Fecal calprotectin as a predictor of gastrointestinal immune-related adverse events (CF-19): A prospective study. [version 1; peer review: awaiting peer review]

Ana Cardesñ̦ Gutiérrez 1, Xabier Mielgo Rubio 1, Manuel Ruiz Muñoz 2, Ruth Martinez Cabañas 3, Diana Moreno Muñoz 1, Susana Hernando Polo 1, Clara Olier Garate 1, Alicia Hurtado Nuño 1, Verónica Sotelo Peña 1, Maria Virginia Sánchez Becerra 1, Andrea María González López 1, Mónica Esteban García 1, Teresa Robles Bermejo 1, Elia Pérez Fernández 3, Juan Carlos Cámara Vicario 1

1 Medical Oncology, Alcorcon Hospital Foundation, Alcorcón, Spain
2 Internal Medicine, Alcorcon Hospital Foundation, Alcorcón, Spain
3 Research Department, Alcorcon Hospital Foundation, Alcorcón, Spain

Abstract

Background: Colitis is a frequent immune-related toxicity, without any biomarker that may predict its onset. It is endoscopically similar to intestinal inflammatory diseases, where fecal calprotectin (FC) is used as a biomarker to early-detect a relapse. We found contradictory evidence about FC and immunotherapy and no prospective study was already published.

Methods: We present an analytical, observational and prospective study of one year's duration. We analyzed FC basal, and then prior to each cycle until the sixth, ending with quarterly follow-up. For evaluating the predictive value of FC we estimated the area under the ROC curve for basal absolute values and for each cycle, and calculated its relative percentage change with respect to basal. We also planned to estimate sensitivity, specificity and predictive values indexes for different cut-off points. Because of lack of recruitment we did a preliminary analysis at the end of the initially estimated period before suggesting its prolongation.

Results: 24 patients (19 male) were included in the study. This included n=15 diagnosed with lung cancer, head and neck, renal, bladder and colorectal cancer (n=2, each), and melanoma (n=1). They were treated with Anti PD-1/PDL-1 mono therapy (n=18), combo with chemo (n=2), or combo with anti-CTLA4 (n=2). Three patients had G1 colitis and two, >=G2, all treated with anti-PD1 and before 6th cycle, as described on literature. ROC curve presents AUC 0.559 (CI95%:0.32-
0.798) and RR for colitis taking FC value is 1.001 for each 10 units (p=0.493).

**Conclusion:** Even though we must take into account the limitations of the study we cannot conclude that FC could be used as a predictor for detecting immune-mediated colitis.

**Keywords**
Immunerelated adverse event (irae), Biomarker predictors, Fecal calprotectin, colitis, immune checkpoint inhibitors
1. Introduction
Immunotherapy has reached oncology to stay, and a new class of immune-mediated adverse effects (irAEs) has emerged, colitis being one of the most frequent.1,2 It is still unknown if any biomarker may predict irAEs onset, even though they resemble classic autoimmune diseases and some data suggests that some of the parameters that we use in this scenario could be useful in predicting this kind of toxicity.

For example, a phase I study found that adding BCG to ipilimumab in stage III-IV melanoma (n = 15), describes a remarkable increase of auto-antibodies (ImmunomeTM protein array) just before the development of severe irAEs.3 However, there are not prospective studies designed to validate this observation. Also, a retrospective study from a French group describes that CRP (C-reactive protein) increase and hemoglobin and albumin decrease are the main alterations that can be observed in patients diagnosed with immune-mediated colitis during anti-CTLA4 treatment (n = 27).4 Furthermore, during ESMO (European Society for Medical Oncology) 2018 Congress some groups showed that the increase in neutrophil/lymphocyte ratio decreases immune-related colitis risk. It was also found that decreasing absolute lymphocyte count and increasing monocytes and eosinophyles correlates with a irAEs risk increase (n = 130).5 However, all these parameters could be affected by other illnesses that may be associated with our oncological patients and for that reason, they do not seem sufficiently conclusive to change our daily-clinical practice.

Focusing our attention on gastrointestinal toxicity and on the French study mentioned before,4 they concluded that endoscopic lessons from immune-related colitis were very similar in comparison with the ones that appear during inflammatory intestinal diseases like Ulcerative Colitis or Crohn’s disease, some also with extra-intestinal manifestation associated. In eight patients of the study, fecal calprotectin (FC) was analyzed, and it was remarkably high in all of them (1755 μg/g [299–12900]), just like in patients diagnosed with intestinal inflammatory diseases.6 Furthermore, their condition is improved if we use the same treatments that are approved for this illness (corticosteroids, infliximab, vedolizumab).7,8 Nevertheless, Berman et al.9 cast FC aside as a predictive tool based on the data of Weber’s study,9 where FC is measured in two groups (ipilimumab + placebo vs ipilimumab + rectal budesonide), but this data could have been affected by the use of corticosteroids in one of the groups. Thus the evidence in the literature is contradictory.

We know that the use of CF as a predictor for intestinal inflammatory disease relapse is globally accepted and may diagnose relapse 3 months before the onset of symptoms (S 78%, E 73%).10 However, there are no prospective studies that analyze FC value as gastrointestinal irAEs predictor.

Taking into account that immune-mediated colitis could be endoscopically compared with inflammatory intestinal diseases, we hypothesize that FC may be useful to predict severe immune-related colitis.

2. Methods
Study population
Between May 2019 and December 2019, recruitment was open to participate in a prospective, analytical, observational and multi-centric study, following approval by our institution’s Committee for Clinical Research (H. Universitario Fundación Alcorcón). None of these dropped out of the study. The follow-up was done by the investigators in every oncology consultation that the patient needed. The data manager of the study registered all clinical and analytical data in an excel database after each appointment. All the clinicians followed the regular clinical practice guidelines. This is an observational study to analyze if fecal calprotectin can be considered as a good biomarker to predict immune related colitis, and the control cases were those that did not developed the event (colitis). The monitoring ended on April 2020, assuring a minimum of 4 months follow-up for all subjects included, making sure that we covered the weeks of higher probability to develop immune-related colitis.

Patients were recruited at the time of their first visit in medical oncology and were included if they were going to receive immunotherapy (both anti-CTCLA4 and anti-PD-1/PDL-1). All tumors and stages could be considered for inclusion. Also, signed informed consent was obtained from all participants.

Key exclusion criterion were previous diagnosis of inflammatory intestinal disease and regular treatment with NSAIDs (Nonsteroidal anti-inflammatory drugs). NSAIDs could alter FC values because they could cause enteropathy themselves. Moreover, NSAIDs are related with higher risk of developing immune-mediated colitis – in one particular study healthy volunteers who took diclofenaco for two weeks experienced FC elevation, which normally occurs two weeks after stopping medication.11 For this reason, we only included patients who had not taken any NSAIDs in the two weeks prior to beginning the study.12 Furthermore, there is evidence that healthy volunteers who received acetylsalicylic acid (100 mg/day) present significant elevation of CF. Those levels were low (<60 μg/g) and considering the importance of antitrombotic treatment in higher risk patients, stopping this treatment is not justified.12 However, we will take this into account.
Study design and procedure
Patients who met the inclusion criteria and signed informed consent had a basal recording taken of their FC, CRP and absolute number of Hemoglobin, Neutrophils, Eosinophils, Lymphocytes and Monocytes. Then, sub-investigators included those parameters in every blood test required by standard clinical practice until cycle number 6. Afterwards, if patients had not developed immune-mediated colitis, they continued their normal follow-up in consultations, and had to repeat these parameters every three months until the end of the study or the development of immune-mediated colitis, as the purpose of this project was predicting the event. The main objective of this study was serial evaluation of FC in every patient who needs immunotherapy from the participant centers. If they developed immune-mediated gastrointestinal toxicity, we used this data in order to analyze if there was a previous elevation of this parameter before the development of any symptoms.

Our hypothesis was that the determination of FC could be used as a predictive marker of immune mediated colitis. If this had been demonstrated, we might be able to propose an early treatment to avoid toxicity ≥ grade 2 that could force the suspension of the treatment, and as a result, deprive patients of its potential benefit.

We also took into account the administration of antibiotics during immunotherapy treatment because it is known that this affects the treatment’s efficiency, and could also affect gut microbiome and in consequence, the development of immune-mediated colitis.

Clinicians who participated in this study did not take the value of FC into consideration. This did not affect their standard clinical practice.

Statistical analysis
Our data was described by absolute and relative frequency for qualitative variables and by mean, standard deviation or median and interquartile range taking into account data distribution for quantitative variables.

For evaluating predictive value of FC we estimated the AUC [Area Under the ROC (receiver operating characteristic) curve] for basal absolute values and for each cycle, and calculate its relative percentage change with respect to basal (Ccycle-Cdx)/Cdx. Also, we estimated sensitivity, specificity and predictive values indexes for different cut-off points.

In order to study whether the change of FC during the first six cycles of treatment differed between groups with or without colitis, we used Mixed Models with time as repeated measure, group as fixed factor and their interaction. A statistically significant interaction effect indicates a different trend in the marker for each group. Analogous methodology was used to study the rest of the remaining laboratory markers.

All statistical tests were 2-sided and probability values of <0.05 were considered statistically significant. All tests were performed using the SPSS 17.0 statistical package for Windows (SPSS Inc, Chicago, IL, USA). The same analysis can be performed on the open-access software R.

We calculated sample size using Epidat 4.2, July 2016. Consellería de Sanidade, Xunta de Galicia, España; Organización Panamericana de la Salud (OPS-OMS); Universidad CES, Colombia. ESMO guidelines estimate an incidence of immune-mediated colitis for anti-CTL4 ranging between 27-54%. So, to estimate a sensitivity about 80% with a precision of 10% and 95% confidence level, we need a sample size of 114 patients.

Unfortunately, recruitment was harder than expected because of the large number of patients that had to be excluded for taking NSAIDs, and also, because of the lack of recruitment from the other centers that were included. Furthermore, some patients complained that having to bring stool samples was too disgusting, and sometimes, they missed the delivery. Other patients did not bring all the samples because of opioid-induced constipation. For this reason, we did a preliminary analysis at the end of the initially estimated period before suggesting its prolongation.

Ethical approval
Ethical approval for this study was granted by the ethical committee for medical research (Comité Ético de Investigación con Medicamentos) of the Hospital Universitario Fundación Alcorcón, and signed by the secretary of this committee.

We assured confidentiality in the data management with an anonymized database with verified entry. Personal data was confidential and biological samples were identified with a unique code and only investigators will have access.

We applied organic law on data protection and digital rights guarantee (Organic Law 3/2018, 5th December, BOE
We asked participants to sign informed consent as established by on biomedical research Law 14/2007 (BOE 4-VII-2007). Also, there were either no benefits related with the participation in this study and no additional risk. The benefit of this study is just the data that could be useful for the scientific community to improve management of immune-related colitis.

Patient consent

Written informed consent for publication of the patients’ details was obtained from the patients.

3. Results

24 patients were included, 19 of those were male. The primary origin of the tumors included were, from higher to lower frequency: lung (n = 15), head and neck, kidney and bladder (n = 2, each), and colorectal and melanoma (n = 1, each). Almost all of the patients received monotherapy with anti-PD-1/PDL-1 (n = 18) -nivolumab, pembrolizumab, atezolizumab, durvalumab-. Four of them, in combination with chemotherapy (carboplatin or cisplatin with pemetrexed and pembrolizumab). Two patients received anti-CTLA4, ipilimumab, in combination with nivolumab.

For 15 patients, immunotherapy was the first line of treatment for their pathology (2nd line n = 7, and 3rd line n = 2), so, for the majority of subjects included, previous treatments could not have disrupted our results by affecting their microbiome.

Only two patients stopped their treatment due to toxicity (one of them because of immune-mediated colitis). Other reasons to stop immunotherapy were progressive disease (n = 4), death (n = 2), COVID-19 (n = 1), and social issues (n = 1).

Focusing our attention on the main objective of this study, we registered three grade 1 colitis and two ≥ grade 2 (one grade 2 and other, grade 3). All colitis observed appeared during anti-PD1 treatment, always before cycle number six, according to the already published temporality, that suggests immune-related colitis peaks between 8-12 weeks.

No patient was using NSAIDs before being included in this study (according to the exclusion criteria). However, four of them took NSAIDs during immunotherapy treatment, and one of them developed grade 1 colitis. The rest of the patients did not suffer from toxicity, but we registered modifications on their FC values during NSAIDs administration.

The ROC curve (Figure 1) presents an AUC 0.559 (IC95%:0.32-0.798) and colitis RR (Relative Risk) taking into account that FC value is 1,001 for each 10 units (p = 0.493). Furthermore, Figure 2 shows the FC value distribution comparing patients who have developed colitis with patients that have not. We did not find a statistical difference. In group

Figure 1. ROC curve of FC as predictive value for immune-related colitis (AUC 0.559).
1 (the ones that did not develop the condition), FC median value was 63.5 mg/kg (31.9-172). In contrast, in the group of patients that developed colitis, FC median value was 124 mg/kg (5-1119). We could not find an explanation for the extreme value that was registered for one of the patients who, in addition, did not develop severe immune-related colitis.

4. Discussion
Taking into account the already published data about FC and the urgent need to find biomarkers that may predict immune-mediated toxicity, this study seemed promising. Also, it is the first prospective study that analyzes this issue, and we expected to find a biomarker that allowed us not to stop potentially effective treatments\textsuperscript{14-16} for patients with limited options. However, some factors may have contributed to not having reached our main objective.

First of all, it is true that the small sample size recruited may reduce the power of the study, and having completed the estimated size and having included patients in all the centers that we planned, would have been extremely interesting to observe the number of immune-related colitis and to show stronger conclusions. However, if we analyze the figures above we cannot find any trend towards statistical significance, and we are afraid that increasing the number of patients included would not produce more significant results. Furthermore, if we analyze the only patient that was diagnosed with severe immune-related colitis we can see that FC was not only not increased before the development of the toxicity, but also, it was even decreasing until very close to the event (Figure 3).

Figure 2. FC distribution value comparing group 1 (no immune-related colitis) with group 0 (immune-related colitis developed).

Figure 3. FC evolution in patient diagnosed from grade 3 colitis on 01/02/2020.
In addition, and after analyzing Figure 3 we find one of the other big problems that we faced during the development of this study was lost values. Oncological patients, due to either their illness, related symptoms, or and palliative treatment as opioids, suffer from constipation. Usually, patients were not able to comply with the timeline, and sometimes, they decided not to bring us the sample because they found it excessively disgusting even though we tried to transmit its importance. These lost values could also have affected to the final results.

Secondly, the exclusion criteria of “not being under NSAIDs” was very problematic during recruitment. The vast majority of potential candidates were under this kind of treatment and taking into account the complexity of oncological patients and that pain is usually their main symptom, NSAIDs are a useful treatment that we cannot easily avoid. FC value is easily altered in this context as we showed before and because of that reason, it was considered to be an exclusion criterion. However, and despite remembering its importance in every visit, some patients started NSAIDs during the course of this study. We registered and analyzed this fact, but luckily, it did not significantly affect our results.

Another fact that may be related to the high variability of FC in our study is the microbiome as well as inflammatory activity related with tumors and with immunotherapy,¹⁷ and its uncertain evolution. There are different ongoing studies that are trying to analyze if the microbiome can modulate or even improve the efficacy of some treatments.¹⁸,¹⁹

To sum up, and taking into account the huge complexity that we had to confront to include patients, the extensive variability of FC, especially in oncological patients, and also, the high number of lost values, we consider that extending this study would not contribute to clarifying results in favor of FC as a predictor biomarker for immune-related colitis.

5. Conclusion
Even though we must take into consideration our study limitations (unicentric, small sample size), we cannot conclude that FC would be useful as predictive biomarker for immune-related colitis development. Also, we consider that increasing our sample size and extending this study would not clarify results in favor of FC. Furthermore, and thanks to the expertise that this study brought to us, we consider that FC is not a good parameter to use in daily clinical practice in this context because of its enormous variability, unpredictability, and also the effort that it entails to measure in oncological patients. We will continue looking for predictive biomarkers able to predict immune-related toxicity in order to try to design studies that may offer early treatment to avoid stopping potentially effective treatments.

Author contributions
Ana Cardeña was the beneficiary of the grant that covered this project and consequently, its main coordinator. She was the protocol designer, the informed consent writer, and an active participant during recruitment, data entry and final analysis. Also, she is the author of the complete manuscript. Xabier Mielgo contributed to the protocol design and was second supervisor of the study activity and during the writing of the manuscript. Manuel Ruiz helped to focus the initial idea and also, participated during designing of the protocol that was sent to apply for the grant and finally, contributor for designing of future studies where we could use FC as predictor. Ruth Martinez contributed during the data entry and designing of CRF. Elia Pérez helped with the statistical design of the protocol and final data interpretation. Juan Carlos Cámara is the head of our investigator team and supervised all the process and also, participated actively in the patient recruitment. The rest of the authors contributed equally during recruitment and management of our patients in consultations and oncology ward.

Data availability
Underlying data
Dryad: Fecal calprotectin as a predictor of gastrointestinal immune-related adverse events (CF-19): A prospective study - Excel data and supplementary tables, https://doi.org/10.5061/dryad.rbnzs7hbk.²⁰

This project contains the following underlying data:

- Excel data: raw data from each of the cycles that was given to each patient.
- Supplementary tables: Summary tables with information about patients characteristics during our study.

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).
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