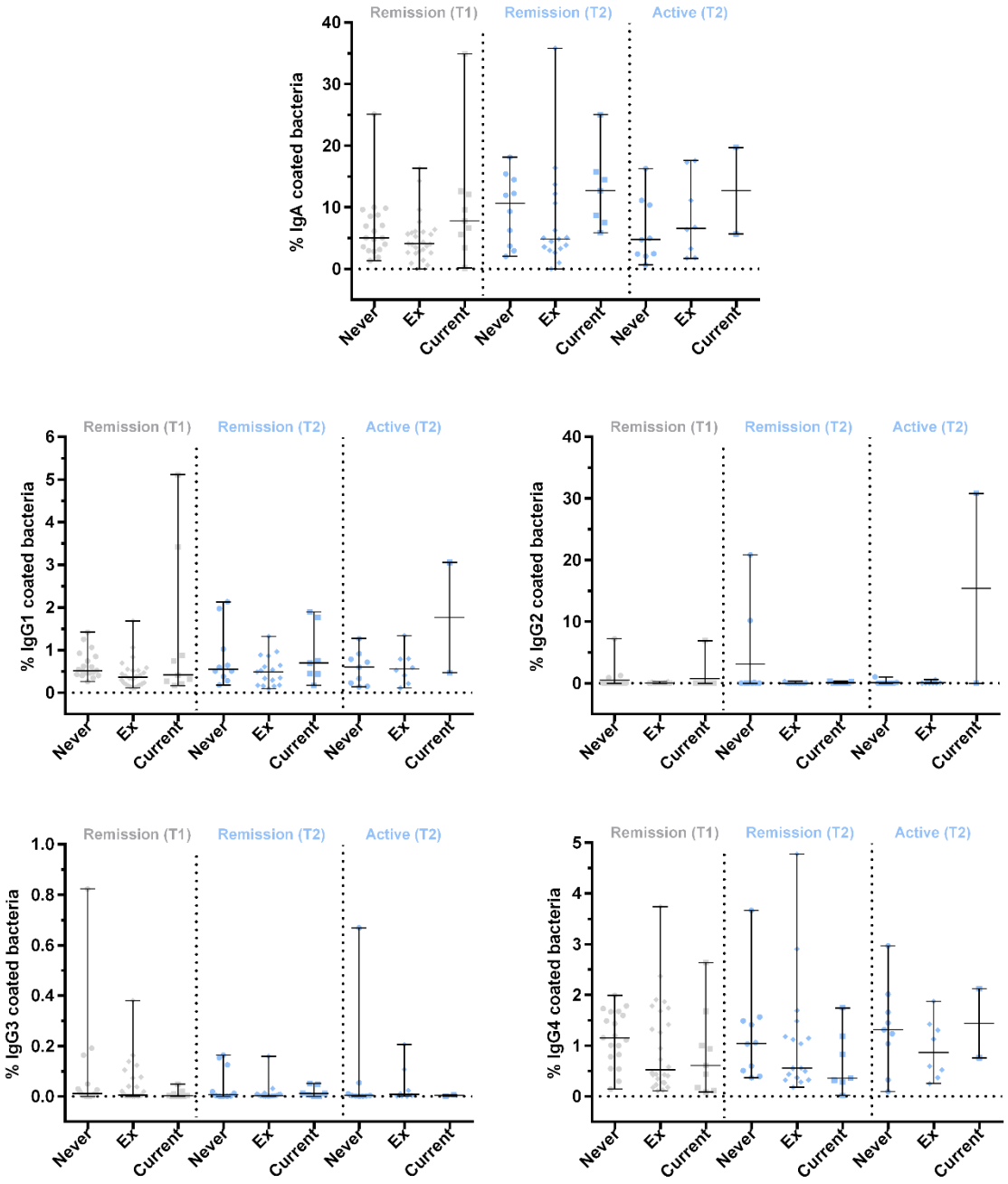

Figure 39. The profile of relative level of bacteria-coating by IgA and IgG subclasses when patients are grouped accordingly to their smoking history.

Data is represented as the relative amount of bacteria-coating by each antibody according to patients smoking history, i.e. never been a smoker (Never), ex-smoker (Ex) and current smoker (Currently-at the time of sample collection). The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.

Table 12. Quantitative data regarding the median levels of bacteria coated by all the antibodies relative to patients smoking history.

Patients are classified accordingly to smoking history, i.e. never been a smoker (Never), ex-smoker (Ex) and current smoker (Currently-at the time of sample collection).The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.

	Remission (T1)			**Remission (T2)**			**Active (T2)**		
	Never	Ex	Currently	Never	Ex	Currently	Never	Ex	Currently
Total bacteria/g faeces	1.14E+10	1.32E+10	1.02E+10	1.11E+10	1.68E+10	7.18E+09	1.97E+10	1.67E+10	4.67E+09
IgA coated bacteria/g faeces	5.74E+08	6.03E+08	9.00E+08	8.39E+08	7.84E+08	1.13E+09	6.32E+08	8.56E+08	3.82E+08
IgG1 coated bacteria/g faeces	6.93E+07	4.19E+07	3.98E+07	5.77E+07	8.18E+07	4.82E+07	8.80E+07	7.20E+07	4.34E+07
IgG2coated bacteria/g faeces	1.49E+06	7.49E+05	4.50E+05	2.90E+06	5.92E+05	4.34E+06	2.03E+06	4.46E+06	2.56E+08
IgG3 coated bacteria/g faeces	4.81E+05	8.98E+05	1.69E+05	3.92E+05	7.80E+05	1.25E+06	5.11E+05	1.97E+06	6.05E+04
IgG4 coated bacteria/g faeces	1.91E+08	1.02E+08	3.23E+07	1.19E+08	9.86E+07	3.14E+07	2.92E+08	1.74E+08	4.67E+07

Table 13. Relative data regarding the median levels of bacteria coated by all the antibodies relative to patients smoking history.

Patients are classified accordingly to smoking history, i.e. never been a smoker (Never), ex-smoker (Ex) and current smoker (Currently-at the time of sample collection). The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.

	Remission (T1)			**Remission (T2)**			**Active (T2)**		
	Never	Ex	Currently	Never	Ex	Currently	Never	Ex	Currently
% IgA coated bacteria	5.06	4.10	7.80	10.66	4.89	12.69	4.76	6.61	12.71
% IgG1 coated bacteria	0.52	0.36	0.42	0.55	0.49	0.70	0.60	0.56	1.76
% IgG2 coated bacteria	0.02	0.01	0.01	0.02	0.01	0.05	0.01	0.02	15.41
% IgG3 coated bacteria	0.01	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
% IgG4 coated bacteria	1.16	0.52	0.61	1.05	0.56	0.36	1.31	0.86	1.44

5. *Gender*

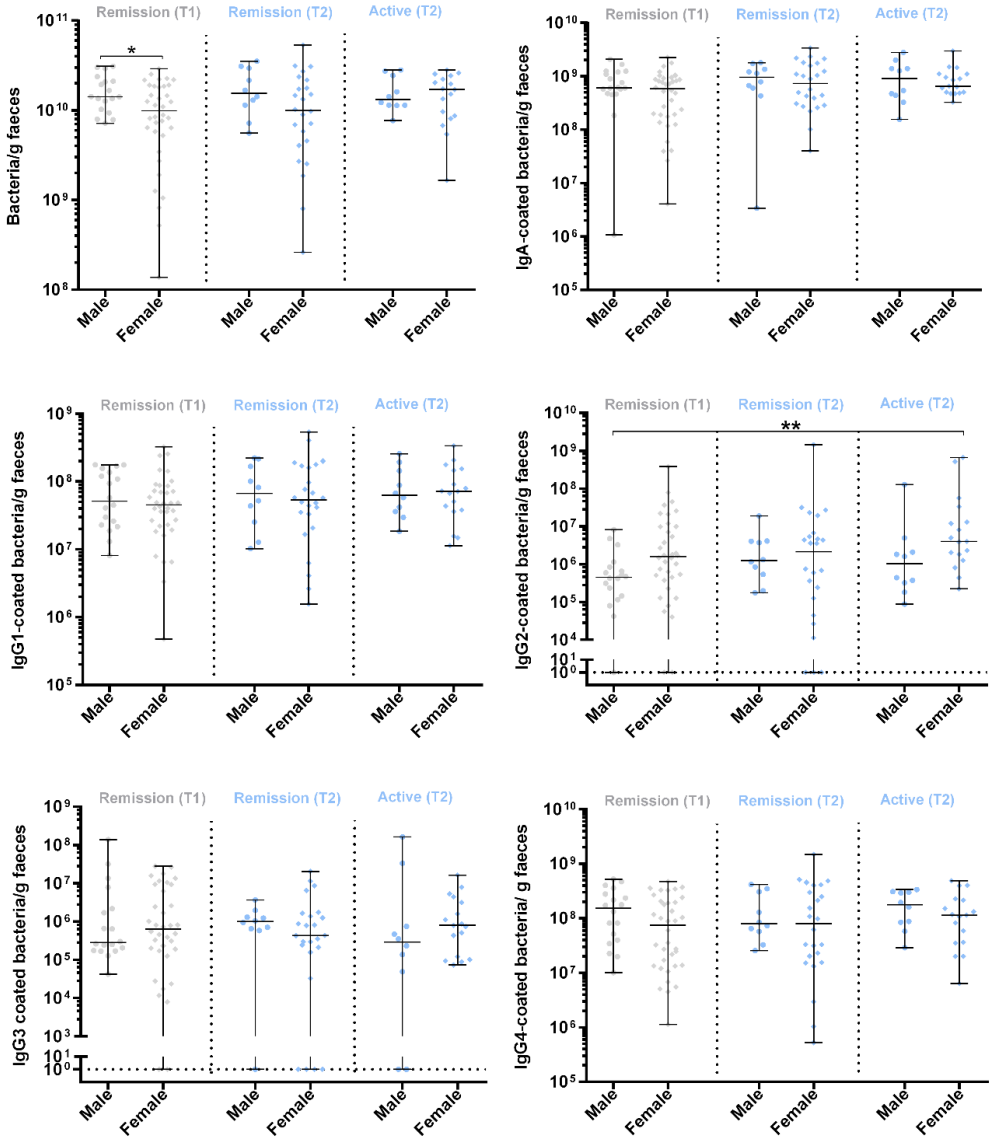

Figure 40. The profile of quantitative amount of bacteria-coating by IgA and IgG subclasses when patients are grouped accordingly to their gender.

*Data is represented as IgA and IgG subclass-coating and/or amount of bacteria/g faeces accordingly to patient's gender i.e., Male or Female. The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. 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. The significant finding is based on an Unpaired T-test. 0.01<*P-value<0.05. Indicated is median + ranges. Data was obtained from GraphPad Prism 5.*

Table 14. Quantitative data regarding the median levels of bacteria coated by all the antibodies relative to patients gender. *Data is represented as the median levels of IgA and IgG subclass-coating and/or amount of bacteria/g faeces accordingly to patient's gender i.e., Male or Female. The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.*

	Remission (T1)		**Remission (T2)**		**Active (T2)**	
	Male	Female	Male	Female	Male	Female
Total bacteria/g faeces	1.42E+10	9.91E+09	1.55E+10	9.96E+09	1.32E+10	1.71E+10
IgA coated bacteria/g faeces	6.11E+08	5.82E+08	9.57E+08	7.32E+08	9.05E+08	6.50E+08
IgG1 coated bacteria/g faeces	5.15E+07	4.53E+07	6.69E+07	5.38E+07	6.29E+07	7.16E+07
IgG2coated bacteria/g faeces	450500	1.61E+06	1.24E+06	2.13E+06	1.03E+06	3.98E+06
IgG3 coated bacteria/g faeces	280000	642000	996500	431500	293500	791000
IgG4 coated bacteria/g faeces	1.53E+08	7.46E+07	7.93E+07	8.03E+07	1.78E+08	1.14E+08

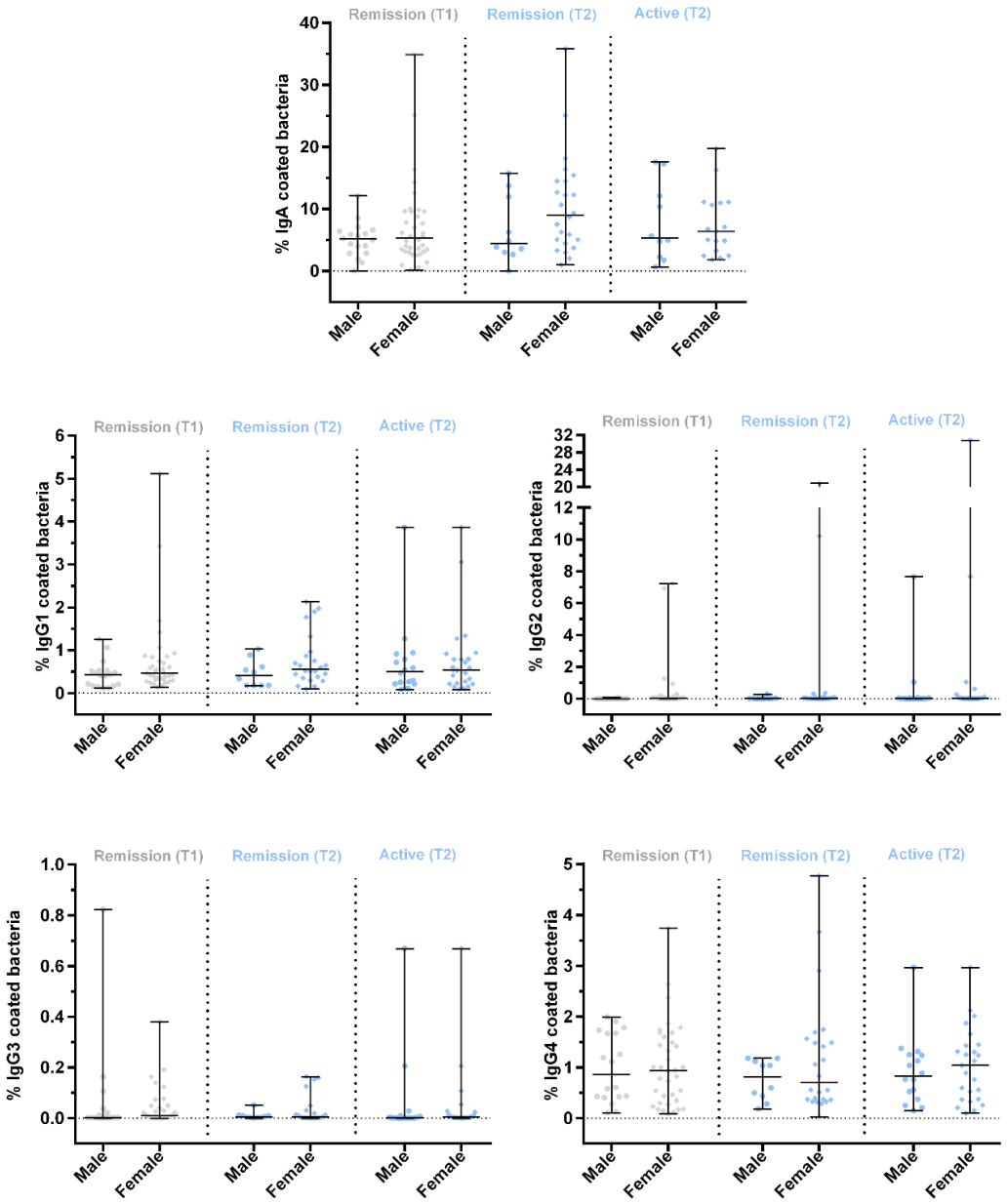

Figure 41. The profile of relative level of bacteria-coating by IgA and IgG subclasses when patients are grouped accordingly to their gender. *Data is represented as the relative amount of bacteria-coating by each antibody according to patient's gender i.e., Male or Female. The plots are divided into three groups where Remission (T1) includes all patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2. Indicated is median ± ranges. Data was obtained from GraphPad Prism 5.*

Table 15. Relative data relating to median levels of bacteria coated in patients grouped accordingly to their gender. *Data is represented as the median levels of IgA and IgG subclass-coating and/or amount of bacteria/g faeces accordingly to patient's gender i.e., Male or Female. The table is divided into three groups where Remission (T1) includes 54 patients in a state of remission in visit 1; Remission (T2) includes the patients whose disease remained in remission in visit 2 and Active (T2) the patients that have entered a state of active disease in visit 2.*

	Remission (T1)		Remission (T2)		Active (T2)	
	Male	Female	Male	Female	Male	Female
% IgA coated bacteria	5.161	5.318	4.391	9.028	5.332	6.427
% IgG1 coated bacteria	0.4255	0.467	0.413	0.556	0.5075	0.545
% IgG2 coated bacteria	0.002	0.0345	0.01	0.016	0.0065	0.016
% IgG3 coated bacteria	0.002	0.0105	0.0055	0.005	0.001	0.004
% IgG4 coated bacteria	0.862	0.9405	0.815	0.696	0.8245	1.037