Title:
Subclinical inflammation with increased neutrophil activity in healthy twin siblings reflect environmental influence in the pathogenesis of inflammatory bowel disease

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Abstract

**Background and aim:** The mechanisms behind increased f-Calprotectin (FC) in healthy relatives of patients with inflammatory bowel disease (IBD) are unknown. Our aims were to explore if there is a subclinical inflammation with increased neutrophil activity in healthy twin siblings in discordant twin pairs with IBD and to assess the influence of genetics in this context.

**Material and method:** Nuclear factor kappaB (NFκB) and neutrophil activity, based on myeloperoxidase (MPO) and fecal-Calprotectin (FC), were analyzed in healthy twin siblings in discordant twin pairs with IBD and compared with healthy controls. NFκB and MPO were assessed by immunohistochemistry and FC by ELISA.

**Results:** In total, 33/34 healthy twin siblings were histologically normal. Increased NFκB was more often observed in healthy twin siblings in discordant twin pairs with CD [13/18 (73%)] and with UC [12/16 (75%)] than in healthy controls [8/45 (18%)]. MPO was more often increased in healthy twin siblings in discordant pairs with CD [12/18 (67%)] than in healthy controls [11/45 (24%)] and FC more often in healthy twin siblings in discordant pairs with UC [14/21 (67%)] than in healthy controls [6/31 (19%)]. Interestingly, the observed differences remained when healthy monozygotic- and dizygotic twin siblings were analyzed separately.

**Conclusion:** We observed increased NFκB, MPO and FC in healthy twins in both monozygotic and dizygotic discordant pairs with IBD. These novel findings speak for an ongoing subclinical inflammation with increased neutrophil activity in healthy first-degree relatives.
INTRODUCTION

The exact mechanisms of the pathogenesis of inflammatory bowel disease (IBD) are still unknown. It has been hypothesized that the inflammation is caused by an abnormal immune response to commensal flora in patients with a genetic predisposition, and that a defective mucosal barrier in combination with environmental exposure precedes the inflammation. (1) A number of biomarkers reflecting the disturbed interaction between microbiota and mucosal immune response have been associated with IBD. Fecal calprotectin (FC), mirroring migration of activated neutrophils and monocytes to the intestine, is one of the most well established biomarkers and correlates with the inflammatory activity in both Crohn’s disease (CD) and ulcerative colitis (UC) in IBD. (2) In vitro studies suggest that cytokine driven neutrophil transmigration and activation happen early in the inflammatory cascade, followed by increase in epithelial permeability and tissue damage. (3-5)

Interestingly, Thjodleifsson et al. observed elevated levels of FC in healthy first degree relatives of patients with CD. (6) Similar data has also been reported in first-degree relatives of patients with UC. (7) We have previously observed a discrepancy in mucosal glycosylation, gut permeability, and expression of anti-\textit{Saccharomyces cerevisiae} antibodies (ASCA) in healthy twin siblings in our cohort of twins with IBD, (8-14) supporting a disturbed interaction between microbiota and mucosal immune response in these healthy first-degree relatives. However, the implications of these findings are unknown. Is there an ongoing subclinical mucosal inflammation in relatives of IBD patients and if so, is this due to shared genetic background?

Twin studies are a powerful tool to disentangle the relative contribution of genetics and environmental exposure to the pathogenesis of IBD. Monozygotic twins have identical genes and usually share environmental factors. Dizygotic twins share environment to the same extent, but on average only half of the genes. The primary aim of our study was to explore if subclinical mucosal inflammation including neutrophil activity exists in healthy twin siblings in a cohort of twins with IBD. Secondly, we wanted to assess the influence of genetics in this context.
Activated NFκB was used as general marker of inflammation and myeloperoxidase (MPO) in combination with FC as specific markers for neutrophil activity.

METHODS

Twins

Twins enrolled in the study were derived from a previously described cohort of twins with IBD in Sweden.(15, 16) In short, twin pairs where at least one twin in each pair had been hospitalized for IBD, were identified by linking the Swedish Twin Registry with the Swedish Hospital Discharge Register. Each twin responded to a questionnaire concerning the diagnosis of IBD and general gastrointestinal symptoms and gave written consent for their medical records to be examined. The diagnosis of IBD was verified and the disease phenotype was classified according to the Montreal classification.(17) Zygosity was assessed by a questionnaire-based method, applied by the Swedish Twin Registry.(18, 19) Twin pairs of the same sex, under the age of 75 years, living within the vicinity of Örebro County, i.e. the middle and southern parts of Sweden, where both twins in each pair had approved further contact, were invited to undergo colonoscopy and to provide stool samples for FC assay. Exclusion criteria was previous extensive IBD related surgical resection, i.e. colectomy. Forty-five twin pairs underwent colonoscopy and were asked to send fecal samples 7-10 days prior to procedure. Twins living outside the vicinity of Örebro and those not willing to undergo colonoscopy were invited to provide stool samples for assessment of FC only.

Immunohistochemistry

Activated NFκB was used as a general marker of inflammation and MPO as a specific marker for detecting neutrophils. Biopsies from controls (n=45) with macroscopically normal mucosa were recruited from the Pathology register, Örebro University Hospital and matched for sex and
age ±5 years. These controls, who agreed to participate, had undergone colonoscopy during the same period as the twins, 2006-2009, to investigate altered bowel habits and/or abdominal pain (n=31), or unexplained rectal bleeding (n=9), or for polyp surveillance (n=5).

From each individual formalin-fixed and paraffin-embedded biopsies from both ascending colon and rectum were used. Four micron sections were stained with immunohistochemistry using the Envision technique (peroxidase). Antigen retrieval was achieved by heating the samples in Envision Flex Target Retrieval Solution High and Low pH in a PT Link (Dakocytomation, Denmark) for 20 minutes. Staining was performed using a Dakos Autostainer Link 48 and DAB+ Envision Flex (Dakocytomation, Denmark). Polyclonal anti-NFκB p65 antibody (ab16502, Abcam, USA) at dilution 1:1000 and polyclonal anti-MPO antibody (rabbit IgG Department of Medical Sciences, Clinical Chemistry, Uppsala University, Sweden)(20) at dilution 1:1600 were used. Slides were incubated with respective antibody for 30 minutes. All slides were stained simultaneously with a control slide exposed to the secondary antibody only.

**Histological assessment and evaluation of staining**

Hematoxylin and eosin stained sections were scored by AS as normal or inflamed(21), where the evaluator was blinded to diagnosis, FC levels and immunohistochemistry. NF-κB and MPO were scored by VHS who was blinded to diagnosis, histology and FC levels. Ascending colon and rectum were scored separately. Staining distribution of NF-κB p65 was localized to cytoplasm and/or nucleus. The intensity of staining was graded semi-quantitatively under high power microscopy (100 x magnification) into four categories, where 0 = absence of staining, 1 = reduced staining, 2 = moderate staining and 3 = strong staining. Similarly, MPO was classified semi-quantitatively according to percentage of stained neutrophils: 0 = 0-10%, 1 =10-50%, 2 = 50-80% and 3 = >80% .

**Fecal Calprotectin**

Stool samples were collected in screw-capped plastic containers, sent the same day by mail to
the laboratory, and stored at –70 °C. Before testing, the samples were thawed over night, prepared according to manufacturer’s protocol and analysed with ELISA (EK-CAL, Bühlmann Lab. AG, Switzerland). FC levels were expressed as micrograms of FC per gram of feces, using <50 µg/g as cut off. The intra- and inter-assay variations were less than 10% for all assays. A previously described control group (n=31) was used as a reference population.(22)

Statistics

Continuous data are presented as median (range). NFκB and MPO activity were dichotomized in low (grade 0-1) or high (grade 2-3). FC was categorized with the predefined cut off at 50 µg/g. Logistic regression model adapted for small samples was used to calculate odds ratio (OR) and corresponding 95% confidence interval (CI). In addition, differences in the presence of FC were analyzed from a quantitative multivariate perspective using regression analysis of a mixed model design with allowance for dependence within the twin pair. For FC levels below the lowest detectable value of 10, 10/√2 served as a substitute.(23) To comply with the statistical assumptions, group comparisons were carried through on the logarithm of FC, and the results were transformed back to the original FC scale. The effect parameter is therefore the ratio of geometric means rather than differences.(24) The ratios with corresponding 95% CI are given for the defined study groups. Statistical significance for the ratio of geometric means as well as the OR was judged against a predetermined cut off of p < 0.05. Calculations were performed in the statistical packages SAS (Version 9.2, www.sas.com) and LogXact (Version 8, www.cytel.com).

Ethical approval

The Örebro County Ethical Committee approved the study.
RESULTS

Twins

In total 104 twin pairs, who had not undergone extensive IBD related bowel resections, were identified. (16) Forty-eight twin pairs under the age of 75 years, living within the vicinity of Örebro were invited to take part in a colonoscopy-based study. Forty-five twin pairs (94%) agreed to participate and underwent colonoscopy (Figure 1). The remaining 59 twin pairs were invited to provide fecal samples for assessment of FC only, 34 of these twins agreed to participate. Thus, in total 124 twins provided stool samples for analyses of FC. Twin pairs can either be concordant, i.e. both twins in each pair suffer from the disease, or discordant, i.e. only one of the twins in each pair is affected. The observation period in the healthy twin siblings was 22 (4-42) years and 25.5 (12-56) years since diagnosis of CD and UC, respectively, in the diseased twin sibling within the discordant pairs.

Figure 1. Study groups of twins.

CD, Crohn’s disease; UC, ulcerative colitis; MZ, monozygotic pairs; DZ, dizygotic pairs.
**Histological assessment**

In total, 17/18 (94%) healthy twin siblings in discordant pairs with CD, 16/16 (100%) healthy twin siblings in discordant pairs with UC as well as all the controls were histologically normal. In addition, 23/28 (82%) of twins with CD and 17/18 (94%) with UC were in histological remission.

**NFκB status**

Increased NFκB activity was more often detected in healthy twin siblings in twin pairs discordant for CD as well as in pairs discordant for UC than in healthy controls (Table 1). The differences in NFκB activity in healthy twin siblings and in healthy controls remained when monozygotic and dizygotic twins were analyzed separately. NFκB was increased in 6/10 healthy twin siblings in discordant monozygotic and in 7/8 discordant dizygotic twin pairs with CD, [OR 6.7 (1.2-40.2)] and [OR 29.5 (3.2-1496)], respectively. Similarly, increased NFκB was observed in 5/9 healthy monozygotic and in 7/7 healthy dizygotic twin siblings in discordant pairs with UC, [OR 5.5 (1.0-35.0)] and [OR 38.1 (5.1-∞)], respectively. NFκB status in the rectum was correlated to its status in the ascending colon (Spearman's rho 0.941, p<0.0001).
Table 1 NFκB and MPO activity in ascending colon, OR and corresponding 95% CI from logistic regression model adapted for small samples

<table>
<thead>
<tr>
<th></th>
<th>NFκB low grade n (%)</th>
<th>NFκB high grade n (%)</th>
<th>OR (95% CI)</th>
<th>MPO low grade n (%)</th>
<th>MPO high grade n (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>37 (82)</td>
<td>8 (18)</td>
<td>1.0 (reference)</td>
<td>34 (76)</td>
<td>11 (24)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>HCD</td>
<td>5 (28)</td>
<td>13 (73)</td>
<td>11.4 (2.9-54.0)</td>
<td>6 (33)</td>
<td>12 (67)</td>
<td>6.0 (1.6-24.6)</td>
</tr>
<tr>
<td>HUC</td>
<td>4 (25)</td>
<td>12 (75)</td>
<td>13.1 (3.0-71.2)</td>
<td>10 (62)</td>
<td>6 (38)</td>
<td>1.8 (0.4-7.3)</td>
</tr>
</tbody>
</table>

HCD, healthy twin siblings in discordant twin pairs with Crohn’s disease; HUC, healthy twin siblings in discordant twin pairs with ulcerative colitis; OR, Odds Ratio; 95% CI, 95% confidence interval.

MPO status

Increased MPO was more frequently observed in healthy twin siblings in discordant twin pairs with CD than in healthy controls but MPO staining in healthy twin siblings in discordant twin pairs with UC did not differ significantly (Table 1). The increased activity in the discordant pairs with CD appeared to be independent of zygosity, since MPO seemed to be increased in both healthy monozygotic and healthy dizygotic twin siblings, [OR 11.7 (1.9-129.1)] and [OR 3.0 (0.9-1730)], respectively. MPO in healthy twin siblings in discordant monozygotic and discordant dizygotic twin pairs with UC did not significantly differ from controls, [OR 3.8 (0.7-22.6)] and [OR 0.5 (0.0-5.1)], respectively. MPO status in the rectum correlated to its status in the ascending colon (Spearman's rho 0.325, p<0.0001).

FC status and levels

FC was assessed in the different groups of twins and compared with the healthy controls. Increased FC, defined as >50µg/g, was observed in 12/32 (37%) of healthy twin siblings in
discordant twin pairs with CD [OR 2.7 (0.8-10.2)] and in 14/21 (67%) healthy twin siblings in discordant twin pairs with UC [OR 7.9 (2.0-36.3)], compared to 6/31 (19%) of healthy controls. Distribution of FC in each twin pair is shown in figure 2. FC was increased in 7/11 (64%) healthy twin siblings in discordant monozygotic twin pairs with UC and in 7/10 (70%) healthy twin siblings in discordant dizygotic twin pairs with UC, [OR 6.9 (1.3-44.1)] and [OR 9.0 (1.5-71.2)], respectively. Furthermore, FC was increased in 7/15 (47%) healthy twin siblings in discordant monozygotic twin pairs with CD and in 5/17 (29%) healthy twin siblings in discordant dizygotic twin pairs with CD, [OR 3.5 (0.8-17.3)] and [OR 1.7 (0.3-8.3)], respectively.

The median (range) concentration of FC was higher in healthy twin siblings in discordant twin pairs with UC compared to healthy controls, 72.0 (<10-317) and 28.0 (<10-225) µg/g, respectively. The observed increased levels of FC remained when healthy twin siblings in monozygotic and dizygotic twin pairs were analyzed separately (Table 2). The median (range) concentration of FC in healthy twin siblings in discordant twin pairs with CD (37.0 (<10-278) µg/g) did not significantly differ from the healthy controls.
**Figure 2.** Distribution of fecal calprotectin (FC) by pairs of twins. Twin pairs are ordered along the Y-axis according to decreasing FC on the left panel of each graph. In twin pairs concordant for the disease, the left panel comprises the twin in each pair with a higher FC and the right panel the twin with lower FC. In twin pairs discordant for inflammatory bowel disease the left panel comprises diseased twin and the right panel the healthy twin sibling.

- a) discordant monozygotic twin pairs with Crohn’s disease (CD),
- b) discordant dizygotic twin pairs with CD,
- c) concordant monozygotic twin pairs with CD,
- d) concordant dizygotic twin pair with CD,
- e) discordant monozygotic twin pairs with ulcerative colitis (UC),
- f) discordant dizygotic twin pairs with UC,
- g) concordant monozygotic twin pair with UC.

Extreme values are shown with numbers.
**Table 2** Ratio of geometric means for fecal calprotectin between selected groups of twins, monozygotic and dizygotic pairs analyzed separately. Estimates from mixed model with type of diagnosis as the only explanatory factor.

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Ratio of means</th>
<th>95% CI for ratio</th>
<th>p-value for testing ratio=1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCD – healthy controls</td>
<td>1.22</td>
<td>0.67 – 2.22</td>
<td>0.48</td>
</tr>
<tr>
<td>HUC - healthy controls</td>
<td>2.49</td>
<td>1.29 – 4.81</td>
<td>0.007</td>
</tr>
<tr>
<td>MZ HCD – healthy controls</td>
<td>1.45</td>
<td>0.75-2.81</td>
<td>0.27</td>
</tr>
<tr>
<td>DZ HCD – healthy controls</td>
<td>1.04</td>
<td>0.51-2.10</td>
<td>0.81</td>
</tr>
<tr>
<td>MZ HUC - healthy controls</td>
<td>2.36</td>
<td>1.13-4.95</td>
<td>0.02</td>
</tr>
<tr>
<td>DZ HUC - healthy controls</td>
<td>2.68</td>
<td>1.15-6.25</td>
<td>0.02</td>
</tr>
</tbody>
</table>

HCD, healthy twin siblings in discordant twin pairs with Crohn’s disease; HUC, healthy twin siblings in discordant twin pairs with ulcerative colitis; MZ, monozygotic; DZ, dizygotic; 95% CI, 95% confidence interval.
DISCUSSION

There has been a great progress in the understanding of the pathophysiology of IBD. However, the increased expression of biomarkers reflecting disturbance in the interaction between microbiota and mucosal immune response in first-degree relatives of patients with IBD is intriguing. Previous studies have reported increased FC concentrations, indicating increased neutrophil and monocyte activity, in first-degree relatives of patients with IBD,\(^{(6, 7)}\) with 49\% of these relatives having a positive FC.\(^{(6)}\) Based on the strong correlation between FC and fecal excretion of Indium-111-labeled neutrophils,\(^{(25)}\) it has been suggested that this observation could reflect a subclinical inflammation in these healthy individuals.\(^{(6)}\) However, previous studies have not explored whether intestinal inflammatory activity, at a macroscopic or microscopic level, really exists in these individuals. In the present study we demonstrated that all except one of the healthy twin siblings in discordant twin pairs with IBD had a histologically normal mucosa without any signs of macroscopic inflammation at endoscopy. Yet, increased NF\(\kappa\)B activity was observed in 74\%, of these healthy twin siblings. Similarly, we observed increased neutrophil activity, as reflected by FC and MPO, in these healthy twin siblings. Interestingly, the observed increased NF\(\kappa\)B activity remained when monozygotic and dizygotic pairs were analyzed separately. Increased NF\(\kappa\)B activity was observed in healthy twin siblings in both discordant monozygotic (60\%) and discordant dizygotic pairs with CD (88\%). Correspondingly, 56\% of healthy twin siblings in discordant monozygotic and 100\% of discordant dizygotic twin pairs with UC had increased NF\(\kappa\)B activity. Consistently, FC was increased in both monozygotic and dizygotic healthy twin siblings in discordant twin pairs with UC. The difference was significant irrespectively of if FC was analyzed as a categorical parameter or as a continuous parameter. Increased neutrophil activity, as reflected by MPO, was also observed in healthy twin siblings in discordant twin pairs with CD and appeared to be independent of zygosity, since MPO seemed to be increased in both monozygotic (80\%) and dizygotic (100\%) discordant pairs. The influence of shared environment during childhood and
adolescence is equal in monozygotic and dizygotic twin pairs in contrast to the influence of genetics which is more pronounced in monozygotic pairs. Subclinical inflammation with neutrophil activity was observed in healthy twin siblings in both discordant monozygotic and discordant dizygotic pairs. Thus, these findings point toward the importance of environmental factors rather than genetics alone in the etiology of this subclinical inflammation. In general, twins in each twin pair were separated around the age of 20 years. Thus, environmental factors driving the subclinical inflammation seem to interact early in life, during childhood or adolescence. These findings question previous observed increased expression of FC in spouses(7), although the literature is not totally consistent.(6)

Furthermore, the correlation between NFκB activity in rectum and right colon suggests that the subclinical inflammation affects the entire colon rather being a local phenomenon. This confirms our previous observations in a small set of unaffected twin siblings belonging to the first Swedish twin cohort with IBD. Only monozygotic twin pairs were included in the previous study, where increased activation of NFκB was observed in rectal biopsies from healthy twin siblings in discordant twin pairs with IBD, using a monoclonal antibody (MAB3026; Chemicon Europe, Chandlers Ford, UK) directed against the p65 subunit.(8)

Similarly, we have previously noted an increased proportion of immunoglobulin G1 producing immunocytes in rectal biopsies from both monozygotic twins with UC and from their unaffected twin siblings.(13) Changes have also been observed in the mucus of the colonic mucosal barrier. Initially, altered mucin glycoprotein profiles were observed in healthy twin siblings in discordant monozygotic pairs with UC.(11) However, more recent data suggest that this is true also for healthy twin siblings in discordant monozygotic twin pairs with CD.(8) Taken together these observations add further support to the hypothesis of an ongoing subclinical mucosal inflammation at a molecular level in healthy first-degree relatives of IBD patients.

Alterations in inflammatory pathways in healthy first-degree relatives of IBD patients have also been reported beyond twin studies. Hollander et al. frequently observed increased gut
permeability in relatives of patients with CD(26), although more recent studies have had
difficulties in reproducing this finding.(14, 27, 28) Similarly, increased concentrations of
serologic markers like ASCA, outer-membrane porin C (OmpC) and anti-neutrophil cytoplasmic
autoantibodies, has been reported in 20-32 % of healthy first-degree relatives.(9) Based on the
observed increased NFκB activity, subclinical mucosal inflammation seems to be a common
phenomenon in healthy twin siblings in discordant twin pairs with IBD. The discrepancy
between 74% of healthy twin siblings with increased NFκB activity and the well-described
figure of 5-10% of family members who develop clinical IBD underscores that these healthy
twin siblings barely will develop IBD. In addition, the median age (57 years) in these twin
siblings is beyond the age-specific peak incidence for IBD. The previously observed
concordance in age at diagnosis in monozygotic twin pairs where both twins suffer from CD or
UC,(29) and the median observation time of 23 years since diagnosis in the diseased twin,
further supports the prospect that these healthy twin siblings will not develop manifest IBD. It
remains to be shown whether these healthy twin siblings have been exposed to protective
environmental factors or have avoided triggering environmental factors that would drive the
subclinical inflammation into manifest IBD. The data raise the very important clinical question
whether the degree of subclinical inflammation in- first-degree relatives is associated with future
risk of manifest IBD.

The limited number of enrolled twin pairs with IBD is probably the greatest limitation of the
study. Even though twins were recruited through linkage of national population-based registries
45 IBD twin pairs only were enrolled and underwent colonoscopy. A substantial number of pairs
were excluded due to previous extensive IBD related surgical resections in the diseased twins.
Thus, the absence of any significant difference in FC between healthy twin siblings in discordant
twin pairs with CD and healthy controls is probably due to the limited number of enrolled twins
and lack of power. Due to costs only twins living within the vicinity of Örebro County were
invited to undergo ileocolonoscopy. In contrast, twin pairs from the more rural northern parts of
Sweden, were invited to provide stool samples for analyses of FC only. However, it is difficult to speculate how this could have influenced or biased the results, since the healthy controls were recruited from the same geographic area as the twins.

In conclusion, a subclinical inflammation, mirrored by increased NFκB activity and increased neutrophil activity, i.e. FC or MPO expression, was observed in healthy twin siblings in both discordant monozygotic and discordant dizygotic twin pairs with IBD. These findings strongly support the hypothesis of an ongoing subclinical mucosal inflammation at a molecular level in healthy first-degree relatives of IBD patients and add important pieces towards our understanding of the pathophysiology of IBD.

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GUARANTOR OF THE ARTICLE

Dr. Jonas Halfvarson, MD, PhD

SPECIFIC AUTHOR CONTRIBUTIONS

VHS, AS, CP analyzed the data, revised the manuscript and approved the final version.

LB analyzed and interpreted the data, revised the manuscript and approved the final version.

AG, NN, AW, JB collected and interpreted the data, revised the article and approved the final version.

YZ, JHN, MC, CT conceived and designed the study, collected, analyzed and interpreted the data, drafted the article and approved the final version.
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POTENTIAL COMPETING INTERESTS

None.

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