EXPRESSION OF TOLL-LIKE RECEPTORS 2 AND 4 IN THE COLONIC MUCOSA OF CHILDREN WITH INFLAMMATORY BOWEL DISEASE

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ABSTRACT

Background: Inflammatory bowel disease is a chronic inflammation of gastrointestinal tract (GIT), it include two forms, Ulcerative Colitis (UC) and Crohn's disease. Patients with IBD have different expressions of Toll like Receptors (TLRs) in comparison with healthy control.

Aim of the study: The aim of this study was to understand the etiology of inflammatory bowel disease and to help in its management through studying the differential expression of Toll-like receptors 2 and 4 in the colonic mucosa of children with IBD.

Patients and methods: The study was conducted as a case control study after taking an informed consent from patient and or their parents (18 children with IBD, 18 children as control). We assessed the TLR2 and TLR4 expression at the colonic mucosa of patients with IBD and control group.

Results: There was a statistical significance increase in expression of TLR2 & TLR4 among cases group compared to control group. There was a statistically significant positive correlation between TLR2 and TLR4 expression also there was a statistically significant positive correlation between both TLR2 & TLR4 and CRP, ESR and Calprotectin in cases group. The study shows that at cut off 1.33 TLR2 had accuracy of 69.4% in diagnosis of IBW while TLR4 at cut off 1.09 had accuracy of 63.9% in diagnosis of IBD.

Conclusion: This study demonstrated that increased TLR2 and TLR4 mRNA expression in the inflamed colonic mucosa of children with IBD supports the potential implication of the innate immune system in the pathology of this disease.

Keywords: Inflammatory bowel disease (IBD), Toll like Receptors (TLRs), Colonic Mucosa.

I. INTRODUCTION:

Inflammatory bowel disease is a chronic inflammation of gastrointestinal tract (GIT), it include two forms, Ulcerative Colitis (UC) and Crohn's disease [1].

IBD showed an increase in prevalence and incidence, so it become a global health problem [2].

The etiology of IBD is exactly remain unclear, but there is close relationship between IBD and immunity [3], genetics [4], Environment [5] and diet [6].

Commensal organisms and food antigens are prevented from entering lamina propria by intact intestinal epithelial barrier [7].

The few commensal organisms or antigens which can pass through the intestinal barrier will be phagocytosed by macrophages without production of pro inflammatory cytokines [8].
If this immunological tolerance towards endogenous microflora has a defect, for example, decrease epithelial function, impaired mucosal clearance [9], decrease of regulatory T-cell and immune suppressive cytokines [10], commensal flora can invade mucosa in large number or persist at lamina propria leading to CD4 T-cell activation [10], increasing permeability and finally chronic inflammation [11].

Toll like receptors (TLRs) are members of the conserved interlukin 1 super family of trans membrane receptors that recognize pathogen-associated molecular pattern (PAMPs) and are a member of pathogen recognition receptors (PRRs). TLRs are 11 in number and are expressed in a variety of cells in gut [13].

Patients with IBD have different expressions of TLRs in comparison with healthy control. TLRs become dimerized and activate the downstream signaling cascade such as inducing a variety of cytokines. TLRs activation led to regulation of the maturation of the dendritic cells and induction of proliferation and differentiation of Th1 and Th2 [14]. There is disturbance at the balance between T regulatory cells and effectors T cells in patient with IBD when the function of T regulatory cells in providing immune tolerance is suppressed and effector T cells like Th1, Th2, Th17 and NK T cells are activated, there is release of inflammatory cytokines and progression of inflammatory bowel disease [15].

We aimed in this study to understand the etiology of inflammatory bowel disease and to help in its management through studying the differential expression of Toll-like receptor2 and 4 in the colonic mucosa of children with IBD.

II. PATIENTS AND METHODS:

2.1. The current study was conducted as a case control study. A total number of 36 children (18 of them were freshly diagnosed inflammatory bowel disease and the other 18 children acted as a control group). They were collected from GIT unit in the Pediatric department at Zagazig University Hospital after obtaining the approval of the institutional review board (IRB) of Zagazig University.

Patient group:
18 children with freshly diagnosed IBD (8 boys, 10 girls, median age 18 years, range 5-16 years).

Control group:
18 children whom macroscopical diagnoses were polyp or mucosal congestion and causes other than IBD. (10 boys, 8 girls, median age 9 years, range 3-13 years).

2.2. A consent form approved by the committee of human rights in research in Zagazig University was obtained from each participant’s parent before the study initiation.

2.3. Patients who were included in this study with IBD who were diagnosed by standard endoscopic and histological examination of each sample and aged below 18 years.

2.4. All patients who were older than 18 years or patients receiving immune suppressive drugs prior to inclusion in the study were excluded from the study.

2.5. The patients who met the inclusion criteria and were suitable candidates for the study have been subjected to: Clinical assessment:

Full detailed history taking.
Personal history, name, age, sex, address.

Complaint:
GIT symptoms (abdominal pain, diarrhea, nausea, vomiting, constipation, perianal disease [16].

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General symptoms (weight loss, fever, anorxia, growth retardation, lethargy \(16\)).

Extra intestinal symptoms (ocular disorder, renal disorder, anemia, arthritis, skin disorder, \(16\)).

Past history for any disease, drug taking, surgery.

Family history of similar condition.

**Full clinical examination:**

General examination

Weight, height, clubbing, oral aphthous ulcer,

Color, skin, joint, peri anal region).

- Abdominal examination:
  1. Focal tenderness.
  2. Rebound tenderness and guarding (perforation, abscess).
  3. Palpation of deep organs.
  4. Percussion for ascites, enlarged organs.

**Investigation:**

1. Lab.
   - CBC \{Hb, WBC, platelet\}.
   - Inflammatory markers (CRP, ESR).
   - Liver profile (Alkaline phosphatase, serum alanine amino transferase ALT, aspartate amino transferase AST).
   - Serum Albumin.
   - Stool examination (occult blood, calprotectin, bacteria).

2. Radiology
   - Abdominal sonar and sometimes MRI enterography.

3. Colonoscopy and biopsy
   - For routine histopathology which is standard for diagnosis and classification of IBD \(16\).
   - For histochemistry TLR2 and TLR4 by polymerase chain reaction – enzyme linked immunosorbent assay.

**Determination of mRNA expression levels (TLR2 & TLR4) in colonic tissues using RT-PCR.**

(1) RNA extraction from tissue:

Extraction of RNA was purified using triazole kit in two steps;

- **Precipitate the RNA:** add 0.5ml of isopropanol to the aqueous phase per 1ml of triazole.
- Incubate for 10 minutes.
- Centrifuge for 10 minutes at 12000xg at 4°C
- Total RNA precipitate forms a white gel-like pellet at the bottom of the tube.
- **Wash the RNA:**
  a. Resuspend the pellet in 1ml of 75% ethanol per 1ml of TRIzol.
  b. Vortex the sample briefly, then centrifuge for 5 minutes at 7500xg at 4°C.
  c. Discard the supernatant with amicropipettor.
  d. Vacuum or air dry the RNA pellet for 5-10 minutes.

(2) **cDNA Reverse Transcription:**
Kit is high Capacity cDNA Reverse Transcription Kit purchased from (Thermo Scientific).

**Assay procedure:**
1. The kit components were allowed to thaw on ice.
2. The master mix for each sample was prepared.
3. The master mix was mixed thoroughly and carefully by vortexing.

The thermal cycler condition program was operated by thermal cycle (Applied Biosystems) to obtain Reverse Transcriptase.

(3) **Primer’s preparation:**
Primers were prepared as follow:
1. Lyophilized primer at -20°C was equilibrated at room temperature.
2. Equilibrated primer was spin down for 3 sec. using spin-centrifuge-vortex.
3. Lyophilized primer was diluted (both forward and reverse) with RNase free water (The volume was added to get 100 pmol/µl stock) and then the tube was gently invert for 2 min at room temperature.
4. Stock primer was diluted with RNase free water buffer (pH 8.0) to get 5 µM and Kept at -20 C° until used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs and probes</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>Forward: 5'-AGTTGATGACTCTACCAGATG-3'</td>
<td>598 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GTCAATGATCCACTTGCC-3'</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>Forward: 5'-CTTATAAGTGCTGAATCCC-3'</td>
<td>680 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TACCAGCAGCAGCTGC TCAG-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-CACCACCATGGAGAAGGCTG-3'</td>
<td>240 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GTG ATGGCATGG ACTGTG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe1: 5'-LCRed460-CCC TGG CCAAGGT CATCCATGA-PH-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe2: 5'-TCCTGCACCACCAACTGCTTAGC-FL-3'</td>
<td></td>
</tr>
</tbody>
</table>

(4) **Real Time PCR Amplification:**
The real-time RT-PCR was performed in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA) using 5x HOT FIRE Pol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia).

**Assay procedure:**
1. The kit components were allowed to thaw on ice.
2. The master mix for each sample was prepared.
3- The master mix was carefully and thoroughly mixed by vortexing. Centrifuge the master mix briefly to collect residual liquid from the walls of the tube.

4- Samples were added to each well in duplicate for each primer (target and endogenous gene).

5- The thermal cycler condition program was operated by Real Time PCR (Stratgen MX 3005p)

(5) Interpretation of the result (Relative quantification method):
The average between both of the house keeping genes (GAPDH and β actin) was used as normalizing to calculate the relative gene expression or fold change. Therefore, the quantities (Ct) of target gene were normalized with quantities (Ct) of housekeeping gene (GAPDH) by used the 2^ΔΔCt method (Livak and Schmittgen, 2001) as follow:

The control group was used as calibrator, while other groups represented as test groups in both target and reference gene.

2- The threshold cycle numbers (Ct) of the target gene were normalized to the threshold cycle numbers (Ct) of reference (ref) gene, in both the test groups and the control group by using the following equations:

ΔCt (test) = Ct (target in test groups) – Ct (ref. in test groups)

ΔCt (calibrator) = Ct (target in control) – Ct (ref. in control)

3- The ΔCt of the test genes were normalized to the ΔCt of the calibrator:

ΔΔCt = ΔCt (test) – ΔCt (calibrator)

4- Finally, fold change of relative gene expression was calculated by the following equation: Fold change = (2^ΔΔCt)

2.9. Statistical analysis:
The collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 24.0. Qualitative data were represented as frequencies and relative percentages. Chi square test was used to calculate difference between qualitative variables. Mann Whitney test was used to calculate difference between quantitative variables in not normally distributed data in two groups. Paired Wilcoxon test was used to calculate difference between not normally distributed data quantitative variables in the same group pre & post treatment. Spearman’s correlation coefficient was used to calculate correlation between quantitative variables. The threshold of significance is fixed at 5% level (P-value): P value of >0.05 indicates non-significant results. P value of <0.05 indicates significant results.

III. RESULTS:
There were no statistical significance differences between cases and control group in age, weight, and BMI or sex distribution (Table 2).

There was a statistically significant increase in frequency of abdominal pain, bloody diarrhea among cases group while in control group there was a statistical significance increase in frequency of bleeding per rectum. Regarding type of disease in cases group 38.9% were Crohn’s disease and 61.1% were Ulcerative colitis as illustrated in figure (1).

There was a statistically significant increase in platelets, CRP & ESR among cases group compared to control group (Table 3).

There was a statistically significant increase in ALT and AST among cases group compared to control group (figure (2)).

There was a statistical significance increase in mean of calprotectin and statistical significance decrease bacteria among cases group compared to control group (Table 4).
By gastroscope 77.8% of the cases were normal, 11.1% had gastritis and 11.1% had patchy of inflammation which agreed with histopathological results were also 77.8% of the cases were normal, 11.1% had H. pylori related Gastritis and 11.1% had chronic inflammation. The most frequent findings in biopsy were Chronic inflammatory infiltrate (61.2%), Architecture changes (50%) and finally PNLs & eosinophil infiltration (33.3%).

There was a statistical significance increase in expression of TLR2 & TLR4 among cases group compared to control group (Table 5).

There was a statistically significant positive correlation between TLR2 and TLR4 expression also there was a statistically significant positive correlation between both TLR2 & TLR4 and CRP, ESR and Calprotectin in cases group (Table 6).

At cut off 1.33 TLR2 had accuracy of 69.4% in diagnosis of IBW while TLR4 at cut off 1.09 had accuracy of 63.9% in diagnosis of IBD (Table 7, Figure 4).

Table (2): Demographic Characteristics of the studied groups:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=18)</th>
<th>Control (n=18)</th>
<th>test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age: (years)</td>
<td>Mean ± SD</td>
<td>10.61±4.07</td>
<td>8.51 ± 3.27</td>
<td>MW</td>
</tr>
<tr>
<td></td>
<td>Range 10</td>
<td>5 - 16</td>
<td>9</td>
<td>1.35</td>
</tr>
<tr>
<td>Weight: (kg)</td>
<td>Mean ± SD</td>
<td>17.38 ± 3.1</td>
<td>18.15 ± 1.21</td>
<td>t</td>
</tr>
<tr>
<td></td>
<td>Range 14.24 - 24.22</td>
<td>16.89 - 21.93</td>
<td>1.96</td>
<td>0.06 NS</td>
</tr>
<tr>
<td>BMI:</td>
<td>Mean ± SD</td>
<td>34.08 ± 15.26</td>
<td>25.44 ± 8.84</td>
<td>MW</td>
</tr>
<tr>
<td></td>
<td>Median 30</td>
<td>18 - 62</td>
<td>14 - 40</td>
<td>1.65</td>
</tr>
<tr>
<td>Sex:</td>
<td>Female 8</td>
<td>10</td>
<td>44.4</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>Male 10</td>
<td>8</td>
<td>55.6</td>
<td>44.4</td>
</tr>
</tbody>
</table>

Table (3) laboratory markers among the studied groups:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=18)</th>
<th>Control (n=18)</th>
<th>Test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs: (x10^3/mm^3)</td>
<td>Mean ± SD</td>
<td>10.11 ± 3.33</td>
<td>8.82 ± 5.22</td>
<td>MW</td>
</tr>
<tr>
<td></td>
<td>Median 10.5</td>
<td>6.66</td>
<td>1.67</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Range 5.9 - 15</td>
<td>4.2 - 25.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Variable</td>
<td>Cases (n=18)</td>
<td>Control (n=18)</td>
<td>Test</td>
<td>P</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>----------------</td>
<td>------</td>
<td>---</td>
</tr>
<tr>
<td><strong>Hb: (gm/dl)</strong></td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>10.78 ± 2.14</td>
<td>7.6 - 14</td>
<td>10.41 ± 1.7</td>
<td>8.5 - 15.3</td>
</tr>
<tr>
<td><strong>Platelets: (x10^11/mm^3)</strong></td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>445 ± 161.82</td>
<td>407.5</td>
<td>233 - 766</td>
<td>249.89 ± 101.37</td>
</tr>
<tr>
<td><strong>CRP: (mg/dl)</strong></td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>82.74 ± 72.61</td>
<td>60.5</td>
<td>1.1 - 202</td>
<td>4.81 ± 4.05</td>
</tr>
<tr>
<td><strong>ESR: (mm/h)</strong></td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>52.72 ± 26.64</td>
<td>49</td>
<td>11 - 90</td>
<td>25 ± 19.4</td>
</tr>
</tbody>
</table>

Figure (2): ALT among the studied groups.

Table (4): Stool analysis among the studied groups:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=18)</th>
<th>Control (n=18)</th>
<th>Test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calprotectin:</strong></td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>895.39 ± 551.83</td>
<td>813</td>
<td>225 - 1800</td>
<td>151.9 ± 121.69</td>
</tr>
<tr>
<td><strong>Bacteria:</strong></td>
<td>-veN (%)</td>
<td>N (%)</td>
<td>&gt;5 N (%)</td>
<td>-veN (%)</td>
</tr>
<tr>
<td></td>
<td>9 (50%)</td>
<td>7 (38.8%)</td>
<td>2 (22.2%)</td>
<td>2 (11.1%)</td>
</tr>
</tbody>
</table>

Figure (3): Histopathological findings among cases group.

Table (5): Toll-like Receptors 2 and 4 expressions among the studied groups:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=18)</th>
<th>Control (n=18)</th>
<th>MW</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histopathology</strong></td>
<td>Normal</td>
<td>H pylori related Gastritis</td>
<td>Chronic inflammation</td>
<td>77.8</td>
</tr>
</tbody>
</table>
TLR2: Mean ± SD 2.09 ± 1.11 1.26 ± 0.73
Median 1.94 1.49
Range 0.18 – 4.55 0.23 – 2.09

TLR4: Mean ± SD 1.95 ± 1.59 1.09 ± 0.81
Median 1.46 0.99
Range 0.017 – 6.79 0.37 – 3.81

Table (6): Correlation between Toll-like Receptors 2 & 4 expression and age, BMI & laboratory findings among the cases group:

<table>
<thead>
<tr>
<th>Variable</th>
<th>TLR2</th>
<th>TLR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.41</td>
<td>0.43</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>0.40</td>
<td>0.42</td>
</tr>
<tr>
<td>BMI (Kg/m$^2$)</td>
<td>0.46</td>
<td>0.38</td>
</tr>
<tr>
<td>WBCs ($\times 10^3$/mm$^3$)</td>
<td>0.41</td>
<td>0.37</td>
</tr>
<tr>
<td>Hb (gm/dl)</td>
<td>0.36</td>
<td>0.37</td>
</tr>
<tr>
<td>Platelets ($\times 10^3$/mm$^3$)</td>
<td>0.22</td>
<td>0.40</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>0.66</td>
<td>0.68</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>0.63</td>
<td>0.70</td>
</tr>
<tr>
<td>S. Albumin (mg/dl)</td>
<td>0.38</td>
<td>-0.44</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>0.37</td>
<td>0.16</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>0.24</td>
<td>0.07</td>
</tr>
<tr>
<td>INR</td>
<td>0.37</td>
<td>0.29</td>
</tr>
<tr>
<td>Calprotectin</td>
<td>0.52</td>
<td>0.49</td>
</tr>
<tr>
<td><strong>TLR4</strong></td>
<td>0.60</td>
<td>0.009**</td>
</tr>
</tbody>
</table>

Table (7): Validity of TLR2 & 4 expression in diagnosis of IBD among the studied groups:

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cut off</th>
<th>AUC</th>
<th>CI (95%)</th>
<th>Sen.</th>
<th>Spec.</th>
<th>PPV</th>
<th>NPV</th>
<th>Accu</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>1.33</td>
<td>0.71</td>
<td>0.53-0.88</td>
<td>88.9%</td>
<td>50%</td>
<td>64%</td>
<td>81.8%</td>
<td>69.4%</td>
<td>0.03*</td>
</tr>
<tr>
<td>TLR4</td>
<td>1.09</td>
<td>0.70</td>
<td>0.52-0.87</td>
<td>72.2%</td>
<td>55.6%</td>
<td>61.9%</td>
<td>66.7%</td>
<td>63.9%</td>
<td>0.04*</td>
</tr>
</tbody>
</table>

Figure (4): ROC curve for Validity of TLR2 & 4 expression in diagnosis of IBD among the studied groups.

IV. DISCUSSION:
The incidence of inflammatory bowel diseases (IBDs) such as ulcerativecolitis (UC) and Crohn’s disease (CD) has been increasing during recent years. Although the etiology of these diseases remains unknown, there is abody
of evidence suggesting that, in genetically susceptible individuals, intestinal inflammation in IBD results from an alteration at the balance between resident microbes in the gut and the host immune response at the mucosal barrier (16).

Ten TLR family members have been identified in humans, each one of them responds to different pathogen associated molecular patterns such as viral dsRNA (TLR-3), peptidoglycan (TLR-2), lipopolysaccharide (LPS) (TLR-4), viral ssRNA (TLR-7/8) flagellin (TLR-5) and unmethylated cytosine–phosphate–guanine (CpG) DNA (TLR-9). While the specific ligand for TLR-10 is still unknown, it has been shown recently to mediate the inflammatory response to both viruses and bacteria (17).

Activation of most TLRs results in the recruitment of the signaling adaptor molecule myeloid differentiation primary response gene 88 (MyD88) and subsequent activation of signaling pathways which culminate in degradation and phosphorylation of inhibitor of kB (IkB), and translocation of the transcription factor nuclear factor kB (NF-kB) to the nucleus. In contrast, TLR-3 recruits the adaptor Toll/interleukin (IL)-1 receptor (TIR) domain-containing adaptor-inducing interferon (IFN)-B (TRIF) to mediate signal transduction. Signaling of TLR through these pathways is important in recruitment of inflammatory cells and in production of inflammatory cytokines in the intestine (17).

TLR activation can, however, be a double-edged sword. Although, TLRs have been included in several gastrointestinal (GI) disorders including, colitis, colon cancer and coeliac disease. The expression pattern of many TLRs in the intestine has been investigated. TLR-4, for example, is expressed normally throughout the intestine at low levels in both lamina propria mononuclear cells and the epithelium, but its expression is reported to be elevated in IBD, indicating that increased TLR-4 expression may participate to the initiation and maintenance of intestinal inflammation. TLR-8 and TLR-2 have also been reported to be augmented in IBD, implying that these TLRs may also contribute to the increased inflammation associated with IBD (17).

It is clear, therefore, that the duration and intensity of the TLR response must be tightly controlled to maintain the balance between appropriate and inappropriate immune system activation in the intestine. Many of the expanding family of TLR inhibitory proteins have been found to be strongly expressed, and to be essential in the regulation of TLRs in the GI tract are namely A20, immunoglobulin receptor related (SIGIRR), peroxisome proliferator-IL-1 receptor-associated (IRAK)-m and Toll interacting protein (TOLLIP) (19).

There were no statistical significance differences between cases and control group in age, weight, BMI or sex distribution.

Our results were in agreement with study of Monastaet al., (20) as they reported that there were no statistical significance differences between cases and control group as regard sex and age distribution.

Also, Gearry et al., (21) reported that there were no statistical significance differences between IBD cases and control group regarding sex and age distribution.

The patients of IBD can present different gastrointestinal symptoms at presentation with a variable severity, including diarrhea, abdominal pain, gastrointestinal bleeding, intra-abdominal abscess, intestinal fistula, or perianal disease. In comparison to the onset of IBD with different gastrointestinal manifestations, certain patients can present an atypical onset with extraintestinal manifestations involving different systems, such as skin, musculoskeletal system, hepatobiliary, and ocular systems (21).

The present study showed that there was a statistically significant increase in frequency of abdominal pain, bloody diarrhea among cases group while in control group there was a statistical significance increase in frequency of bleeding per rectum. Regarding type of disease in cases group 38.9% were Crohn’s disease and 61.1% were Ulcerative colitis. In the study performed on Chinese pediatric patients with IBD, the most encountered clinical manifestations were abdominal pain in patient with CD and diarrhea in patient with UC (22).

The current study showed that there was a statistically significant increase in platelets, CRP, ESR, ALT and AST among cases group compared to control group.
In the study of Mărginean et al., (22), they found that C-reactive protein was normal in both their patients with UC at the time of diagnosis, even though the symptoms were much more severe than in the 2 patients diagnosed with CD, where C-reactive protein was elevated.

Stool should be examined for occult blood, ova, bacterial pathogens (including Clostridium difficile), and parasites. Fecal calprotectin, a neutrophil-derived protein with high concentrations in the setting of intestinal inflammation, is used as a useful biomarker, with 68% specificity and 98% sensitivity in children with suspected IBD (23).

In the study in our hands, there was a statistical significance increase in mean of calprotectin and statistical significance decrease bacteria among cases group compared to control group.

Our results were supported by study of Holtman et al., (24) as they reported that the best marker-fecal calprotectin-improved the area under the curve of symptoms by 0.26 (95% CI, 0.21-0.31). The second-best marker-erythrocyte sedimentation rate-improved the area under the curve of symptoms by 0.16 (95% CI, 0.11-0.21).

The present study showed that there was a statistical significance increase in frequency of pancolitis among cases group while in control group there was a statistical significance increase in frequency of polyp in colon scope. By gastroscope 77.8% of the cases were normal, 11.1% had gastritis and 11.1% had patchy of inflammation which agreed with histopathological results were also 77.8% of the cases were normal, 11.1% had H pylori related Gastritis and 11.1% had chronic inflammation. The most frequent findings in biopsy of colon were architectural collapse and structural changes (77.8) Chronic inflammatory infiltrate (72.2%), and finally diffuse mucosal ulceration (38.3%).

In the study of Ashton et al., (25), in CD there are, however, statistically significant increases in extent of disease in the stomach, ileum, and throughout the large bowel at all time points of study, compared to diagnosis. there is possibility that this reflects the transmural and systemic inflammation seen in CD not seen in UC or IBDU (inflammatory bowel disease unclassified). Granulomatous disease (upper or lower GI) was present in 66.1% of initial FU (follow-up) endoscopy cases and 56.4% of most recent FU endoscopy cases.

Evidence is emerging that the underlying mechanisms of intestinal epithelial tolerance versus intolerance towards luminal bacterial ligands are mediated through Toll-like receptors (TLRs) and other pattern recognition receptors of the innate immune system. Mammalian TLRs composed of a family of (so far) 11 individual type I transmembrane receptors, which recognize pathogen-associated molecular patterns (PAMPs). TLR2 interacts with peptidoglycan, a component of a cell walls of bacteria, as well as additional constituents of Gram-positive bacteria, fungi and mycobacteria. TLR3 recognizes double-stranded RNA and initiates responses to the standard immunostimulantpolyinosine-polycytidylic acid and immune specific viral pathogens. The most important TLR4 ligand is lipopolysaccharide (endotoxin, LPS), which is a major composition of the Gram-negative bacterial outer membrane. Interaction of TLRs with PAMPs triggers a complex signaling pathway that stimulate several transcription factors, such as nuclear factor κB, which in turn induce the activation of the inflammatory genes such as IL-1, TNF, IL-8 and IL-6 (17).

The current study showed that there was a statistical significance increase in expression of TLR2 & TLR4 among cases group compared to control group. There was a statistically significant positive correlation between TLR2 and TLR4 expression also there was a statistical significance positive correlation between both TLR2 & TLR4 and CRP, ESR and Calprotectin in cases group. There was a no statistical significant positive correlation between TLR2 and TLR4 expression independently and any of clinical or laboratory findings in the control group.

Using ROC curve; at cut off 1.33 TLR2 had accuracy of 69.4% in diagnosis of IBW while TLR4 at cut off 1.09 had accuracy of 63.9% in diagnosis of IBD.

Our results were supported by study of Szebeni et al., (18) as they aimed to illustrate the alteration of TLR2, TLR3 and TLR4 expression in the samples of colonic biopsy taken from children with active IBD either freshly diagnosed (fdIBD) or relapsing after treatment (rIBD).

They found higher TLR4 and TLR2 mRNA expression and protein levels in controls. In the non-inflamed colonic mucosa of children with rIBD and fdIBD, TLR4 and TLR2 mRNA expression and protein levels were similar to controls. TLR4 and TLR2 mRNA expression and protein levels also did not differ between children with rIBD.
fdIBD in either inflamed or non-inflamed colonic mucosa. TLR3 mRNA expression and protein levels stay unchanged in all groups studied. We observed no difference in the mRNA expression and protein levels of, TLR3 TLR2 and TLR4 in the colonic mucosa of children with UC or CD.

Previous studies have found an increased expression of TLR2 and TLR4 on both mRNA and protein levels in inflammatory bowel disease and colonic diarrhea. TLR4 expression is also increased in experimental and human necrotizing enterocolitis (18).

V. CONCLUSION:
From the results of this study, we concluded that increased TLR2 and TLR4 mRNA expression in the inflammed colonic mucosa of children with IBD supports the potential implication of the innate immune system in the pathology of this disease.

Conflict of Interest: No conflict of interest.

REFERENCES.