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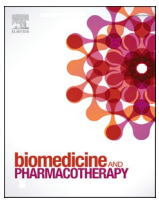

NLRP3 promoter methylation as a predictive biomarker for glucocorticoid response in patients with inflammatory bowel disease

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ABSTRACT

Glucocorticoids are used for inflammatory bowel disease (IBD) therapy; however nearly 50 % of IBD patients exhibit resistance or dependence. This study evaluates the relationship between methylation level at two CpG sites (cg21991396 and cg00448525) within *NLRP3* promoter and glucocorticoid response of 94 IBD pediatrics (39 with Crohn's disease (40.4 %)) and 47 IBD adults (26 with Crohn's disease (55.3 %)). Disease activity scores were collected before the treatment, after the first full-dose reduction and after 3 months of therapy. Patients with active disease despite receiving a standard dose of prednisone were considered resistant, while those who initially responded but relapsed upon dose reduction were classified as dependent. The DNA methylation was investigated through sodium bisulfite conversion followed by pyrosequencing. In IBD adults, methylation levels at both *NLRP3* CpG sites increased with patients' age ($p = 0.0038$ and $p = 0.0018$, respectively). In IBD pediatrics, the methylation level at both CpG sites negatively correlated with the disease activity score before treatment ($p = 0.031$ and $p = 0.072$, respectively) and after 1 month of therapy ($p = 0.037$ and $p = 0.067$, respectively). Furthermore, poor glucocorticoid response after one month of therapy in pediatric patients was associated with lower methylation levels at both CpG sites ($p = 0.045$ and $p = 0.038$, respectively). Crohn's disease patients had higher percentage of good responders compared to ulcerative colitis patients ($p = 0.06$). These findings indicate that *NLRP3* methylation might change through patients' lifespan and could have different clinical implications for pediatric and adult IBD forms.

1. Introduction

Inflammatory bowel diseases (IBD), mainly comprising Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract with uncertain and multifactorial etiologies, which include genetic predisposition, mucosal barrier dysfunction, disturbances in the gastrointestinal microbiota, dysregulated immune responses and environmental factors [1]. The peak age of

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onset occurs in adolescence and young adulthood, with approximately 25 % of IBD cases occurring in individuals under 20 years of age [2]. Compared to patients with adult-onset, younger IBD subjects present a more aggressive disease with extensive anatomic involvement and more active disease course, necessitating more intensive treatment strategies [1]. Although a curative therapy for IBD has not yet been identified, current treatment strategies focus on inducing and maintaining disease remission while managing symptoms. These approaches often involve the use of immunomodulators, including aminosalicylates, glucocorticoids (GCs), and biological drugs. GCs play a pivotal role in managing moderate-to-severe IBD, but their effectiveness varies significantly between individuals, leading to patients classification as GC-sensitive or GC-resistant within the first 30 days of treatment [3]. GC resistance is relatively common, particularly in younger patients; however, a reliable predictor of GC response has not yet been identified [4].

Recent studies have shown that demographic factors, such as gender, can influence GC response in pediatric patients,[5] as well as variability in the development of adverse reactions in adults [6]. Despite that the molecular mechanisms underlying GC resistance in IBD are still not well understood [7], the promoter methylation level of the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome has been identified as a possible biomarker of GC response in different inflammatory diseases and cancerous conditions, such as idiopathic nephrotic syndrome and acute lymphoblastic leukemia.8 [9], NLRP3 is a multiprotein complex composed of apoptosis-associated speck-like protein containing a CARD domain (adipose-derived stem cell [ASC]) and procaspase-1, that mediates activation of caspase 1, a crucial enzyme in the secretion of the pro-inflammatory cytokines interleukin 1β (IL-1β) and IL-18, which represent important inflammatory players [10]. The NLRP3 inflammasome expression is crucial for the intestinal homeostasis maintenance and the presence of abnormal levels of this complex is one of the main clinical manifestations of IBD patients; also the presence of polymorphisms conferring a hypofunctional NLRP3 phenotype is associated with the development of CD [11]. However, the effects of NLRP3 methylation levels on GC response in IBD have not been investigated yet.

Due to the dynamic changes in epigenetic factors such as DNA methylation during different developmental stages (from youth to adulthood) [12], this study aims to investigate the potential role of *NLRP3* methylation in the GC response in both pediatric and adult patients with IBD undergoing GC therapy.

2. Material and methods

2.1. Pediatric patients cohort

The samples used in this study were collected from previous studies and stored in a biobank at the Gastroenterology Unit of the Pediatric Hospital "Burlo Garofolo" of Trieste, Italy. This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the local ethical committee (Prot 2198; approval date September 2013). The inclusion criteria were: previous IBD diagnosis and initial treatment with prednisone for 4 weeks at the standard dose (1–2 mg/kg/day) and 8–11 weeks of tapering. The GC treatment was used as remission induction therapy in patients with ileo-colic or colonic CD when exclusive enteral nutrition was not an option and in patients with moderate or severe UC. Disease activity was evaluated according to the pediatric Crohn's disease activity index (PCDAI) or the pediatric ulcerative colitis activity index (PUCAI). Clinical response to GC was assessed after 4 and 12 weeks of therapy. Patients were considered GC responsive when a drop of at least 50 % from the basal clinical score was observed. Patients with an active disease despite prednisone at a daily dose of up to 2 mg/kg over a period of 4 weeks were defined as GC resistant, whereas patients who initially responded to prednisone therapy but were unable to sustain remission within 3 months after the beginning of therapy while receiving reduced dose of steroids, were considered GC-dependent. Both GC-resistant and GC-dependent patients were classified as "poor-responders", whereas the responsive subjects were classified as "good responders".

In case of clinical score unavailability, the physician global assessment definitions of "remission" and "response" or "persistent active disease" were used.

2.2. Adult patients cohort

The samples used in this study were collected at the Clinic for Gastroenterology and Hepatology, University Clinical Center Serbia, Belgrade, Serbia. The analysis of these samples was approved by the local ethical committee (Prot 432/7; approval date February 2020). All patients included in the study received systemic GC for the induction of clinical response and remission. The inclusion criteria required patients to meet the following conditions: be over 18 years old at the time of diagnosis, have a prior diagnosis of either UC or CD, have no history of treatment with immunosuppressive or immunomodulatory therapies (including GC, azathioprine, methotrexate, or biologics), and have undergone prednisone treatment at a standard dose of 1 mg/kg/day for two weeks, followed by a tapering regimen. For each adult patient, blood samples were collected during the active phase of the disease, just before the commencement of GC therapy. The global clinical activity assessment of UC was determined using the Mayo score, while the Crohn's Disease Activity Index (CDAI) was used in CD patients. Response to GC therapy in adult IBD patients was obtained at 2 weeks, before the full-dose reduction had been performed, and after 3 months of GC therapy. The primary endpoint was the clinical response to GCs with a decrease of IBD activity index (Mayo score or CDAI) of more than 50 %. The secondary endpoint included a reduction in elevated inflammatory biomarkers such as C-reactive protein. To analyze the correlation between methylation levels and clinical activity scores in adult IBD patients, CDAI and Mayo scores were first normalized to account for their different ranges (CDAI ranges from 0 to 600, while Mayo ranges from 0 to 12). The normalization was performed as follows: clinical activity score / maximal value of the score * 100. Patients with persistent active manifestations of IBD despite having the GC standard treatment of 1 mg/kg per day for 2 or more weeks were considered GC-resistant. IBD patients were considered GC-dependent if they initially responded to prednisone therapy, but were unable to sustain remission within 3 months after the beginning of therapy while receiving reduced dose of steroids.

2.3. Total DNA extraction

Genomic DNA was extracted from patients' peripheral blood using commercial kits (Merck, Milan, Italy for the pediatric cohort or QIAamp DNA Blood Mini Kit, Qiagen, Hilden, Germany for the adult cohort) according to the manufacturer's instructions.

2.4. DNA bisulfite conversion for methylation analysis

For the methylation analyses, patients' genomic DNA was treated with sodium bisulfite using the EZ 96-DNA methylation kit (Zymo Research, Irvine, CA, USA), following the manufacturer's standard protocol to convert the DNA not-methylated cytosine residues to uracil, while 5-methylcytosine residues remain unchanged.

2.5. Pyrosequencing analysis for DNA methylation

The methylation was investigated in two CpG sites: cg21991396 (chr1:247,418,115–247,418,116) and cg00448525 (chr1:247,418,106–247,418,107) located in the promoter of *NLRP3*. To analyze the methylation of the candidate CpG sites pyrosequencing assay was performed, using the PyroMark Q96 MD (Qiagen, Inc.; Germantown, MD). Methylation was then quantified with Pyro Q-CpG (version 1.0.9; Biotage, Inc.). Table 1 provides the list of the primers and sequencing

Table 1
Primers and sequencing probes for pyrosequencing analysis.

Genomic location	Oligos	Sequence 5' – 3'
GRCh38:1:247417985:247418210:1	P.F.	5'-AAGTAAAGAGTTAGAGTTTTTAGTTTGGAG−3'
	P.R.	5'-[BIO]ACAAACAAATCCATAAACAAAATTATATTAC−3'
	S.P.	5'-TTGTTTAATGTTATAGTTTYGTAATTAA−3'

List of primers and sequencing probes for pyrosequencing analysis. P.F., primer forward; P.R., primer reverse; S.P., sequencing probe, [BIO], 5'-biotinilated primer; GR38, genome reference 38.

probes specific for the bisulfite converted DNA sequence designed using the PyroMark Assay Design Software 2.0 (Qiagen, Inc.; Germantown, MD). The methylation percent calculated as ratio mC:C at each CpG site was used to determine the samples' DNA methylation.

2.6. Statistical analyses

Statistical analyses were performed using the software R, version 3.6.1.

Analysis of association between GC response and *NLRP3* methylation level was performed by logistic regression. For these analyses, response to prednisone was considered as the dependent variable and level of methylation as the independent variable while the clinical or demographic data were used as covariates of interest (i.e., age, gender and type of IBD). For the analyses considering continuous variables parametric or non-parametric tests were applied, as appropriate. Before applying parametric tests, normality of the continuous variable was assessed with the Shapiro-Wilk test. All p-values < 0.05 were considered statistically significant.

3. Results

3.1. Patients' characteristics

A total of 94 IBD pediatric patients were enrolled by the Gastroenterology Unit of the Pediatric Hospital "Burlo Garofolo'' in Trieste: median age 13 interquartile range (IQR) 7.57–15.31, 43 were females (45.7 %), 39 with Crohn's disease (CD, 40.4 %). Disease activity scores were collected before GC treatment (PUCAI 36.2 ± 20.4 and PCDAI 34.5 ± 12.5), after 1 (PUCAI 8.4 ± 11.2 and PCDAI 11.7 ± 10.2) and 3 (PUCAI 13.2 ± 22.8 and PCDAI 5 ± 3.95) months of therapy.

A total of 47 adult IBD patients were enrolled at the Clinic for Gastroenterology and Hepatology, University Clinical Center Serbia in Belgrade: median age 40, IQR 29–51.5; 22 were females (46.8 %), 26 with CD (55.3 %). Disease activity clinical scores (CDAI for CD, Mayo for UC) were collected before GC treatment (CDAI median = 274, IQR 221–380; Mayo median = 8, IQR 7–9), after 2 weeks (CDAI median = 136, IQR 120–217.5; Mayo median = 6, IQR 4–7), and after 3 months of GC therapy (CDAI median = 10.5, IQR 5.3–15.8; Mayo median = 3, IQR 3–8).

3.2. The association of NLRP3 methylation level with demographic and clinical characteristics of pediatric and adult IBD patients

In both IBD cohorts, pediatric and adult, no differences between genders in methylation level of *NLRP3* CpG sites were observed. The *NLRP3* methylation levels were comparable between the two cohorts (Supplementary Figure 1). In pediatric patients, the methylation level at analyzed positions did not correlate with age. Interestingly, in the adult IBD group, methylation levels at both CpG sites significantly increased with patients' age (Spearman $\rho = 0.4$, $p = 0.0038$; Spearman $\rho = 0.4$, $p = 0.002$, respectively, Fig. 1). However, combining the pediatric cohort with the adult cohort, no significant correlation between age and methylation levels of both CpG sites was found (Supplementary Figure 2).

In the pediatric cohort, the methylation level of both *NLRP3* CpG sites negatively correlated with the disease activity score before treatment (n = 35, Spearman's $\rho = -0.4$, $p = 0.031$ and $\rho = -0.3$, $p = 0.072$, respectively, Fig. 2) and after 1 month of therapy (n = 28, Spearman's $\rho = -0.4$, $p = 0.037$ and $\rho = -0.3$, $p = 0.067$, respectively, Fig. 3); however no association between *NLRP3* methylation level and disease score was found at 3 months.

In the adult patient cohort, no significant correlations were found between clinical activity scores and the level of methylation at both *NLRP3* CpG sites, neither before nor after 2 weeks and 3 months of treatment (Supplementary Figure 3).

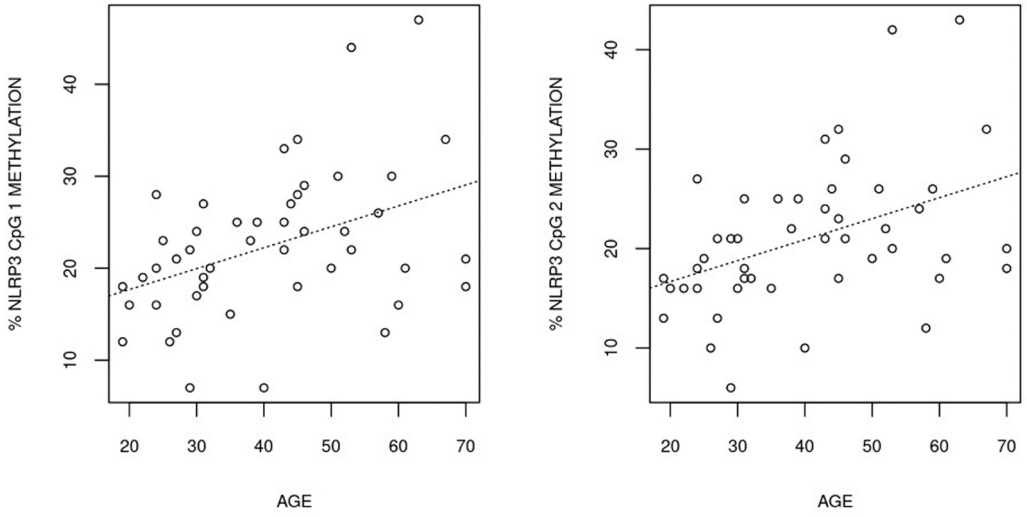

Fig. 1. *NLRP3* methylation and age in IBD adult patients. Correlations between *NLRP3* methylation level of both CpG sites and adult IBD patients' age (n = 47, Spearman $\rho = 0.4$, $p = 0.0038$ and Spearman's $\rho = 0.4$, $p = 0.0018$, respectively).

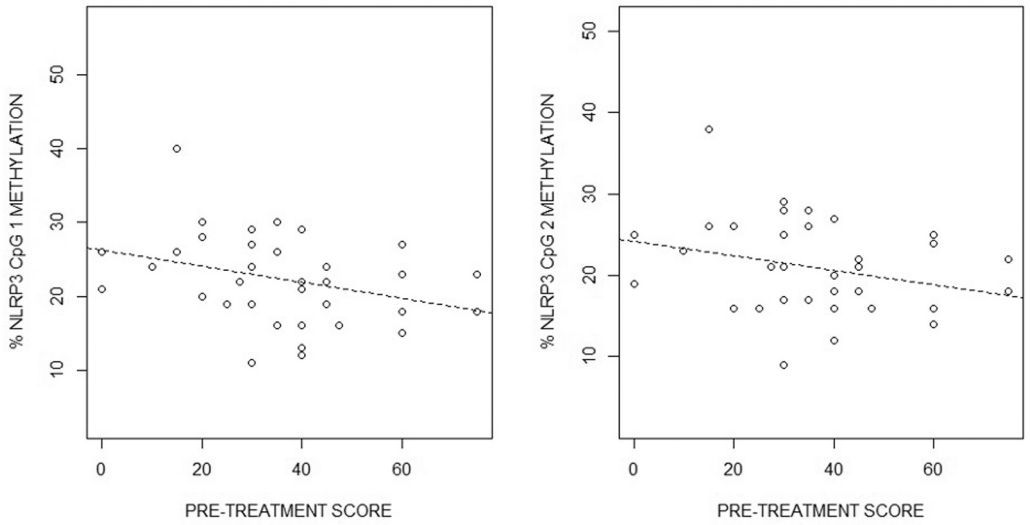

Fig. 2. *NLRP3* methylation level and pre-treatment disease score in IBD pediatric patients. Correlations between *NLRP3* methylation level of both CpG sites and disease activity score before treatment (n = 35, Spearman's $\rho = -0.4$, $p = 0.031$ and $\rho = -0.3$, $p = 0.072$, respectively).

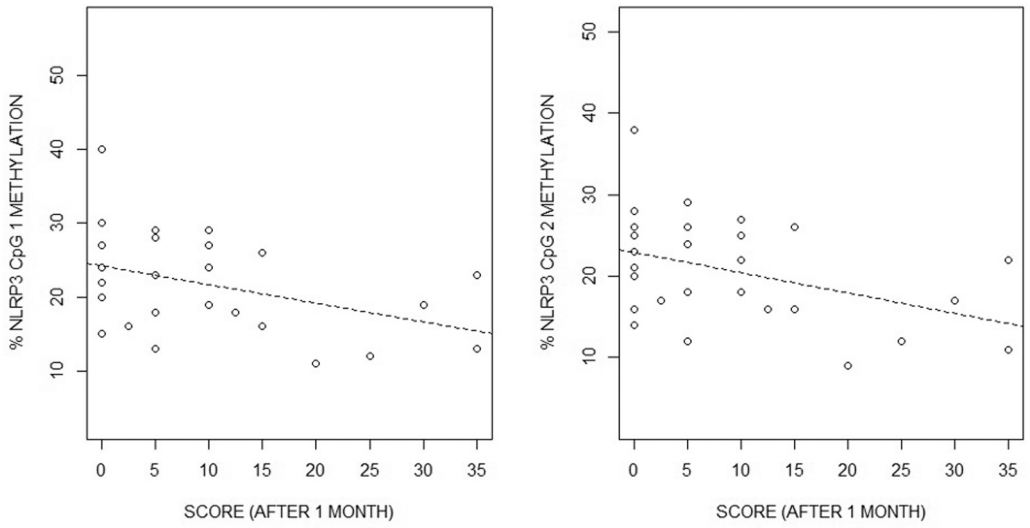

Fig. 3. *NLRP3* methylation level and disease score at 1 month in IBD pediatric patients. Correlations between *NLRP3* methylation level of both CpG sites and disease activity score after 1 month of therapy (n = 28, Spearman's $\rho = -0.4$, $p = 0.037$ and $\rho = -0.3$, $p = 0.067$, respectively).

3.3. GC response and NLRP3 methylation level in IBD pediatric patients

After 1 month of treatment, 83 (88.3 %) IBD pediatric patients were GC-sensitive and 11 (11.7 %) were GC-resistant. No association was found between patients' response after 1 month of GC therapy and patients' age, gender or IBD type. The GC response at 1 month was significantly associated with the methylation level of both *NLRP3* CpG sites (logistic regression $p = 0.045$ and $p = 0.038$, respectively, Fig. 4), indicating a higher methylation level in GC-sensitive patients compared to GC- resistants.

After 3 months of treatment, 32 (36 %) IBD pediatric patients were GC good responders (GC-sensitive) and 57 (64 %) were considered poor responders (GC-resistant or GC-dependent). No associations between GC response and patients' age or gender were detected, whereas it was found that CD patients seemed to respond better to GC therapy (logistic regression $p = 0.06$, Supplementary Figure 4) compared to UC patients. The GC response at 3 months was not associated with *NLRP3* methylation level.

The GC response was not found to be associated with the concomitant therapy with other immunomodulators alone or combined with parenteral nutrition.

3.4. GC response and NLRP3 methylation level in IBD adult patients

After 2 weeks of treatment, 40 (85.1 %) adult IBD patients were GC-sensitive and 7 (14.9 %) were GC-resistant. Patients' age, gender or IBD type were not associated with their response to GC therapy after 2 weeks of treatment. A logistic regression model was fitted to analyze the relationship between *NLRP3* methylation and GC patients' response using age as a co-variable. In contrast to the pediatric group, the GC response was not dependent on the methylation level at both *NLRP3* CpG sites ($p = 0.95$ and $p = 0.93$, respectively, figure Supplementary 5).

After 3 months of treatment, 26 (55.3 %) adult patients were classified as good responders, while 21 (44.7 %) were considered as poor responders. No associations between GC response at 3 months and patients' age, gender, or IBD type were detected. Additionally, GC response was not dependent on the *NLRP3* methylation level at both CpG sites after 3 months of the therapy.

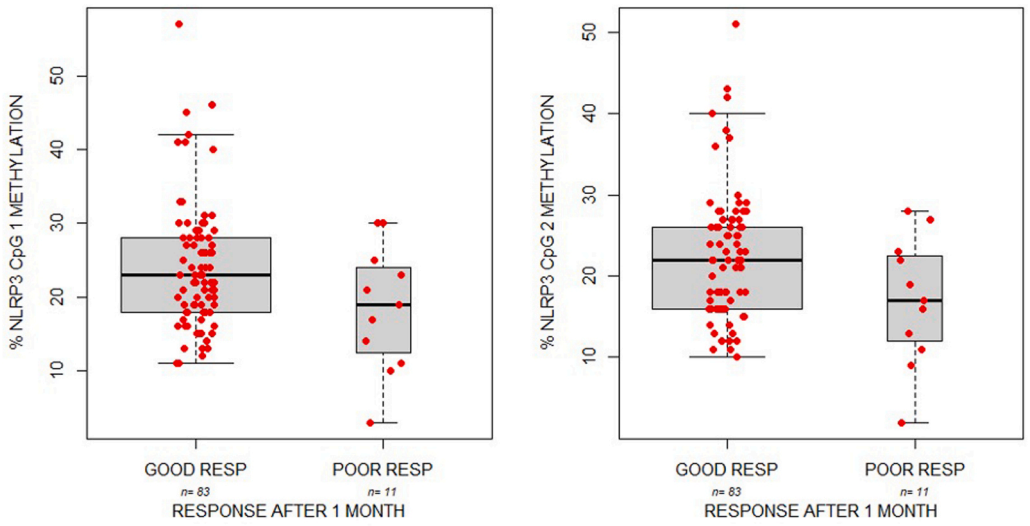

Fig. 4. *NLRP3* methylation level and glucocorticoid response at 1 month in IBD pediatric patients. *NLRP3* methylation level of both CpG site and the IBD pediatric patients' response after 1 month of GC therapy (logistic regression $p = 0.045$ and $p = 0.038$, respectively).

4. Discussion

This study investigated the *NLRP3* promoter methylation as a potential biomarker for GC response in both pediatric and adult IBD patients, because it had been previously identified as a promising predictor of GC response in pediatric patients with acute lymphoblastic leukemia [9] and idiopathic nephrotic syndrome [8] treated with GC therapy. The methylation of *NLRP3* significantly affects the inflammasome expression and activity [13], which is important for the IBD mucosal immune response [14]. In the current study, two CpG sites (cg21991396 and cg00448525) in the promoter of *NLRP3* were investigated showing similar methylation levels, suggesting their potential role as possible transcription modulators of NLRP3 inflammasome.

IBD children underwent methylprednisolone therapy at standard dose for 1 month before starting tapering, whereas adults received the full dosage only for 2 weeks, followed by tapering. The GC response was assessed at 4 weeks and 3 months in pediatric patients, whereas it was determined at 2 weeks and 3 months in the adult cohort. Consistently, the GC response was assessed at the end of the GC full dose exposure and after tapering. Still, difference in treatment protocols poses a substantial challenge in comparing outcomes between adult and pediatric IBD.

The methylation levels of these two CpG sites were negatively correlated with the disease score before starting GCs and after 1 month of therapy in pediatric IBD patients, confirming a higher NLRP3 inflammasome activation (lower methylation level of *NLRP3*) in correspondence of a greater disease severity. We showed that *NLRP3* methylation levels were associated with GC response at 1 month of therapy, underlying its potential to predict the anti-inflammatory effects of GCs in IBD pediatric patients. However, no association between *NLRP3* methylation level and GC response was detected in adult patients, which could be a consequence of the relatively small number of enrolled adult patients compared to pediatric cases. Indeed, the cohort of adult patients is approximately half the size of the pediatric cohort, and this could be considered as a study limitation. It is also important to highlight that adult patients might undergo several immunosuppressive treatment's cycles during their life and this could affect the epigenetics [25]. Moreover, it is known that epigenetic factors change through the patients' lifespan [12]. *NLRP3* methylation could be considered a reliable GC response predictor in younger subjects, who may present more severe disease, associated with increased inflammatory levels [15]. Consistently, this study showed a positive correlation between age and *NLRP3* methylation level in adult IBD patients, indicating higher NLRP3 activity in younger subjects. The impact of age in the disease clinical features has been demonstrated: pediatric patients with early onset show a more acute and severe disease [16] and require higher drug concentrations due to pharmacokinetics differences [17,18]. Older adult patients usually require longer GC therapy and show lower treatment responsiveness compared to younger individuals, increasing the risk of concomitant morbidity and adverse events [19,20]. Because pediatric IBD showed similar methylation levels than adult, the lack of association between age and *NLRP3* methylation level in the pediatric IBD cohort could depend on the disease characteristics. For instance, the genetic impact on the disease pathogenesis may be more important than other factors in pediatric IBD, whereas it is less important in adults [20]. The analyzed pediatric cohort is larger than the one of IBD adults and the difference in the sample size could explain why no significant correlation between age and *NLRP3* methylation levels was observed when combining the two cohorts.

GCs are widely used in IBD despite of their variable interindividual response. Patients can be sensitive, resistant or can become GC dependent after drug tapering. The problem of GC poor response is particularly common in younger subjects [4]. However, the molecular mechanisms undergoing this condition are not completely understood. *In vitro* evidences reported that NLRP3 inflammasome activation lowered GC receptor concentration, increasing GC resistance [8]. Also the presence of genetic variants in the GC receptor (GR/*NR3C1*) gene were associated with GC dependency and resistance in pediatric IBD patients [21]. In the analyzed pediatric cohort, after 3 months of GC therapy, only 36 % of patients were considered GC sensitive and classified as good responders, in accordance with similar results obtained in previous studies [4,5]. In the current study no associations between patient's gender and GC response were found both in pediatrics and adults; consistently, no previous studies showed an effect of sex in the drug response in adult subjects with IBD, whereas previous studies on pediatric patients detected an impact of gender in the GC response at 1 month [5,24]. Indeed, further studies on IBD patients will be required to confirm the potential role of gender in the GC treatment response. Although the GC response at 3 months seemed to be associated with the disease type: a trend was observed, indicating that CD patients may respond better than subjects with UC. Similarly, previous data have showen a better response of CD patients compared to UC [4,22], because UC is usually characterized by a more severe inflammation and higher *NLRP3* hypomethylation and inflammasome activity [23]. Together, the reported evidence suggested the importance of epigenetics in the regulation of inflammasome levels and highlighted the ability of *NLRP3* methylation to predict the anti-inflammatory GC response in IBD pediatric patients.

5. Conclusion

In conclusion, these data show that methylation level at two CpG *NLRP3* sites changes with patients' age. In IBD pediatric patients *NLRP3* methylation levels before treatment are related to disease severity and are predictive for the GC response at 1 month. On the contrary, *NLRP3* methylation level is not associated with patients' disease severity or GC response in adult IBD. Presented results indicate that epigenetic factors change through the patients' lifespan and could have different clinical implications for pediatric and adult IBD forms.

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CRediT authorship contribution statement

Gabriele Stocco: Writing – review & editing, Project administration, Funding acquisition. **Giuliana Decorti:** Writing – review & editing. **Sanja Dragasevic:** Writing – review & editing, Resources. **Branka Zukic:** Writing – review & editing, Resources, Funding acquisition. **Erika Cecchin:** Writing – review & editing, Methodology, Investigation. **Matteo Bramuzzo:** Writing – review & editing, Resources, Funding acquisition. **Davide Selvestrel:** Writing – review & editing, Methodology, Investigation. **Marianna Lucafò:** Writing – review & editing, Supervision, Project administration, Formal analysis, Data curation, Conceptualization. **Giulia Zudeh:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Biljana Stankovic:** Writing – review & editing, Resources, Formal analysis, Data curation. **Monica D'Andrea:** Writing – review & editing, Investigation. **Nikola Kotur:** Writing – review & editing, Resources.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Matteo Bramuzzo reports financial support was provided by Italian National Ministry of Health. Gabriele Stocco reports financial support was provided by Italian National Ministry of Health. Branka Zukic reports financial support was provided by European Commission. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.117824.

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