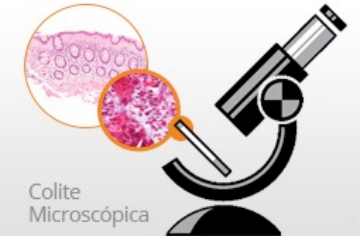
CLINICAL STUDY PROTOCOL

Grupo de Estudo da Doença Inflamatória Intestinal (GEDII)



Title: An epidemiological study to determine the prevalence of Microscopic Colitis and describe its clinical and histological features among patients with symptoms of chRonic watery diarrhea submitted to colonoscopy Attending to the Portuguese gastroenterology setting

Study code: **MICRA**

Type of study: Observational

Date of protocol: *12th November 2014*

Version no.: 1

Study Sponsor:	Grupo de Estudo da Doença Inflamatória Intestinal (GEDII)
Name of Scientific Coordinator	Prof. Fernando Magro
Signature and Date	_____ _____
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PRINCIPAL INVESTIGATOR SIGNATURE PAGE *(to be signed by the PI from each participating center)*

Study Title: An epidemiological study to determine the prevalence of Microscopic Colitis and describe its clinical and histological features among patients with symptoms of chronic watery diarrhea submitted to colonoscopy attending to the Portuguese gastroenterology setting.

Study Code: MICRA

Protocol Version/Date: 1, 12th November 2014

Center Name: _____

Principal Investigator:

Name:

Academic degree:

Address:

Phone:

Email:

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 of Signature

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1. Sinopse

Title:	An epidemiological study to determine the prevalence of **Mi**croscopic **C**olitis and describe its clinical and histological features among patients with symptoms of ch**r**onic watery diarrhea submitted to colonoscopy **a**ttending to the Portuguese gastroenterology setting
Study Code:	**MICRA**
Scientific Coordinator:	Prof. Fernando Magro
Disease/Condition:	Microscopic Colitis
Rational:	There's a lack of epidemiological data regarding Microscopic Colitis (MC) in Portugal, including prognosis factors and therapies used to treat this condition. Therefore, it becomes pertinent to characterize this population and, simultaneously, to evaluate the key characteristics that distinguish this clinical entity from other conditions among a population who attends a gastroenterology setting with symptoms of chronic watery diarrhea. This study aims to estimate the prevalence of MC and its subtypes among patients who attend a gastroenterologist appointment due to chronic, watery diarrhea of unknown etiology and who are eligible to colonoscopy. In addition, socio-demographic, clinical characteristics and treatment patterns will be described for patients with confirmed diagnosis of MC and these features will be compared with non-MC cases. Furthermore, this study will prospectively assess the clinical and histological progression of MC patients during a follow up period of 2 years. Potential biomarkers for this condition will also be explored.
Research hypothesis:	No research hypothesis was defined
Primary Objectives:	To determine the prevalence of MC among patients who attend a specialist appointment with symptoms of chronic, watery diarrhea of unknown etiology for more than 4 week and undergo colonoscopy.
Secondary Objective(s):	• To determine the prevalence of MC subtypes (collagenous colitis, lymphocytic colitis and incomplete colitis) • To compare the socio-demographic, clinical and histological characteristics and previous therapies of patients with confirmed diagnosis of MC (MC patients) versus patients without diagnosis of MC (non-MC patients) • To describe the therapeutic attitude regarding MC patients over a period of 24 months • To describe the clinical activity of MC patients over a period of 24 months. • To describe the histological features of MC patients 24 months after diagnosis. • To explore the association of socio-demographic, clinical and histological characteristics with clinical activity among MC patients followed for 24 months. • To explore the association of socio-demographic, clinical characteristics with histological activity among MC patients followed for 24 months. • To explore the correlation of treatments used by MC patients with clinical activity. • To explore the correlation of treatments used by MC patients with histological activity. • To explore the correlation between fecal calprotectin, EPC and EPX levels with clinical activity among MC patients
Study Design:	This is a multicenter, cross-sectional, observational study aiming to determine the prevalence of MC among a consecutive sample of subjects who attend a specialist consultation with symptoms of chronic watery diarrhea and who are eligible to colonoscopy. In addition, the study design contemplates a 24-month prospective

	component with repeated measures for all MC patients with confirmed diagnosis of MC in order to investigate the clinical and histological progression. There is no imposed experimental intervention or treatment in this study. All therapeutic decisions and management of subjects will be done according to routine clinical practice.
Inclusion Criteria:	1. Patients who signed the informed consent; 2. Male or female patients, 18 years or older; 3. Patients with chronic or intermittent, watery diarrhea of unknown etiology for more than 4 weeks; 4. Patients who are eligible for complete colonoscopy according to physician's clinical criteria.
Exclusion Criteria:	1. Patients with history of IBDs or any other known intestinal disease. 2. Any condition that precludes the patient to undergo complete colonoscopy. 3. Patients who are being treated with any investigational agent; 4. Pregnancy 5. Patients who are not willing to comply with routine clinical appointments or procedures
Expected number of subjects:	300
Expected number of sites:	Approximately 10 centers are expected to participate.
Subject selection:	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 of interest. All eligible subjects will undergo a colonoscopy according to the routine practice for this type of population.
Exposure of interest:	Not applicable
Main data collected:	At first appointment socio-demographic, clinical, endoscopic, histologic data will be collected for all eligible patients. Patients with confirmed diagnosis of MC will be followed during 24 months. During this period, clinical and histological data will be collected.
Endpoints	**Primary endpoint:** Number of patients with confirmed diagnosis of MC (any subtype) over the total number of colonoscopies performed in all patients included in the study. **Secondary endpoints:** •Proportion of patients with confirmed diagnosis of each subtype of MC (CC, LC and IC) •Age, gender, BMI, smoking status, duration of symptoms, stool frequency and consistency (type), familial history of IBDs, comorbidities, previous pharmacological/ non-pharmacological therapies and laboratory parameters will to be compared between MC patients and non-MC patients. •Treatments (pharmacological/non-pharmacological) prescribed by specialist for patients with MC diagnosis •Number of stools and type (Bristol stool form scale), urgency and abdominal pain over the last 7 days before each time point of assessment for patients with MC diagnosis. •Proportion of MC patients with clinical remission at each data collection time point (month 3, 6, 9, 12, 15, 18, 21 and 24) Clinical remission: mean of < 3 stools per day and mean of < 1 watery stool per day •Proportion of MC patients with histological remission at month 24. See section 8.3.2 for definition of histological remission •Age, gender, BMI, smoking status, duration of symptoms, stool frequency and consistency, familial history of IBDs, age at diagnosis of MC, time to diagnosis of MC, comorbidities, pharmacological/non-pharmacological therapies, laboratory parameters and histological features, compared with clinical activity status (active/non active) described for patients with MC diagnosis. •Age, gender, BMI, smoking status, duration of symptoms, stool frequency and

	consistency, familial history of IBDs, age at diagnosis of MC, time to diagnosis of MC, comorbidities, pharmacological/non-pharmacological therapies, laboratory parameters, compared with histological features, described for patients with MC diagnosis. •Area under the ROC curve of fecal calprotectin, ECP and EPX levels with clinical activity at each data collection time points among MC patients. •Area under the ROC curve of fecal calprotectin ECP and EPX levels with histological activity at each data collection time points among MC patients.
Statistical methods	The association between two quantitative variables will be performed through Pearson correlation coefficient or Spearman correlation coefficient, in case the normality assumption is not verified. The association of two categorical variables will be tested through the Chi-Square test or Fisher Exact test (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 (if applicable). The proportion of patients with clinical remission and histological remission (overall, by MC subtype and colonic segment) at each data collection time points 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. Repeated Measures Analysis or Generalized Estimated Equations will be used to investigate the evolution profile of clinical and histological characteristics throughout the data collection time points (time-effect). Generalized Estimated Equations will be used to explore the association between clinical (as independent variable) and histologic features (as dependent variable) observed within-patients.
Overall Study Duration:	The overall duration of the study is three years (1 year of recruitment + 2-year observation period).
Study timelines:	The study is expected to start during the 1th Quarter of 2015. Study closure is expected to occur in the 2st Quarter of 2018.

2 INTRODUCTION

2.1 MICROSCOPIC COLITIS

Microscopic colitis (MC) is a chronic inflammatory bowel disease, characterized by watery, non-bloody diarrhea with macroscopically normal colonic mucosa and typical microscopic features. Other clinical findings of MC may include nocturnal diarrhea, faecal incontinence, abdominal pain and mild weight loss.[1,2]

No unifying mechanism has been identified as being responsible for the pathophysiological abnormalities found in MC and the disease is likely to have multifactorial causes.[3] Several risk factors such as cigarette smoking, especially among young patients,[4] have been shown to be associated with the increased incidence of the disease. Diversion of the fecal stream has sometimes resulted in histological improvements,[5] suggesting that the disease is caused by an abnormal response to luminal antigens. A wide variety of drugs have been proposed as potential triggers or etiological factors for MC, but none has been conclusively implicated. These include the non-steroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors (PPIs), selective serotonin reuptake inhibitors (SSRIs) and statins. However, all these drugs are widely used, therefore, it is expected that patients with MC might frequently use them coincidentally. Some authors point out that drug-induced MC cases are more likely due to an idiosyncratic reaction.[6,7]

The diagnosis of MC rests on characteristic pathological findings in biopsies from normal or oedematous colonic mucosa. Although the topographic distribution of MC remains controversial, rectal biopsies are not sufficient.[6,8] Some studies demonstrate that biopsies from the right and transverse colon are necessary for the diagnosis of MC, while others demonstrate that biopsies from the descending and sigmoid colon suffice for diagnosing this disease.[8-12] However, colonoscopy should be preferred because it is essential to rule out malignant colonic disease. Diarrhea caused by other diseases should be excluded; these diseases include bile acid diarrhea, coeliac disease, and lactose malabsorption.[13]

Two distinct histological types of MC, lymphocytic colitis (LC) and collagenous colitis (CC), have been described, but overlapping features are often present; indeed, some authors think that LC and CC are two histological subtypes of the same disease.[3,14]

Abnormal chronic inflammation in the lamina propria is a typical histological characteristic but does not allow a distinction between the subtypes of MC.[15] CC is characterized by a thickened subepithelial collagen layer, i.e., > 10 μm in well-oriented biopsies cut perpendicularly to the surface,[16] and lymphocytic colitis (LC) is characterized by an increased number of intraepithelial lymphocytes (IEL), i.e., ≥ 20 IEL per 100 epithelial cells. Colonic biopsies are considered normal in the absence of chronic inflammation in the lamina propria, when the number of surface epithelial cells is ≤ 5 IEL/100, and the collagen band is ≤ 5 micrometers.[10,17]

Recent studies have shown that histological features in the individual patient are inconsistent over time, as findings of MC interchange with chronic (non-specific) inflammation or incomplete signs of MC at prior or repeat colonoscopy, and the overlap between CC and LC is significant.[8,18,19] This histological interchange between MC subtypes and the incomplete identification of some patients have led to the introduction of a third subtype designated as incomplete MC (IC). The histological criteria for IC are an abnormally thickened collagenous band (> 5 and < 10 μm), and /or an increased number of intraepithelial lymphocytes (> 5 and < 20 per 100 epithelial cells) without reaching the thickness or number required for the diagnosis of CC or LC.[1]

Epidemiologic studies show that MC is almost as common as classic IBD (i.e. Crohn's disease and ulcerative colitis).[13,20-22] The incidence of MC appears to be rising and in some countries it may be diagnosed in 10% of patients investigated for chronic non-bloody diarrhoea, and in 20% or more of such patients older than 70 years.[13,20] After a period of rising incidence figures, the data have been more divergent in recent studies. Incidence rates of 2.6/100,000 to 10.8/100,000 inhabitants have been reported for CC, and an annual incidence rate of 2.2 to 14 per 100,000 inhabitants for LC.[13,20-22]
Patients with MC are predominantly elderly women, and the average age at diagnosis is approximately 65 years, but a recent study shows that the age at diagnosis is increasing and a large proportion are now diagnosed in their seventies. This may be attributable to the rising of life expectancy observed over the last decades.[23] The female predominance is very clear in MC, but is less pronounced for LC than for CC.[21]
The incidence of IC has been described in only one consecutive case series in which a seven-fold increase was found for both IC and LC from 1999-2001 to 2008-2010 as compared to a 3-fold increase for CC.[24]

2.2 BIOMARKERS IN MICROSCOPIC COLITIS

Given the invasive nature of endoscopy, the implementation of an easy, non-invasive method to support the pre-diagnostic screening and monitoring of disease activity is essential.
An increased prevalence of autoimmune serological tests has been found in MC patients but no clinically useful marker for the disease has yet been identified.[3]
The faecal markers evaluated so far in MC include a variety of substances/proteins that either leak from or are generated by the inflamed mucosa in the gut. Proteins studied include lactoferrin, myeloperoxidase (MPO), calprotectin, eosinophil cationic protein (ECP) and eosinophil protein X (EPX), as indicators of neutrophilic, macrophage and eosinophil activation. Likewise, studies of tryptase, occult blood and cytokines have been performed.[25] Fecal calprotectin has been shown to be useful in the diagnosis of IBD, correlates with mucosal disease activity and can help to predict response to treatment or relapse. In IBD, the presence of active gut inflammation is associated with migration of leucocytes, including neutrophils, to the gut mucosa.[26] As a result the faecal stream contains increased levels of these inflammatory proteins including calprotectin. Faecal calprotectin has been shown to differentiate quiescent from active disease in both patients with CD and UC.[27,28]
Overall, 52 patients with MC have been investigated for faecal calprotectin in five different studies, of whom 71% (n=37) had positive samples.[29-33] In two cohorts calprotectin was significantly increased compared to healthy controls and decreased significantly when remission was induced. On the other hand, in the two studies no correlation was found between calprotectin levels and disease activity (measured by bowel frequency), stool consistency and histological grade of inflammation in mucosal biopsies.[32,33]
Overall ECP and EPX have been examined in 15 and 33 patients with active CC, respectively.[32,34,35] In the study of Lestejo et al., 2006, 18 patients with CC were compared with patients with IBDs and healthy controls in order to elucidate whether EPX in combination with other faecal markers could be used to distinguish between these disorders. Eleven patients (61%) had EPX values above the upper limit of normal, and values were significantly higher than in IBDs and healthy controls.[34]
In the study of Wagner et al., 2011, ECP and EXP were positive in 11 and 8 out of 12 patients with active CC disease, respectively, and became normal in all patients when remission was induced with budesonide.[32]
In spite of the small samples analyzed, ECP and EXP may be promising faecal markers.[36]

The association of faecal calprotectin, ECP and EXP with clinical and histological features in MC patients will be explored in this study.

2.3 RATIONALE

There's a lack of epidemiological data regarding MC in Portugal, including its incidence, prognosis factors and therapies used to treat this condition. Therefore, it becomes pertinent to characterize this population and, simultaneously, evaluate the key characteristics that distinguish this clinical entity from other conditions among a population who attends a gastroenterology setting with symptoms of chronic watery diarrhea. An observational study using sound epidemiological methods can provide valuable information regarding these aspects.
This study aims to estimate the prevalence of MC and its subtypes among patients who attend to the gastroenterologist appointment due to chronic, watery diarrhea of unknown etiology and who are eligible to colonoscopy. In addition, socio-demographic, clinical characteristics and treatment patterns will be described for patients with confirmed diagnosis of MC and these features will be compared with non-MC cases. Furthermore, this study will prospectively assess the clinical and histological progression of MC patients during a follow up period of 2 years. Potential biomarkers for this condition will also be explored.

No research hypothesis is predefined.

3 OBJECTIVES

3.1 PRIMARY OBJECTIVE

To determine the prevalence of microscopic colitis (MC) among patients who attend a specialist appointment with symptoms of chronic, watery diarrhea of unknown etiology for more than 4 weeks and undergo colonoscopy.

3.2 SECONDARY OBJECTIVES

- To determine the prevalence of MC subtypes (CC, LC and IC)
- To compare the socio-demographic, clinical and histological characteristics and previous therapies of patients with confirmed diagnosis of MC (MC patients) versus patients without diagnosis of MC (non-MC patients)
- To describe the therapeutic attitude regarding MC patients over a period of 24 months
- To describe the clinical activity of MC patients over a period of 24 months.
- To describe the histological features of MC patients 24 months after diagnosis.
- To explore the association of socio-demographic, clinical and histological characteristics with clinical activity among MC patients followed for 24 months.
- To explore the association of socio-demographic, clinical characteristics with histological activity among MC patients followed for 24 months.
- To explore the correlation between the treatments used by MC patients and clinical activity.
- To explore the correlation between the treatments used by MC patients and histological activity.

- To explore the correlation between fecal calprotectin, EPC and EPX levels and clinical activity among MC patients.
- To explore the correlation between fecal calprotectin, EPC and EPX levels and histological activity among MC patients.

4 STUDY DESIGN

This is a multicenter, cross-sectional, observational study aiming to determine the prevalence of MC among a consecutive sample of subjects who attend a specialist consultation with symptoms of chronic watery diarrhea. In addition, the study design contemplates a 24-month prospective component with repeated measures for all patients with confirmed diagnosis of MC.

There is no imposed experimental intervention or treatment in this study. All therapeutic decisions and management of subjects will be done according to routine clinical practice.

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 of interest. All eligible subjects will undergo a colonoscopy according to the routine practice for this type of population (Figure 1). Colonic specimens from all segments will be collected for subjects with macroscopically normal colonoscopies as part of the differential diagnosis of MC. Subjects with abnormal findings in colonoscopy suggestive of disorders other than MC will not be included in the prospective component of the study. These patients will continue to be managed according to the center's routine procedures.

The subjects with confirmed diagnosis of MC (upon histology assessment) will be followed over a period of 24 months in this study in order to investigate the clinical and histological progression. The data collection time points over this period will reflect this routine schedule. According to previous epidemiological studies on MC, it is estimated that 10-15% of patients with chronic, watery diarrhea will have a confirmed diagnosis of MC.[23,37]

Patients without confirmed histological diagnosis of MC will not be followed up during the prospective part of the study. These patients will continue to be managed according to the center's routine procedures.

Socio-demographic and clinical characteristics of patients without diagnosis of MC (either based on macroscopic or histological assessment) will be directly compared with MC patients.

Overall, 300 subjects will be included in this study. The recruitment period will last one year but may be extended if the target number of participants is not achieved within the defined timeframe.

A total of 10 centers in Portugal are expected to participate. Subject recruitment will be competitive across participating centers.

The physician should include the eligible patients consecutively as they attend to the consultation.

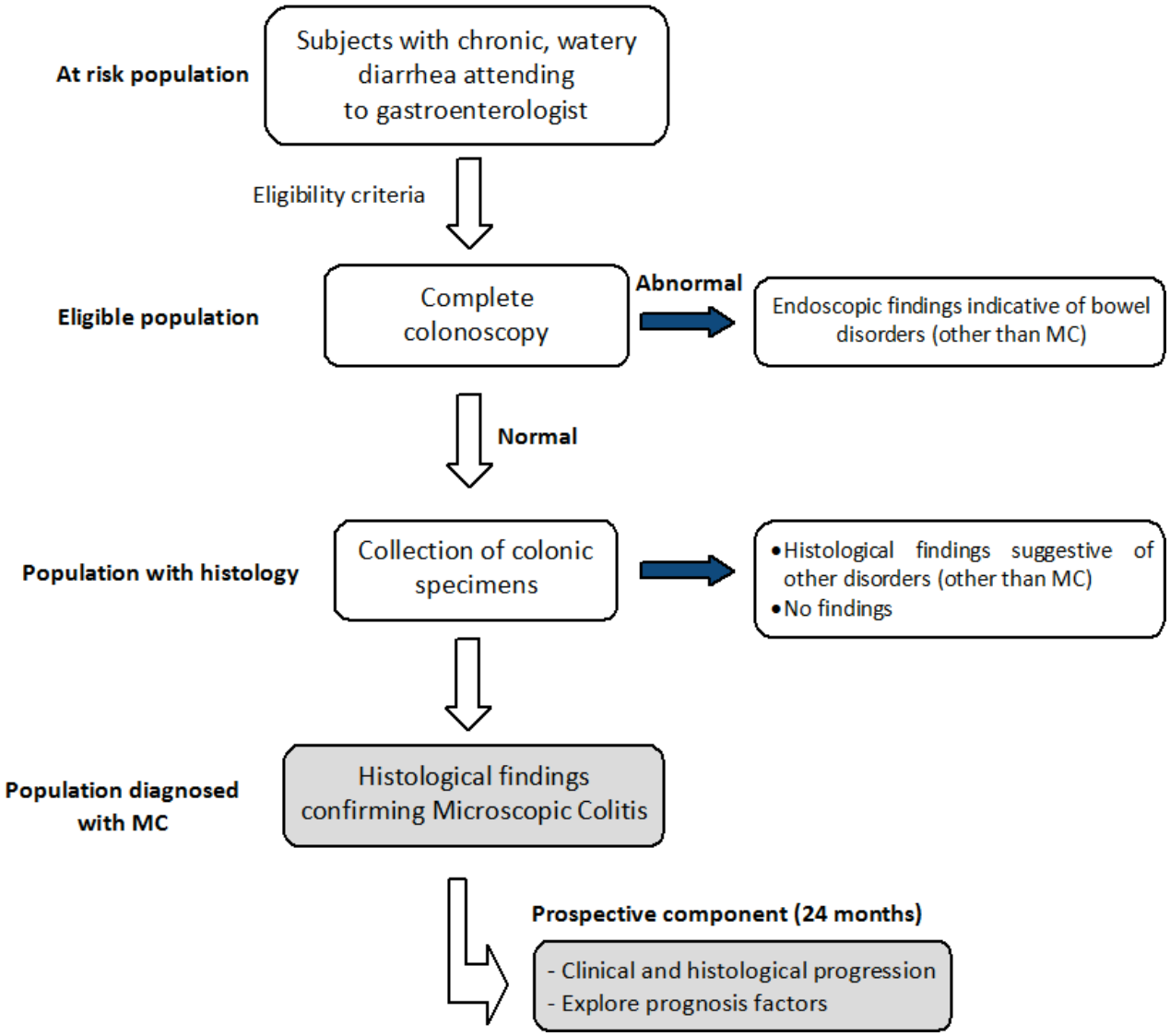

Figure 1 – Study Flowchart

5 STUDY TIMELINES

The study is expected to start during the 1th Quarter of 2015.

The overall duration of the study is three years (1 year of recruitment + 2-year observation period). Study closure is expected to occur in the 2nd Quarter of 2018.

6 STUDY POPULATION

6.2 INCLUSION CRITERIA

Study subjects must fulfill the following criteria:

1. Patients who signed the informed consent
2. Male or female patients, 18 years or older;
3. Patients with chronic or intermittent, watery diarrhea of unknown etiology for more than 4 weeks
4. Patients who are eligible for complete colonoscopy according to physician's clinical criteria

6.3 EXCLUSION CRITERIA:

Subjects will be excluded if at least one of the following criteria is met:

1. Patients with history of IBDs or any other known intestinal disease.
2. Any condition that precludes the patient to undergo complete colonoscopy.
3. Patients who are being treated with any investigational agent;
4. Pregnancy
5. Patients who are not willing to comply with routine clinical appointments or procedures.

6.4 DISCONTINUATION FROM OBSERVATION PERIOD (MC PATIENTS)

In this study, patients with confirmed diagnosis of MC will be followed up to a maximum period of 24 months. However, observation may be stopped prior to the 24 months for different reasons including, but not limited to:

- Adverse event
- Violation of the study protocol
- Lost to follow up
- Patient withdrawal of consent
- Death

In the case observation period is stopped prior to the 24 months, the date of study discontinuation and the reason for discontinuation should be recorded in the electronic Case Report Form (eCRF).

7 INFORMATION TO BE COLLECTED

7.1 VARIABLES TO BE CAPTURED

Baseline characteristics

The following socio-demographic, clinical characteristics will be collected for all eligible subjects.

- Date of birth
- Sex
- Height
- Weight
- Smoking status
  - non-smoker: never smoked before or smoked a little occasionally
  - former smoker: patients who had quit smoking more than 6 months before inclusion in the study;
  - current smoker: greater than 7 cigarettes (or approximately half a pack) per week for at least 6 months and had reported any cigarette smoking in the previous 6 months.
- History of bowel surgery
- Familial history of IBDs
- Date of start of first symptoms of diarrhea (at least the month and the year when the patient first experienced non-bloody, watery diarrhea)
- Acute onset of symptoms (yes/no)
- Symptoms (to be captured by the physician by interviewing the patient and referring to the previous 7 days):
  - number of stools per day (the mean number of stools and watery stools per day will be calculated by summing the number of stools [any type] and watery stool that occurred over the week before the time point of assessment divided by 7 days).
  - type of stool (1 to 7 - Bristol Stool Chart)
  - nightly diarrhea
  - urgency (0 = no urgency, 1 = need to defecate within 30 minutes, 2=immediate need to empty the bowel, 3 = fecal incontinence - have to go immediately to the toilette)
  - difficulties staying continent with a respective bowel movement
  - abdominal pain (0 = no pain, 1 = mild, 2 = moderate, 3 = severe pain)
- Unexplained weight loss (> 4.5 kg) in the previous 6 months.
- Comorbidities, including the following conditions of interest: heart disease, hypertension, malignant disease, bile acid malabsorption, celiac disease, rheumatoid arthritis, Diabetes mellitus I, Sjögren's syndrome, Raynaud's syndrome, psoriasis, thyroiditis, hyperthyroidism, hypothyroidism, other auto-immune disorders.
- Pharmacological therapies (within the previous 3 months before study inclusion), including the following therapies of interest: NSAIDs, salicylic acid, proton pump inhibitors, SSRIs and statins.
- Laboratory parameters (prior to colonoscopy) - hemoglobin, platelets, leukocytes, neutrophils, eosinophiles, basophiles, monocytes, lymphocytes, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), iron and transferrin.
- Fecal sample - for assessment of calprotectin, ECP and EPX levels.
- Result of colonoscopy: normal/abnormal - describe whether other findings may have caused diarrhoea (e.g., polyps > 2 cm, tumors, Crohn's disease, ulcerative colitis, ischemic colitis, other).

For patients with macroscopically normal colonoscopy:

- Date of sampling of colonic specimens (this date will correspond to the date of MC diagnosis, if confirmed).
- Date of histological assessment
- Histological evidence of MC (yes/no). The following features will be captured:
    - Geboes Index (grade for each of the colon segments: rectum, sigmoid, descending, transverse and ascending) – see Section 7.4.2.
    - Thickness of the subepithelial collagen layer (not observed, ≤5 μm; > 5 and < 10 μm; ≥ 10 μm)
    - IEL in the surface epithelium (not observed, < 5 IEL/100 surface epithelial cells, > 5 and < 20 IEL/100 surface epithelial cells, ≥20 IEL/100 surface epithelial cells);
- If a diagnosis of MC was not confirmed, was other diagnosis established? Yes/no - if yes, describe.

Characterization of MC patients

- MC histological assessment: location of microscopic findings (by colon segment), type of MC (lymphocytic, collagenous, incomplete colitis) - see histological assessment in section 7.4.
- Therapeutic attitude upon MC confirmation
    - Antidiarrheal medication (e.g., loperamide or diphenoxylate/atropine),
    - Bismuth subsalicylate or aminosalicylates, e.g. 5-aminosalicylic acid
    - Cholestyramine
    - Corticosteroids (budesonide, prednisolone)
    - Immunosuppressive agents, e.g. azathioprine, 6-mercaptopurine or methotrexate
    - Surgical intervention (e.g. ileostomy)
    - Other interventions (pharmacological or non-pharmacological)
- Dispensing of patient diary card to document the number of stools including the type according to Bristol stool form scale (see section 7.2) in the week prior to the next appointment. In addition, the use of additional pharmacological or other therapies will be recorded by the patient.

Follow up assessments of MC patients (as per routine practice) - month 3, 6, 9, 12, 15, 18, 21 and 24.

The following will be assessed throughout the 24-month prospective component of the study - see **Appendix.**

At every data collection time point

- Weight (kg)
- Symptoms (to be captured by the physician by checking the patient diary and interviewing the patient, referring to the previous 7 days):
    - number of stools per day (the mean number of stools and watery stools per day will be calculated by summing the number of stools [any type] and watery stool that occurred over the week before the time point of assessment divided by 7 days).
    - type of stool (1 to 7 - Bristol Stool Chart)
    - nightly diarrhea
    - urgency (0 = no urgency, 1 = need to defecate within 30 minutes, 2=immediate need to empty the bowel, 3 = fecal incontinence - have to go immediately to the toilette)

- difficulties staying continent with a respective bowel movement
- abdominal pain (0 = no pain, 1 = mild, 2 = moderate, 3 = severe pain)

- Laboratory parameters – hemoglobin, platelets, leukocytes, neutrophils, eosinophiles, basophiles, monocytes, lymphocytes, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), iron and transferrin.
- Concomitant medications
- Changes in concomitant illness (worsening of previous conditions, new condition)

At diagnosis of MC and every 3 months up to month 24:

- Fecal sample – for assessment of calprotectin, ECP and EPX levels.

In addition, at Month 24 only:

- Date of colonoscopy with sampling of colonic specimens
- Result of colonoscopy: normal/abnormal - describe
- Date and result of histological assessment :
  - Geboes Index (grade for each of the colon segments: rectum, sigmoid, descending, transverse and ascending) – see Section 7.4.2.
  - IEL in the surface epithelium (not observed, < 5 IEL/100 surface epithelial cells, > 5 and < 20 IEL/100 surface epithelial cells, ≥20 IEL/100);
  - Thickness of the subepithelial collagen layer (not observed, <5 μm; > 5 and < 10 μm; ≥ 10 μm).

7.2 SYMPTOMS ASSESSMENT

The symptoms will be assessed throughout 24 months (approximately every 3 months) for all patients with confirmed diagnosis of MC (any type). The symptoms include:

- Stool frequency (number of stools per day).
- Stool consistency (type of stool according to the Bristol Stool Chart – Figure 1)
- Nightly diarrhea
- Urgency (0 = no urgency, 1 = need to defecate within 30 minutes, 2 = immediate need to empty the bowel, 3 = fecal incontinence - have to go immediately to the toilette),
- Difficulties staying continent with a respective bowel movement (yes/no)
- Abdominal pain (0 = no pain, 1 = mild, 2 = moderate, 3 = severe pain)

The Bristol Stool Chart is used to monitor change in intestinal function and is widely used both in clinical practice and research. This scale classifies the form of human faeces into seven categories (figure 2).

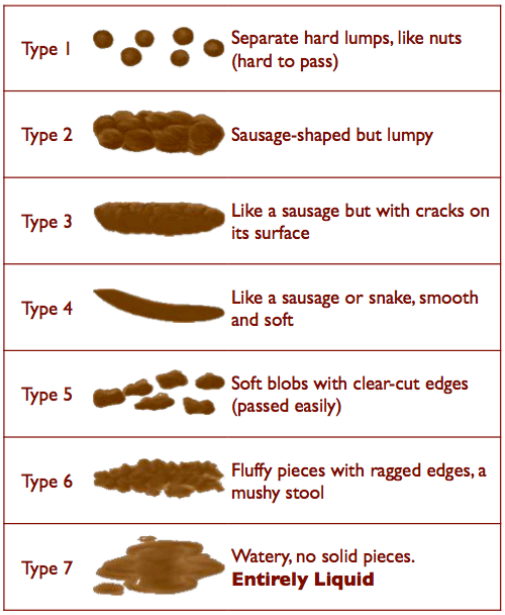

Figure 2 – Bristol Stool Chart

During the first appointment of the study, the symptoms will be captured by the physician into the eCRF by interviewing all the eligible patients who will report to the previous 7 days. During the subsequent appointments the symptoms will **only** be recorded by the MC patients, using the patient diary. The patient diary should be completed on a daily basis for the period of 7 days before the next appointment to the specialist. The symptoms to be captured in the diary include the total number of stools and its type (according to Bristol Stool Chart) and therapies used (pharmacological/non-pharmacological) between appointments. The physician should clarify with the patient any inconsistency or missing information found in the patient diary.
The patient will be given at each appointment the necessary number of diaries to cover the period until the next scheduled appointment.
In case the patient forgets or loses the diary at one appointment, the investigator and the patient will have to estimate the number of stools and watery stools and this situation should be recorded in the eCRF (as estimated result) due to missing diary.

At each appointment, the investigator calculates the mean number of stools and of watery stools per day according to the formula:
The number of stools (any type) and watery stools in the week prior to the appointment will be divided by number of days with entries.

The number of stools and the type will not be evaluable if less than 5 days of entries are found and there is no possibility to collect any further information regarding that week. The day corresponding to the preparation of endoscopy will not be used to assess these symptoms.

7.3 ENDOSCOPIC ASSESSMENT

All eligible patients will undergo a complete colonoscopy as part of the routine procedures for differential diagnosis. The findings of the colonoscopy will be collected into the eCRF.
During the complete colonoscopy biopsies will be taken from the colonic segments for histological examination – see section 7.4.

7.4 HISTOLOGICAL ASSESSMENT

7.4.1 Sampling and shipment procedures

The sampling of specimens for screening of MC is as follows:

- Four biopsies will be collected from each colon segment: rectum, sigmoid, descending, transverse and ascending.
- The biopsies will be fixed in buffered formalin 4% and will be sent to central lab (pathologist) through courier at room temperature along with a study specific form. All materials and courier arrangements will be provided by the Central Lab.

The central pathologist will confirm the diagnosis of microscopic colitis.

Contact of central pathologist:
Profª. Doutora Fátima Carneiro
Serviço de Anatomia Patológica
Hospital de São João
Alameda Prof. Hernâni Monteiro.
4200-319 Porto.

7.4.2 Histological criteria for diagnosis of MC and subtypes

The diagnosis of MC and subtypes (confirmed/not confirmed) can only be established if the histological findings are present in at least one biopsy of at least two segments of the colon. For each segment the worst values of both biopsies are to be used for evaluation. The values used for evaluation have to be marked.

The histopathological criteria used for confirmation of Microscopic colitis (MC) regardless of the subtype are:

a) Abnormal intraepithelial lymphocytes (IEL) > 5 IEL per 100 surface epithelial cells of the colonic mucosal membrane, **and/or**
b) A subepithelial collagen layer > μ5 m

Colonic biopsies are considered normal when the number of surface epithelial cells is ≤ 5 IEL/100 surface epithelial cells, the collagen band is ≤ 5 μm and Geboes grade < 3.1.

The histopathological criteria used for confirmation of Lymphocytic colitis (LC) are:

a) Abnormal intraepithelial lymphocytes (IEL) ≥ 20 IEL per 100 surface epithelial cells of the colonic mucosal membrane
b) A subepithelial collagen layer < 10 μm

The histopathological criteria used for confirmation of Collagenous colitis (CC) are:

a) An irregularly thickened subepithelial collagen layer ≥ 10 μm

The histopathological criteria used for confirmation of Incomplete colitis (IC) are:

a) Thickened subepithelial collagen layer (> 5 μm and < 10 μm) **and/or**

b) Abnormal IEL (> 5 and < 20 lymphocytes per 100 surface epithelial cells of the colonic mucosal membrane).

Geboes index, is a validated score for evaluating histologic disease activity and is presented in Table 1. Active histologic disease can be defined as a Geboes grade ≥3.1 (presence of epithelial neutrophils with or without crypt destruction or erosions).

Table 1 - Geboes score for assessment of histologic disease activity for each segment of the colon

		Colonic segments				
Structural (architectural changes)	**Grade 0**	**R**	**S**	**D**	**T**	**A**
	Subgrades					
No abnormality	0.0					
Mild abnormality	0.1					
Mild or moderate diffuse or multifocal abnormalities	0.2					
Severe diffuse or multifocal abnormalities	0.3					
Chronic inflammatory infiltrate	**Grade 1**					
	Subgrades					
No increase	1.0					
Mild but unequivocal increase	1.1					
Moderate increase	1.2					
Marked increase	1.3					
Lamina propria neutrophils and eosinophils	**Grade 2**					
	2A *Eosinophils*					
No increase	2A.0					
Mild but unequivocal increase	2A.1					
Moderate increase	2A.2					
Marked increase	2A.3					
	2B Neutrophils					
No increase	2B.0					
Mild but unequivocal increase	2B.1					
Moderate increase	2B.2					
Marked increase	2B.3					
Neutrophils in epithelium	**Grade 3**					
	Subgrades					
None	3.0					
< 5 % Crypts involved	3.1					
< 50 % Crypts involved	3.2					
> 50 % Crypts involved	3.3					
Crypt destruction	**Grade 4**					
	Subgrades					
None	4.0					
Probable - local excess of neutrophils in part of crypt	4.1					
Probable - marked attenuation	4.2					
Unequivocal crypt destruction	4.3					
Erosion or ulceration	**Grade 5**					
	Subgrades					
No erosion, ulceration, or granulation tissue	5.0					
Recovering epithelium + adjacent inflammation	5.1					
Probable erosion focally stripped	5.2					
Unequivocal erosion	5.3					
Ulcer or granulation tissue	5.4					

R - rectum; S - sigmoid; D - descending; T - transverse; A – ascending

7.5 ASSESSMENT OF BIOLOGICAL MARKER

In this study, fecal calprotectin, eosinophil cationic protein (ECP) and eosinophil protein X (EPX) will be analyzed for all patients included in the study (with or without MC). For patients with confirmed diagnosis of MC these markers will be assessed at diagnosis and every 3 months up to month 24 (prospective component). The fecal samples will be analyzed by a Central Laboratory (Dept. de Farmacologia FMUP) using Quantum Blue. Report with the results will be provided to Investigators by mail.

7.5.1 Assessment of fecal calprotectin levels

The collected stools sample will be sent immediately to the laboratory at room temperature for extraction. Stools can be stored up to 6 days at room temperature. After 6 days, stools must be extracted. One gram of stool is enough for calprotectin detection.
Stools will be weighted and extracted with extraction buffer delivered with commercial kit.
After extraction and before storage, samples will be centrifuged (5min at 3000xg). Once centrifuged, supernatant must be transferred into a fresh tube. Undiluted extracts can be stored at -20°C for at least 4 months. Extracts must be diluted prior to analysis.
Calprotectin from diluted samples will be determined with an ELISA assay. Calprotectin wells are coated with monoclonal antibodies against calprotectin. Calprotectin present in patient samples binds to calprotectin antibody in the coating. After washing the unbound components, an anti-human calprotectin enzyme-labeled (calprotectin conjugate) is added to give a complex conjugate calprotectin antibody. After incubation, unbound conjugate is washed and removed, and the complex is incubated with a developing solution. Stops solution ends reaction and fluorescence in the wells is read. A standard curve allows the determination of calprotectin concentration in the sample.

7.5.2 Assessment of fecal EPC and EPX levels

Stools samples will be collected and immediately frozen at -20ºC or stored in a refrigerator for a maximum of 12h before freezing. Later, feces samples will be thawed overnight in the refrigerator. Approximately 0.1–1 g of feces will be weighted and diluted five times by adding 4 volumes (vol/wt) of an extraction buffer. The mixture will be homogenized until a homogenous solution is obtained.
An aliquot of 0.5 ml of the homogenate will be diluted 20 times in extraction buffer. After incubation at 6°C for 30 min and mixing, the homogenate will be centrifuged at 2000xg for 30 min at 5°C. The supernatant without particles will be thereafter transferred to fresh tubes and frozen at -20°C for later analysis.
Eosinophil cationic protein (ECP) from fecal samples will be determined with a fluoroenzymeimmunoassay. Anti-ECP, covalently coupled to ImmunoCAP, reacts with the ECP in the patient sample. After washing, enzyme labelled antibodies against ECPare added to form a complex. After incubation, unbound enzyme-anti-ECP will be washed away and the bound complex will then be incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate will be measured. To evaluate the test results, the response for the patient samples are transformed to concentrations with the use of a calibration curve.
Eosinophil protein X (EPX) will be determined as described for Eosinophil cationic protein (ECP).

8 ENDPOINTS

8.1 PRIMARY ENDPOINT

Number of patients with confirmed diagnosis of MC (any subtype) over the total number of colonoscopies performed in all patients included in the study.

8.2 SECONDARY ENDPOINTS

- Proportion of patients with confirmed diagnosis of each subtype of MC (CC, LC and IC)
- Age, gender, BMI, smoking status, duration of symptoms, stool frequency and consistency (type), familial history of IBDs, comorbidities, previous pharmacological/ non-pharmacological therapies and laboratory parameters to be compared between MC patients and non-MC patients.
- Treatments (pharmacological/non-pharmacological) prescribed by specialist for patients with MC diagnosis
- Number of stools and type (Bristol stool form scale), urgency and abdominal pain over the last 7 days before each time point of assessment for patients with MC diagnosis.
- Proportion of MC patients with clinical remission at each data collection time point (month 3, 6, 9, 12, 15, 18, 21 and 24)
  Clinical remission: mean of < 3 stools per day and mean of < 1 watery stool per day
- Proportion of MC patients with histological remission at month 24.
  See section 8.3.2 for definition of histological remission
- Age, gender, BMI, smoking status, duration of symptoms, stool frequency and consistency, familial history of IBDs, age at diagnosis of MC, time to diagnosis of MC, comorbidities, pharmacological/non-pharmacological therapies, laboratory parameters and histological features, compared with clinical activity status (active/non active) described for patients with MC diagnosis.
- Age, gender, BMI, smoking status, duration of symptoms, stool frequency and consistency, familial history of IBDs, age at diagnosis of MC, time to diagnosis of MC, comorbidities, pharmacological/non-pharmacological therapies, laboratory parameters, compared with histological features, described for patients with MC diagnosis.
- Area under the ROC curve of fecal calprotectin, ECP and EPX levels with clinical activity at each data collection time points among MC patients.
- Area under the ROC curve of fecal calprotectin ECP and EPX levels with histological activity at each data collection time points among MC patients

For definition of clinical and histological activity see section 8.3.1 and 8.3.2.

8.3 DEFINITIONS OF INTEREST

8.3.1 Clinical outcomes (applicable to all MC subtypes – CC, LC and IC)

- Clinical activity: mean of ≥ 3 stools per day or mean of ≥ 1 watery stool per day.[38]

- Clinical remission: mean of < 3 stools per day and mean of < 1 watery stool per day.[38]

The mean number of stools and watery stools per day will be calculated by summing the number of stools (any type) and watery stool that occurred over the week before the time point of assessment divided by 7 days.[38]

8.3.2 Histological outcomes

Collagenous colitis (CC)

- Histological remission:
a) Normal thickness of subepithelial collagen layer (≤ 5 µm)[39], **and**
b) No/mild lamina propria inflammation (Geboes grade < 3.1)[40]

Lymphocytic colitis (LC)

Histological remission:
a) Reduction in the number of IEL ≤ 20 IEL/100 surface epithelial cells[41] **and**
b) No/mild lamina propria inflammation (Geboes grade < 3.1)[40]

Incomplete colitis (IC)

- Histological remission:[1]
a) Normal IEL (≤ 5 IEL/100 surface epithelial cells) **and**
b) Normal thickness of subepithelial collagen layer (≤ 5 µm), **and**
c) No/mild lamina propria inflammation (Geboes grade < 3.1)[40]

Histological activity includes (for all subtypes):

- No change in histological features since diagnosis
a) No change in lamina propria inflammation (Geboes grade ≥3.1)
b) No change in collagen layer thickness, and
c) No change in number of IELs in the surface epithelium.

None of these parameters show any increase in any segment assessed.

- Histological aggravation since diagnosis
a) Any increase in any segment in lamina propria inflammation (Geboes grade ≥3.1),
b) Any increase in collagen layer thickness, **and/or**
c) Any increase in number of IELs in the surface epithelium.

9 STATISTICAL ANALYSIS

9.1 GENERAL CONSIDERATIONS

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 statistical analysis will be performed through frequency tables for qualitative variables and tables with descriptive statistics for quantitative variables.

The association between two quantitative variables will be performed through Pearson correlation coefficient or Spearman correlation coefficient, in case the normality assumption is not verified.
The association of two categorical variables will be tested through the Chi-Square test or Fisher Exact test (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 (if applicable).

The proportion of patients with clinical remission and histological remission (overall, by MC subtype and colonic segment) at each data collection time points 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.
Repeated Measures Analysis or Generalized Estimated Equations will be used to investigate the evolution profile of clinical and histological characteristics throughout the data collection time points (time-effect).
Generalized Estimated Equations will be used to explore the association between clinical (as independent variable) and histologic features (as dependent variable) observed within-patients.

The association analysis of fecal calprotectin, EPC and EPX with clinical activity

- The association between fecal calprotectin levels EPC and EPX with clinical activity (remission/active disease) will be analyzed through Area Under the ROC Curve at each data collection time point (diagnosis and every 3 months up to month 24).

The association analysis of fecal calprotectin EPC and EPX with histological features

- The association between fecal calprotectin, EPC and EPX levels with histological features (collagen thickness [≤ 5 µm; > 5 µm], inflammation [Geboes grade < 3.1; grade ≥ 3.1] and IEL count [≤ 5 IEL/100; > 5 IEL/100 surface epithelial cells) will be analyzed through Area Under the ROC Curve at each data collection time point (diagnosis and month 24).

9.2 SAMPLE SIZE

This study will include 300 patients with chronic diarrhea allowing the estimation of a prevalence of 10-15% [23,37] with an error margin of less than 4.5% and with 95% confidence interval. Assuming the lowest rate of the prevalence interval (10%), approximately 30 patients with MC are expected to be followed in the prospective component study.

10 ETHICAL AND LEGAL ASPECTS

10.1 ETHICS

The study will be conducted according to the ethics principles originated from the Declaration of Helsinki and to the Portuguese Clinical Research law (Law #21/2014, 16th April 2014).[42]
A copy of the protocol, proposed informed consent form and other written subject information will be submitted to the competent Ethics Committee for written approval. A copy of the written approval of the protocol and informed consent form must be received by the Investigator before recruitment of subjects and data collection.
The investigator will submit and, where necessary, obtain approval from the competent Ethics Committee for all subsequent protocol amendments and changes to the informed consent document.

10.2 INFORMED CONSENT

Before any protocol specific procedures are performed, the investigator is responsible for obtaining written informed consent from the subject (or authorized representative) and after an adequate and clear explanation of the aims, methods, anticipated benefits, and potential hazards of the study.
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.

10.3 STUDY DISCONTINUATION CRITERIA

Premature termination of this 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, GEDII or designee will promptly notify the appropriate IEC and regulatory authority (if applicable).

11 QUALITY CONTROL

The study will involve a GEDII monitor who will be responsibility 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.
During 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 allow the appropriate regulatory authorities access to source documents to perform this verification but always in the presence and through the investigator.
Prior to study start, 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.

All investigators and study staff will receive training on the protocol and other protocol-related procedures prior to start of activities. The training will be provided by the GEDII or its representative.

12 DATA HANDLING

12.1 CONFIDENTIALITY

The investigator is responsible for ensuring that the subject's confidentiality is maintained.
Questionnaires, database and other documents generated in this study will be identified by a unique subject identification number only. This 2-digit number will be assigned sequentially by each investigator, based on subject's recruitment schedule (e.g.: first subject will be assigned No. 01, the second subject will be No. 02 and so on). Each center will also be assigned a predefined two-digit number.
The study protocol will be submitted to the National Data Protection Committee (Comissão Nacional de Protecção de Dados) for the purpose of data processing under the scope of "Lei de Protecção de Dados de Carácter Pessoal Dec. 67/98 de 26 de Outubro".

12.2 DATA COLLECTION

All study data will collected into an electronic CRF and will be obtained from medical charts, local and central laboratory reports and, when relevant, will be complemented by subject's interview or other medical sources (as appropriate).
The investigator will be responsible for ensuring that all findings and data are accurately and reliably recorded in the case report form.

All eligible subjects who are not enrolled in the study will be recorded in a specific form. No personal data will be collected in this form, only the date of assessment of eligibility criteria and reason for non-enrollment. This form will be kept exclusively at each site.

12.3 STUDY ARCHIVE

The investigator will keep an adequate archive of all study documentation with access restricted to study team. The study archive will be kept at each site for at least 15 years from the study close out.

12.4 PUBLICATION POLICY

All documents and results generated from this clinical study are the exclusive property of the 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 by the Coordinating Investigator in national and international meetings and will published in international papers.
The study results can only be published after the clinical study is terminated, the data analysis is completed and **only** upon the agreement of the study's scientific board. The publication should include the results from all the centers which have participated in the clinical investigation,
The publication of results should be agreed by all participating investigators and in strict adherence to the principles originated from the Helsinki Declaration and to the Portuguese Clinical Research law (Law #21/2014, 16th April 2014).[42]

Authorship criteria

For all publications related to this study, the GEDII will comply with recognized ethical standards concerning publications and authorship established by the International Committee of Medical Journal Editors (*Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals - Updated December 2013*).

For all publications related with this clinical study, the order of the authors is as follows:

- The first author position will correspond to the Coordinating Investigator;
- The subsequent author's positions will correspond to the principal investigator from each center, who will be ranked decreasingly according to the number of patients included in the study, as far as the number of co-authors allowed by the journal is not exceeded;
- All the participating investigators not figuring in the authorship (due to journal's limitations in the number of co-authors) will be cited in the acknowledgment section of the publication.
- The last author position will correspond to GEDII (on behalf of GEDII).

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14 APPENDIX – CRONOGRAM

	All	24-month prospective part Only for patients with diagnosis of microscopic colitis							
Variables	**BSL**	**M3**	**M6**	**M9**	**M12**	**M15**	**M18**	**M21**	**M24**
Date of birth	X								
Sex	X								
Height	X								
Weight	X	X	X	X	X	X	X	X	X
Smoking status	X								
Medical history	X								
Comorbidities	X								
Symptoms of diarrhea	X	X	X	X	X	X	X	X	X
Prior therapies	X								
Colonoscopy	X								X
Colonic specimen collection	X								X
Histology	X								X
Therapeutic attitude regarding MC (pharmacological/non pharmacological)		X	X	X	X	X	X	X	X
Laboratory parameters[2]	X	X	X	X	X	X	X	X	X
Fecal sample (calprotectin, ECP and EPX levels)	X	X	X	X	X	X	X	X	X
Dispensing of patient diary	X[1]	X	X	X	X	X	X	X	

BSL = baseline: date of the study's first appointment; M= month; ECP= eosinophil cationic protein; EPX= eosinophil protein X

[1] The patient diary will be delivered as soon as the MC diagnosis is confirmed. The diary will be returned by the patient at each appointment and a new diary will be dispensed.

[2] hemoglobin, platelets, leukocytes, neutrophils, eosinophiles, basophiles, monocytes, lymphocytes, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), iron and transferrin.