Gut microbiome and IBD therapy: an interplay?

Study code: IBD(OME)

Study type: Observational

Protocol date: 22/10/2020

Protocol version: 2.0

Study Sponsor: Grupo de Estudo da Doença Inflamatória Intestinal (GEDII)

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Contact: fm@med.up.pt

National Scientific Coordinator Signature Page

Study title: Gut microbiome and IBD therapy: an interplay?

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Centre name: Select study center.

National Scientific Coordinator:

Name: Fernando Magro

E-mail: fm@med.up.pt

I, the undersigned, am responsible for overseeing and coordinating the conduct of this study on a national level.

I understand and will conduct the study according to the protocol, any approved protocol amendments, and all applicable Health Authority requirements and national laws.

I will not deviate from the protocol without prior written permission from the GEDII, except where necessary to prevent immediate danger to the subject.

_____ ____ / _____ / ____

Signature *Date*

Principal Investigator Signature Page

To be signed by the PI of each participating centre

Study title: Gut microbiome and IBD therapy: an interplay?

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Protocol version: 2.0

Centre name: Select study center.

Local Scientific Coordinator:

Name: Insert first and last name.

E-mail: Insert e-mail.

I, the undersigned, am responsible for the conduct of the study at this site and affirm that I understand and will conduct the study according to the protocol, any approved protocol amendments, and all applicable Health Authority requirements and national laws.

I will not deviate from the protocol without prior written permission from the GEDII, except where necessary to prevent immediate danger to the subject.

_____ ____ / _____ / ____

Signature *Date*

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1. Study Synopsis

Title	**Gut microbiome and IBD therapy: an interplay?**
Study code	IBD(OME)
National scientific coordinator	Fernando Magro (MD, PhD)
Principal investigator	To be designated in each participating hospital centre
Disease/condition	Crohn's disease and Ulcerative colitis
Rational	Recent evidence suggests that monoclonal antibodies influence gut microbiome dynamics but, on the reverse, microbiome itself may influence biologic therapy's outcome. However, to date, the relationship between gut microbiome and monoclonal antibodies in IBD patients remains poorly explained. Likewise, no information is available in the literature relating tofacitinib and gut microbiome.
Research hypothesis	Gut microbiome may be an important therapy-response biomarker, being useful to predict the outcome following therapy with monoclonal antibodies and tofacitinib.
Objectives	This study aims to analyse the association between gut microbiome and immunosuppressive therapy in IBD patients; the specific aims are: ▪ To evaluate the prospective changes of gut microbiome (bacteria and fungi) of IBD patients receiving biological drugs (infliximab, ustekinumab and vedolizumab) and tofacitinib ▪ To monitor serum drug levels and anti-drug antibodies, in patients receiving biologics, and to relate their levels with gut microbiome characteristics ▪ To monitor adverse events and the use of health resources ▪ To assess the clinical outcomes (response, remission and relapse) ▪ To study the association between microbiome diversity and relative abundance (at baseline and throughout the treatment) and the clinical outcomes, exploring the role of microbiome as a predictive biomarker
Study design	Observational, prospective cohort of IBD patients.
Inclusion criteria	All the following criteria must be met: ▪ Patients aged 18 years or older ▪ Patients with active luminal Crohn's Disease (moderate to severe) or active ulcerative colitis (moderate to severe) ▪ Patients who will initiate biologic agents (infliximab, vedolizumab or ustekinumab, reference products or biosimilars) or tofacitinib (for UC) at the time of inclusion: both anti-TNF-naïve or patients previously treated with monoclonal antibodies ▪ Patients who have been submitted to colonoscopy at the baseline ▪ Patients who have signed the informed consent

Exclusion criteria	Patients will be excluded if **at least one** of the following criteria is met: ▪ Failure to meet any of the inclusion criteria ▪ Antibiotics and probiotics' use in the four weeks prior to inclusion ▪ Patients under current treatment or treated in the four weeks prior to inclusion with the following pharmacological classes: biguanides (metformin), gliptins (DDP-4 inhibitors), incretin mimetics (GLP-1 analogues), or GLP-2 analogues ▪ Patients who have any condition precluding treatment with biologics or with tofacitinib ▪ Patients under treatment with any investigational agent ▪ Patients with active cancer ▪ Patients who are pregnant or breastfeeding ▪ Patients who are not willing to comply with appointments or procedures
Expected number of patients	**300 patients**: 180 with Crohn's Disease (60 receiving infliximab, 60 ustekinumab, and 60 vedolizumab); 120 with Ulcerative Colitis (40 receiving infliximab, 40 vedolizumab, 20 ustekinumab and 20 tofacitinib).
Expected number of sites	Nine Portuguese Hospital Centres.
Data collection	Data will be collected at baseline (prior treatment start), day 1 (treatment start), and at each drug infusion or follow-up visit (timepoints will vary depending on the treatment scheme). The last visit requiring data collection will occur between 48 and 56 weeks after treatment start. Main data to collect: ▪ Anthropometric measures ▪ IBD location, activity and disease course ▪ Extra-abdominal manifestations ▪ Concomitant pharmacological therapies (for IBD and other conditions) ▪ Dose of biologic agent or tofacitinib ▪ Routine laboratory parameters (haemoglobin, leukocytes, neutrophils, eosinophils, lymphocytes, C-reactive protein, ferritin, iron, transferrin and albumin) ▪ Stool samples (faecal calprotectin levels and gut microbiome [fungi and bacteria]) ▪ Blood samples (drug concentration and anti-drug antibodies levels) ▪ Adverse events ▪ Status: ongoing/discontinuation. If discontinued, reason
Study timeline	Study start: first trimester of 2021 Recruitment: one year (2021) Follow-up: one year per patient Study closure: last trimester of 2023 Overall duration: three years

2. Summary

The aetiology of Inflammatory bowel disease (IBD) involves the complex interaction between several factors, among which genetic susceptibility, dysregulated immune response and environmental factors. Recently, much emphasis is being placed on the major role of intestinal microbial community in IBD. Also, current evidence suggests that not only pharmacological therapies influence gut microbiome as, in reverse, microbiome itself may influence the drugs' outcome. However, despite the development of rapid microbiome sequencing techniques the relationship between microbiome and the immunosuppressive therapy for IBD remains poorly explained. The ability to predict the efficacy of biological agents and tofacitinib would allow adopting a more targeted approach, therefore reducing costs and the potential incidence of adverse events. In this context, this project aims to comprehensively study the changes in the gut microbiome of IBD patients receiving biological therapy (anti-TNF, anti-integrin and anti-interleukin) or tofacitinib and to explore the role of microbiome as therapy-response biomarker.

3. Introduction

Inflammatory bowel disease (IBD), which comprises Crohn's disease (CD) and ulcerative colitis (UC), is a chronic and disabling condition with accelerating incidence, following the industrialization and westernization of society[1]. Currently, the incidence rates in Europe range from 0.7 to 9.8 cases per 100,000 person-years in CD and 1.5-20.3 cases per 100,000 person-years in UC[1]. The most consensual model regarding IBD aetiology suggests the existence of a complex interaction between host's genetic susceptibility, external environment and abnormal immune response to the luminal microbial content[2]. The therapeutic landscape for IBD is rapidly evolving with the development of novel treatment options including targeted monoclonal antibodies and small molecules.

The rapidly evolving genotyping and sequencing techniques allowed to decipher gut microbiome of IBD patients. Several studies have shown an association between IBD and the disruption of intestinal micro-ecological balance, with differences in microbial composition, with a decreased alpha microbial diversity (species richness at a local scale) and a shift towards pro-inflammatory microbial species[3,4]. Indeed, the relative abundance of Bacteroidetes (specially *Bacteroides fragilis*) and Proteobacteria (particularly *Escherichia coli) has been described to be increased in IBD while the amount of* Firmicutes (mainly *Roseburia*, *Faecalibacterium prausnitzii* and other short chain fatty-acids [SCFA] producing species) is decreased[5]. Besides that, changes in bacterial function in IBD patients have already been reported, resulting in a decrease in the butyrate levels. Besides being the main energy source for colonic epithelial cells, this SCFA also induces Treg cells to differentiate, participating in immune response[6]. Regarding the fungal microbiome, a decrease in the

proportion of *Saccharomyces cerevisiae*, an increase in the Basidiomycota/Ascomycota ratio and in the proportion of *Candida albicans* were found in IBD patients[7].

It is important to take into account, however, that microbiome is inherently modifiable. The implication of this interplay between human organism and microbes is that nutritional modifications and pharmacological therapies that are targeted at the host will also significantly impact on the gut microbiota[5]. Similarly, it is hypothesized that the gut microbiome of the patients will influence the outcome of pharmacological therapies[8].

The advent of monoclonal antibody therapies has revolutionised inflammatory bowel disease (IBD) treatment and delivered great benefits to patients. Currently, seven agents are approved: four are anti-TNF-α agents (infliximab, adalimumab, golimumab and certolizumab); two are anti-integrin (natalizumab and vedolizumab) while ustekinumab targets interleukin-12/23 pathways[9]. Tofacitinib, an oral small-molecule that targets Janus kinase (JAK) 1, 2, and 3, was approved in 2018 for the treatment of moderate-to-severe active UC with inadequate response, loss of response or intolerance to corticosteroids, immunosuppressive agents and/or biological therapies. JAK inhibitors present several advantages in comparison with monoclonal antibodies, among which: oral administration, predictable pharmacokinetics with a reduced plasma half-life, rapid onset of action and quick clearance (beneficial in cases of severe infections and need for surgery and the lack of immunogenicity[10].

Despite the existence of several alternatives, to date, the different biologic agents and tofacitinib result only result in approximately 40% remission rates after one year of therapy, highlighting a persistent therapeutic gap[11]. The fact that only a subgroup of patients responds to therapy confirms the need to identify biomarkers for therapeutic response which would allow to reduce costs and the potential incidence of adverse events[12]. Thus far, the biomarkers implemented in the clinical management of IBD concern the differentiation between IBD types (CD or UC), the assessment of disease activity and/or prognosis[13]. However, the use of predictive biomarkers for therapy-related outcomes is still scarce[14].

A few recent studies aimed to evaluate the longitudinal dynamics of gut microbiome of IBD patients receiving monoclonal antibodies and to analyse the association between baseline gut microbiome composition and clinical outcomes following biologic therapy. Globally, these studies report that higher microbial diversity at baseline and/or throughout the treatment was associated to response to infliximab[15,16], adalimumab[17], vedolizumab[8] and ustekinumab[18]. Also, throughout the treatment, the relative abundances of microorganisms belonging to the genera *Escherichia* and *Enterococcus* were reduced while the abundance of SCFA producing bacteria increased. On the other hand, information regarding the relationship between fungal microbiome and biologic therapy in IBD is almost inexistent. Concerning the use of gut microbiome as a biomarker, the global conclusions are that the incorporation of both clinical and microbiome data may be useful to predict

the outcome with biological therapies; however, the existing evidence is heterogeneous and scarce. Indeed, ten[8,15–23] studies on this thematic were published between 2014 and 2018 and their characteristics are notably different. Among other aspects, studies differ regarding the type of IBD, number of patients and their age as well as the monoclonal antibody used, hampering results comparison and the translation of such information into the clinical practice.

Concerning tofacitinib, to date no literature exists regarding the its effects on gut microbiome of IBD patients. However, a preclinical study with an experimental model of collagen-induced rheumatoid arthritis reported that treatment with tofacitinib was associated with an increase in microbial diversity and in the relative abundance of the phyla Firmicutes and Actinobacteria[24].

In this context, this project aims to study the interplay between biologic therapy and tofacitinib and gut microbiome in a more comprehensive way, using emerging bioinformatics tools to explore its use as a therapy-response biomarker.

4. Objectives

The main objective of this project is to study the association between gut microbiome and immunosuppressive therapy in IBD patients. The project will be divided in five specific aims:

1. To understand the prospective changes of gut microbiome of IBD patients (bacteria and fungi) receiving biological drugs targeting tumour necrosis factor alpha (TNF-α) [infliximab], interleukin 12 and interleukin 23 [ustekinumab] and $\alpha_4\beta_7$ integrin [vedolizumab], or receiving a small molecule that targets the JAK-STAT signaling pathway [tofacitinib]
2. To monitor serum drug levels and to evaluate the development of anti-drug antibodies, in patients receiving biologics, and to relate their levels with gut microbiome characteristics
3. To monitor the occurrence of adverse events and the use of health resources, consultations, admission to emergency rooms, hospitalizations and surgeries throughout the treatment
4. To assess the clinical outcomes (response, remission and relapse) following therapy with monoclonal antibodies or tofacitinib
5. To study the association between microbiome diversity and relative abundance (both at baseline and throughout the treatment) and the clinical outcomes following therapy with biological agents or tofacitinib, therefore exploring the role of microbiome as a predictive biomarker

5. Research plan, methods and tasks description

5.1. Study design

This project will be a prospective observational study, thereby not imposing any experimental intervention or treatment. All therapeutic decisions and management will be done according to routine clinical practice. The enrolment period of the prospective component of the study will start on day of the study visit of the first included patient and will run, within the defined period, until the expected number of patients is attained.

5.2. Study timelines

The study is expected to start during the second trimester of 2020 and the overall expected duration is three years: one year of recruitment; one-year observation period; and one year to achieve the target number in case the number of participants had not been achieved within the defined timeframe and to work on data analysis and manuscripts drafting.

Patients will be observed, and data will be collected at: baseline (prior treatment initiation), day 1 (treatment start) and then at different timepoints: before infusion of the intravenous drugs (infliximab and vedolizumab; weeks will be defined according to the treatment scheme) or at each follow-up consultation, every 8 weeks, (for patients treated with subcutaneous infliximab, tofacitinib, ustekinumab).

The last visit requiring data collection will occur between 48 and 56 weeks after treatment start.

5.3. Population and sampling

It is expected that patients will be recruited from nine participant hospitals as they attend their routine medical examination. All subjects who meet eligibility criteria and give their written informed consent to participate will be consecutively enrolled and characterized regarding several socio-demographic and clinical characteristics.

Overall, 300 IBD patients will be included in this study:

- 180 patients with Crohn's Disease: 60 receiving infliximab, 60 ustekinumab, and 60 vedolizumab
- 120 with Ulcerative Colitis: 40 receiving infliximab, 40 vedolizumab, 20 ustekinumab and 20 tofacitinib

The recruitment period is expected to last one year but may be extended if the target number of participants is not achieved within the defined timeframe.

5.4. Inclusion criteria

For inclusion, all the following criteria must be met:

- Patients aged 18 years or older
- Patients with **active luminal Crohn's Disease** (moderate to severe, with or without fistulisation and/or perianal disease) or **active ulcerative colitis** (moderate to severe)
- Patients who will **initiate biologic agents** (infliximab, vedolizumab or ustekinumab, reference products or biosimilars) or tofacitinib (for UC) at the time of inclusion according to physician's criteria: both anti-TNF-naïve or patients previously treated with monoclonal antibodies
- Patients who have been submitted to **colonoscopy at the baseline** (4 weeks before or after week 0)
- Patients who have signed the informed consent

5.5. Exclusion criteria

The patients will be excluded If at least one of the following criteria is met:

- Failure to meet any of the inclusion criteria
- Antibiotics and probiotics' use in the four weeks prior to inclusion
- Patients under current treatment or treated in the four weeks prior to inclusion with the following pharmacological classes: biguanides (metformin), gliptins (DDP-4 inhibitors), incretin mimetics (GLP-1 analogues), or GLP-2 analogues
- Patients who have any condition precluding treatment with biologics
- Patients under treatment with any investigational agent
- Patients with active cancer
- Patients who are pregnant or breastfeeding
- Patients who are not willing to comply with routine clinical appointments or procedures

There will be no exclusion in terms of race, sex or disease status. Patients with mental and learning disabilities are included provided they are deemed competent to provide informed consent; however, patients with significant cognitive impairment will be excluded from the study.

5.6. Discontinuation from the observation period

IBD patients receiving monoclonal antibodies or tofacitinib will be followed up for 12 months. However, observation may be prematurely stopped for different reasons including (but not limited to):

- Protocol violation
- Failure to submit blood and stool samples as indicated at each phase of the study
- Need for use antibiotic and/or probiotics during the study
- Cancer diagnosis
- Death
- Pregnancy
- Patient withdrawal of consent or loss to follow up

In case of drop-out, the date of study discontinuation and the associated reason will be recorded in an electronic Case Report Form (CRF).

6. Data collection

All data will be obtained from medical charts, laboratory reports and, when relevant, complemented with subject's interview or other medical sources. The investigator will be responsible for ensuring that all data are accurate and reliably recorded in the case report form (CRF) and that the subject's confidentiality is always maintained.

6.1. Baseline characteristics

The following data will be collected for all eligible patients before starting therapy:

- Date of birth
- Sex
- Residence (district)
- Anthropometric parameters (height and weight)
- Smoking status:
    - Non-smoker: never smoked before or smoked very occasionally
    - Former smoker: patients who stopped smoking more than 6 months before inclusion
    - Current smoker: more than 7 cigarettes per week in the least 6 months (≤ 10 cigarettes/day, 11 to 20 cigarettes/day or > 20 cigarettes/day)
- Family history of IBD

- Date of IBD diagnosis (based on clinical, endoscopic, histologic and radiographic criteria)
- Symptoms at disease onset (abdominal, constitutional, abdominal + constitutional, anal disease, acute abdomen, fever, anaemia, extra-intestinal manifestations, abdominal mass)
- Clinical characteristics:
    - IBD location/behaviour, according to the Montreal Classification
        - CD patients: regarding location as ileal (L1), colonic (L2), ileocolonic (L3) ± upper gastrointestinal tract (L4); regarding behaviour as non-stricturing and non-penetrating (B1), structuring (B2) and penetrating (B3), ± perianal disease (p)
        - UC patients: regarding extent as ulcerative proctitis (E1), left-sided UC (E2) or pancolitis (E3); regarding severity as moderate UC (S2) or severe UC (S3)
    - Disease activity:
        - CD patients: Harvey Bradshaw Score (5 items: general well-being, abdominal pain, number of liquid or soft stools per day, abdominal mass and complications)
        - UC patients: Partial Mayo Score (3 items: stool frequency, rectal bleeding, physician's global assessment)
    - Endoscopy findings:
        - All eligible patients must have undergone endoscopic studies within 4 weeks prior starting treatment with monoclonal antibodies or tofacitinib, as part of routine practice; disease activity will be classified using the Simple Endoscopic Score (for CD patients) and the Mayo Score (for UC patients); 4 biopsies are expected to be performed in the lesioned colonic segments, with 4 additional biopsies from adjacent non-lesioned colonic segments, for a total of 8 biopsies in IBD patients
        - This procedure does not pose additional risks to the patients, is not interventional in nature, and does not deviate from standard clinical care[25,26]
- Extra-abdominal manifestations
- Comorbidities, including heart disease, hypertension, bile acid malabsorption, celiac disease, rheumatoid arthritis, *diabetes mellitus* type I, Sjögren syndrome, psoriasis, thyroiditis, hyperthyroidism or hypothyroidism or other auto-immune disorders
- History of bowel surgery for IBD
- Previous IBD pharmacological therapies for IBD
- Current pharmacological therapies for IBD, including: 5-aminosalicylate, corticosteroids, thiopurines (azathioprine or 6-mercaptopurine), methotrexate, cyclosporine, tacrolimus, mycophenolate mofetil

- Current medical treatments for other conditions, including NSAIDs, PPIs, SSRIs or statins. The use of antibiotics in the last year will also be recorded
- Name of the biologic agent (brand or biosimilar) or tofacitinib, dosage and date of start

6.2. Data collected on each evaluation

The following data will be collected during induction and maintenance phases on the timelines defined in *section 5.2* and schematically presented in *section 15.2*:

- Anthropometric parameters (height and weight)
- Disease activity (as described above)
- Disease course (relapse of disease activity and need for therapy adjustment; changes in dosage and frequency of administration of monoclonal antibody; changes in dose of tofacitinib; changes in other IBD therapies)
- Concomitant non-IBD pharmacological therapies, if changed since previous data collection point
- Extra-abdominal manifestations
- Routine laboratory parameters (haemoglobin, leukocytes, neutrophils, eosinophils, lymphocytes, CRP, iron, transferrin, ferritin and albumin)
- Central Laboratory Parameters:
    - Stool samples: assessment of faecal calprotectin levels and gut microbiome
    - Blood samples: assessment of the level of biological drug and level of anti-drug antibodies
- Use of health resources (surgeries, consultations, admission to emergency rooms, hospitalizations related or not with IBD)
- Adverse events (AE):
    - Corresponding to any harmful manifestation in a patient (that does not necessarily have a causal relationship with the treatment): information on drug, therapy duration, event description (characteristics and duration), action taken, outcome, causality assessment and severity. The following cases will be considered serious AE: event associated to life-threatening risk or leading to death; event that prolongs hospitalization, causes persistent or significant disability; if there is suspicion of transmission of an infectious agent through medication; another situation considered severe by the investigator
    - All adverse events must be reported to the pharmacovigilance unit
- Status: ongoing/discontinuation. If discontinued, reason

- Data regarding the **endoscopic studies** performed between week 48 and 56, according to routine practice, will be recorded and classified using the Mayo score (for UC) or the Simple endoscopic score (for CD)

7. Experimental procedures and data analysis

7.1. Material required for samples collection

- Stool sampling: stool collection kit, tube with ethanol (for gut microbiome analysis), tube without preservative (for faecal calprotectin determination), biohazard bag with date and time label.
- Blood sampling: serum-separating tubes.

7.2. Samples collection, storage and transfer

Blood samples will be collected prior to infusion at the scheduled hospital appointment (for intravenous drugs) or at the follow-up visit (for oral and subcutaneous drugs) into gel tubes for coagulation (serum-separating tubes). One hour after collection, the sample will be centrifuged, and the serum transferred to tubes stored at -20° C. Blood samples must be transferred to the central laboratory, under refrigeration.

At each visit, the physician will provide the stool collection device and explain the stool kit assembly instructions (detailed description of the procedure in Figure 1). Fresh stool samples from IBD patients will be collected at home. Samples must be collected to two tubes: one containing ethanol 100% for DNA preservation, and other without preservative. Samples must be stored at home at room temperature and delivered to the clinician within 24h-48h. At the hospital, stool samples must be kept at room temperature for the maximum of 48h until shipment to the central laboratory. Exceptionally, stool samples may be handled at the participating centre in the 48h following the infusion/follow-up visit.

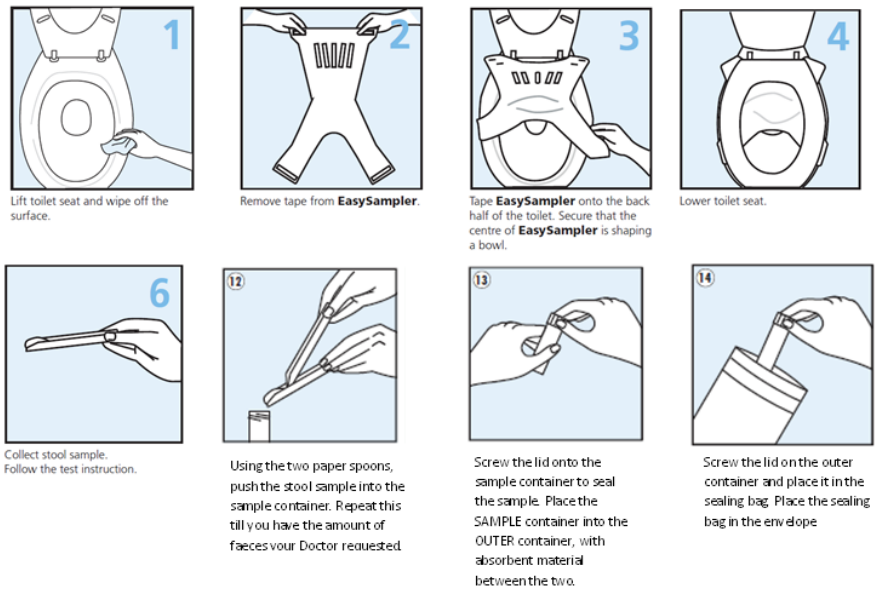
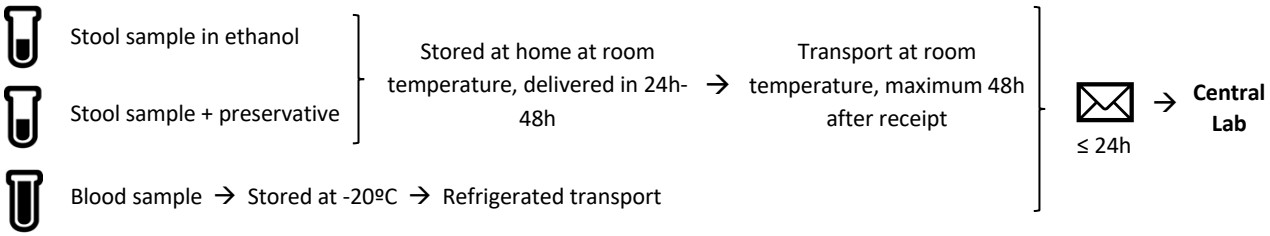


Figure 1. Collection of stool samples, using a sample collector device.

All blood and stool samples must be shipped to the Central Laboratory (GEDII, Centro de Investigação Médica, Faculdade de Medicina da Universidade do Porto) for analysis; maximum transit time for all samples of 24 hours. Samples storage and transfer is summarized in Figure 2.



7.3. Gut microbiome analysis

Gut microbiome of IBD patients is not routinely evaluated in the clinical practice. In this study, stool samples will be analysed using the MiSeq® System, a DNA-to-data next-generation sequencing (NGS) platform, integrating cluster generation, amplification, sequencing, and data analysis into a single instrument (Illumina).

7.3.1. Stool microbiome DNA assessment

In the central laboratory, stool samples will be stored at -80 °C after removing the preservative and will remain frozen until further processing. Microbial DNA will be isolated using a commercial kit, following manufacturers' instructions. The DNA concentrations of the extracts will be measured fluorometrically and DNA will be stored at -80°C until further use.

7.3.2. Gut microbiome sequencing

The microbial profiles of the DNA extracts will be analysed as described in the gene-sequencing protocols (Illumina). The protocol will target the 16S rRNA gene (for bacteria) and the internal transcribed spacer (ITS) ribosomal RNA (for fungi) and will follow the Illumina Metagenomic Sequencing Library Preparation guide[27]. Quality of the raw sequence data will be checked with the FastQC quality-control tool, and the datasets will be analysed with QIIME 2.0 pipeline (Quantitative Insights Into Microbial Ecology; http://qiime.org), as described previously[28–30], using the GreenGenes database[31]. Quality filters will be applied, and the sequences will be clustered into operational taxonomic units (OTUs; sometimes referred to as phylotypes), which provide a working name for groups of related bacteria. This OTUs selection will be performed using the QIIME reference optimal picking, using Usearch (V.7.0.1090) to perform the clustering at 97% of similarity. Samples

with less than 10 000 counts will be removed. OTUs that are not present in at least 1% of our samples or with a low abundance (<0.01% of the total counts) will be filtered out.

One distinctive characteristic of the microbiome data is over-dispersion: while some taxa are common among samples, many others are present at much lower abundances or were never recorded, leading to zero-inflated distributions[32]. The tools for microbial community analysis available on the QIIME platform bypass those characteristics and rely on nonparametric tests to compare species across different conditions[33].

7.3.3. Analysis of Longitudinal Trajectory of the Gut Microbiome

The longitudinal trajectory of microbiome will be analysed as described by Ananthakrishnan et al. (2018)[8]. The log_2 fold change (FC) in the relative abundance between evaluation time-points will be registered. The change will be considered significant if a *taxon* experiences consistent ≥ 1.5 FC in over 80% of the samples. A persistent index, using Bray-Curtis dissimilarity, will be developed to measure the degree of persistency of the effect of the immunosuppressive agents under study on microbial taxa.

7.3.4. Prognostic models based on gut microbiome

Bayesian networks include estimation techniques for inferring complex networks from noisy data and allow to predict clinical outcomes of relevance. In this study, an integrative Bayesian approach will be used to evaluate the association between taxa abundance data (at baseline and throughout the treatment) and other available covariates (baseline epidemiologic and clinic characteristics of IBD patients), following the described by Wadsworth et al. (2017)[33]. Different data-mining techniques (decision trees, association rule mining and Bayesian networks) will be used to develop prognostic models based on gut microbiome. These analyses will be performed in adequate software including IBM SPSS Statistics, R and Waikato Environment for Knowledge Analysis (Weka).

7.4. Assessment of faecal calprotectin levels

In IBD, the presence of active gut inflammation is associated with migration of leucocytes to the gut mucosa. As a result, the faecal stream contains increased levels of inflammatory proteins among which calprotectin. It is well stablished that faecal calprotectin correlates with IBD disease activity, being useful to predict response to treatment or relapse[34]. For faecal calprotectin levels determination, the collected stool samples will be sent immediately to the central laboratory for extraction; where those can be stored up to 6 days at room temperature. Stools will be extracted with a commercial kit and stored at -80ºC, for at the most 4 months.

Calprotectin from extracted samples will be determined using an automated clinical chemistry analyser, a turbidimetric immunoassay, fCAL turbo.

7.5. Assessment of serum drug levels and antidrug antibodies

At the central laboratory, the drug levels will be ascertained using commercial kits; the concentration of anti-drug antibodies will be determined with ELISA assays, employing the quantitative enzyme immunoassay technique. For each monoclonal antibody, the methodologies previously described in the literature will be followed: Magro et al. (2018)[35] for infliximab; Ungar et al. (2018)[36] for vedolizumab; and Battat et al. (2017)[37] for ustekinumab.

7.6. Safety Analysis

Whenever an adverse event (AE) occur, the severity, degree of relationship with study drug, duration, actions taken, and outcome will be registered. All deaths will be listed, being our not attributed to an AE. The incidence of AE and serious AE will be presented. The occurrence of AE by severity and degree of relationship with study drug will be summarized by total number of observations (n) and relative frequency (%).

8. Statistical analysis

The sample size is not based on formal statistical assumptions; 300 patients are expected to be recruited.
All quantitative variables will be summarized through descriptive statistics namely mean, median, standard deviation and range (minimum and maximum) and qualitative variables though absolute (n) and relative frequencies (%) and 95% confidence intervals (if applicable).The comparison of two independent samples in respect to quantitative variables will be performed through t-test for independent samples or the Mann-Whitney non-parametric test, according to the assumption validations of the statistical test; differences with $p<0.05$ will be considered significant. The proportion of patients with clinical response or achieving remission at each data collection time point will be summarized using 95% confidence intervals. Area under the ROC curve will be used to correlate numerical variables with binary categorical variables. A high discriminatory ability will be considered for values > 0.75.
Concerning the gut microbiome data, statistical analyses of the gene sequence data will be performed with QIIME statistical tools (an open-source bioinformatics pipeline to analyse raw DNA sequencing data). All analyses will be made from the randomly subsampled OTU table, with rarefaction level matching the sample with the lowest total OTU count. To study the bacterial diversity of the samples, α and β-diversity metrics will

be computed, and α-rarefaction plots will be generated. Statistically significant differences will be assessed, applying nonparametric methods and considering $p<0.05$ as statistically significant. To test for statistically significant differences in taxonomic richness, *i.e.*, in the OTU abundances, Kruskal-Wallis test will be applied. Taxonomic levels phylum and genus will be studied, and false discovery rate (FDR)-adjusted $p<0.05$ will be considered as statistically significant. To analyse the differences in the overall bacterial diversity across the samples, weighted UniFrac distance matrices will be generated from the randomly subsampled OTU table, and principal coordinate analysis (PCoA) plots will be obtained.

9. Ethical issues

This study will be conducted in accordance with the ethical principles originated from the Declaration of Helsinki and the Portuguese Clinical Research Law (Law 21/2014, 16th April 2014), after approval by the Ethics committees of all participating hospitals.

The investigators will be responsible for ensuring that data are accurate and reliably recorded in the case report form (CRF). The data obtained from the clinical records will be kept on file and may only be used by the study investigators and the ethics committee of the Institution where the study is conducted. The questionnaires, databases and other documents generated in this study will be identified only by an automatically generated alphanumeric code. All information will be confidential, and the anonymity of patients will always be ensured. When necessary, the investigator will submit and, obtain approval from the competent Ethics Committee for all subsequent protocol amendments.

10. Informed consent

Prior to the inclusion of the patient, the investigator is responsible for obtaining written informed consent from the subject (or authorized representative) after an adequate and clear explanation of the aims, methods, anticipated benefits, and potential hazards of the study, in accordance with the institutional review board regulations of the seven participating institutions.

The informed consent process should be documented in the subject's medical charts, and the informed consent form should be signed and personally dated by the subject (or authorized representative, if applicable) and by the person who conducted the informed consent discussion (not necessarily an investigator). The original signed informed consent form should be retained in accordance with institutional policy, and a copy of the signed consent form should be provided to the subject or authorized representative.

11. Study discontinuation criteria

Premature termination of the study may occur because of a regulatory authority decision, change in opinion of the ethics committee, or at the discretion of the GEDII. If GEDII decides to terminate prematurely the study, the appropriate Ethics Committee and regulatory authority (if applicable) will be promptly notified.

12. Quality control

The study will involve a GEDII monitor who will be responsible to ensure that the study is conducted according to the protocol and to the local regulatory/ethical requirements. Before the start of activities, a study monitor will conduct "initiation visits" at the sites in order to train the investigational team on the protocol and other protocol-related procedures. Throughout the study, the monitor will also be responsible for conducting periodic monitoring visits at the sites to ensure that the protocol is being followed and the data recorded is accurate and collected according to the defined procedures.

The sites may be subject to review by the Independent Ethics Committee and/or to quality assurance audits performed by GEDII designated representative, and/or to inspection by appropriate regulatory authorities. The Investigator(s) and their relevant staff should be available during the monitoring visits and possible audits or inspections. The investigator and institution will give the appropriate regulatory authorities direct access to the source documents in order to perform this verification.

Prior to study start, all investigators and study staff will receive training on the protocol and other protocol-related procedures; this training will be provided by the GEDII or its representative. The investigator is required to sign a protocol signature page confirming his/her agreement to conduct the study in accordance with this protocol and to give access to all relevant data and records to monitors, auditors, Ethics Committees, and regulatory authorities, as required.

13. Study archive

The study coordinator will keep an archive of all study documentation with access restricted to study team.

14. Results dissemination

All documents and results generated from this study are property of Coordinating Investigator and GEDII. Any related publications must be previously approved in written by the Coordinating Investigator and GEDII. The results of the study will be presented in national and international meetings and will be published in

international papers. The publication of results must be accepted by all participating investigators and in strict adherence to the principles originated from the Helsinki Declaration and Portuguese Clinical Research Law (Law 21/2014, 16th April 2014)[32]. Any investigator involved in the study who intends to develop any sub-analysis using the collected data may submit a proposal to the study sponsor (GEDII).

15. Appendix

15.1. Data collection timepoints

Information to collect	Baseline#	Induction phase	Maintenance phase	Final visit
300 patients: 180 with CD (60 receiving IFX, 60 UST, and 60 VEDO); 120 with UC (40 receiving IFX, and 40 VEDO, 20 UST, 20 TOFA)	Prior to treatment start	IFX/VEDO: day 1#, weeks 2 and 6 UST/TOFA: day 1, week 8	IFX-iv/VEDO: every 4/4, 6/6, or 8/8 weeks; UST: 4/4 or 8/8 weeks; TOFA/IFX-sc 8/8 weeks	Between 48 and 56 weeks
Date of birth and sex	x			
Anthropometric parameters[1]	x	x	x	x
Smoking status	x			
Date of IBD diagnosis	x			
Symptoms at disease onset[2]	x			
IBD location/behaviour[3]	x			x
IBD clinical score/disease activity[4]	x	x	x	x
History of bowel surgery	x			
Familial history of IBDs	x			
Extra-abdominal manifestations	x	x	x	x
Past medical history	x			
Previous or current pharmacological therapies	x	x	x	x
Endoscopic findings[5]	x			x
Dosage of biologic agent, date of start	x	x	x	x
Routine laboratory parameters[6]		x	x	x
Level biological drug and anti-drug antibodies		x	x	x
Faecal calprotectin levels		x	x	x
Gut microbiome (bacteria and fungi)		x	x	x
Use of health resources[7]		x	x	x
Adverse events		x	x	x

In some clinical settings, the baseline and day 1 visits **may be the same**.

[1] Height, weight, body mass index (BMI) | [2] Abdominal, constitutional, abdominal and constitutional; anal disease, acute abdomen, fever, anaemia, extra-intestinal manifestations, abdominal mass | [3] According to the Montreal Classification | [4] According to the Harvey Bradshaw Score (for CD) and Partial Mayo Score (for UC) | [5] Endoscopy findings will be classified using the Mayo score (for UC) or the Simple endoscopic score (for CD) | [6] Haemoglobin, leukocytes, neutrophils, eosinophils, lymphocytes, CRP, iron, transferrin, ferritin and albumin | [7] Surgeries, consultations, admission to emergency rooms, hospitalizations; related or not with IBD

Abbreviations: Crohn's disease (CD), infliximab (IFX), infliximab intravenous (IFX-iv), infliximab subcutaneous (IFX-sc), tofacitinib (TOFA), ulcerative colitis (UC), ustekinumab (UST), vedolizumab (VEDO)

15.2. Project timeline

Tasks	2021				2022				2023			
	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec
Patients recruitment												
Observation period												
Data analysis, manuscripts drafting												

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