

## Paraoxonase Gene Expression in Pediatric Inflammatory Bowel Disease

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Received date: February 25, 2014, Accepted date: June 10, 2014, Published date: June 17, 2014

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### Abstract

**Background:** Oxidative stress plays a role in the pathogenesis of inflammatory bowel disease (IBD). The Paraoxonase (PON) genes, expressed in the human intestine, are thought to prevent oxidative stress and modulate inflammation. We investigated the effect of IBD and steroids on PON gene. We hypothesized that PON gene expression is decreased in IBD patients and that steroid treatment would return it to normal.

**Methods:** Pediatric patients diagnosed with IBD, were enrolled and matched to control subjects. For *in vitro* studies, human epithelial colorectal adenocarcinoma (Caco-2) cells were treated with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and dexamethasone. PON genes expression was evaluated by quantitative real-time PCR for both the biopsies and the Caco-2 cells.

**Results:** PON gene expression was decreased in intestinal biopsies from medication naïve IBD patients when compared to controls ( $p < 0.05$ ). Biopsies from IBD patients on steroids exhibited up regulation of PON gene expression ( $p < 0.05$ ). Caco-2 cells treated with H<sub>2</sub>O<sub>2</sub> had decreased PON gene expression compared to controls ( $p < 0.05$ ). Dexamethasone increased PON gene expression in Caco-2 cells ( $p < 0.05$ ).

**Conclusion:** Our data suggests that decreased PON expression in IBD patients is a consequence of oxidative stress which plays a role in the pathogenesis of IBD. Further, steroids counteract the effect of oxidative stress by up regulating PON gene expression. PON genes may be targets for the management of intestinal diseases like IBD.

**Keywords:** Inflammatory bowel disease; Paraoxonases; Oxidative stress; Dexamethasone; Caco-2; H<sub>2</sub>O<sub>2</sub>

### Introduction

Inflammatory Bowel Disease (IBD) is a chronic relapsing inflammatory condition that affects the gastrointestinal tract. It is comprised of two major phenotypes, Crohn's disease (CD) and ulcerative colitis (UC) [1]. The pathogenesis of IBD is believed to be multifactorial and includes genetic predisposition, immune dysregulation, dysbiosis, and barrier dysfunction caused by different factors one of which is oxidative stress [2].

Oxidative stress arises when there is marked imbalance between the production of reactive oxygen species (ROS) and their removal by antioxidants. Both animal and human studies link oxidative stress and IBD [3]. The most striking evidence in animal studies comes from genetic knockout mice lacking glutathione peroxidase (GPX). These animals develop a crypt destructive colitis similar to UC as early as 11 days of age [4]. Human studies show the presence of excessive reactive oxygen metabolites and antioxidant imbalance in the human gastrointestinal tract [5-7].

In IBD, ROS are produced in abnormally high levels and this leads to destruction of the mucosal barrier [4]. The damaged intestinal mucosal barrier allows highly immunogenic luminal bacterial to enter the normally sterile submucosa layer. Bacterial antigens initiate an immune response and a destructive cascade follows [8].

The paraoxonase (PON) gene family includes PON 1, PON 2, and PON 3. They are all located on chromosome 7 in humans. All PON genes encode for proteins that have an antioxidative function. Among all of them, PON 1 is the most studied. It is expressed in liver, intestines, lungs, and brain. It was first studied for its organophosphatase activity, but it has several other enzymatic activities, including peroxidase and arylesterase like activity which act to detoxify reactive oxygen species, and lactonase which acts to inhibit bacterial quorum sensing by degrading the bacterial signal molecules such as homoserine lactones [9].

In a recent study, upon inactivation of PON 2 in epithelial cells, an increase in superoxide dismutase, decrease in catalase activity, and up regulation of pro-inflammatory factors (TNF  $\alpha$ , IL6, and monocyte chemo attractant protein1) were observed suggesting that PON 2 functions as an antioxidant and anti-inflammatory agent [10]. Another study showed that PON 2 protects intestinal CaCo-2 cells against iron-ascorbate induced oxidative stress [9-11]. In addition to its antioxidant and anti-inflammatory roles, PON 2 has the highest lactonase activity among the PON genes. PON 3 acts as an antioxidant, and also has lactonase activity.

Previous studies have shown decreased PON 1 and 3 gene expression in diseased intestinal tissue of patients with inflammatory conditions such as IBD. Those studies were performed on adults, age >18 years. Patients who participated in the studies were those with known or suspected IBD. No studies were performed on medication naïve patients. Precourt et al. studies the effect of oxidative stress on PON gene expression in CaCo-2 cells, using iron-ascorbate [12]. Lim

et al. used NIH3T3 cells to study the effect of dexamethasone only on the expression of PON 2 mRNA [13]. In this study we investigate both effects of oxidative stress using H<sub>2</sub>O<sub>2</sub> and steroids on all PON gene expression in human intestinal tissue as well as CaCo2 cell line.

Our goal was to study the PON gene expression in intestinal tissue of pediatric patients with IBD. Our hypothesis was that medication naïve patients with IBD have decreased PON gene expression and thus lack their protective antioxidant and anti-inflammatory effects.

## Materials and Methods

### Patients

The study was approved by the Children and Youth Institutional Review Board, University at Buffalo, Women and Children's Hospital, Buffalo, NY.

All patients, 1-21 years, were enrolled at the time of upper endoscopy and colonoscopy performed from 2010-2012. The procedures were performed to evaluate symptoms of abdominal pain, diarrhea, bloody stools, weight loss, polyposis screening, or as a follow up for established IBD. IBD patients included in the study were those who had clinical and radiographic findings consistent with IBD, and their procedure revealed typical endoscopic and histologic features of IBD. We included medication naïve patients as well as patients on steroids. Two patients who had indeterminate colitis were excluded from the study, none of the screened patients had infectious colitis.

Age and sex matched control subjects were selected from those who had normal biopsies from colonoscopy, normal imaging studies, and negative IBD panel (including perinuclear anti neutrophil cytoplasmic antibody, Anti-Saccharomyces cerevisiae antibodies, outer membrane protein C, myeloperoxidase, and serine protease antibodies) and no other indicators of IBD. Those patients were diagnosed with constipation, reflux, or irritable bowel syndrome.

Biopsies from the terminal ileum (TI) and sigmoid colon (SC) were obtained from each patient. Weight and height were obtained at the time of procedure. Body mass index (BMI; kilograms per square meter) and BMI Z score and height Z score were determined using EPI-INFO anthropometric software. Disease activity was scored using the Pediatric Crohns Activity Index [14] or the Pediatric Ulcerative Colitis Activity Index [15].

### Gene expression analysis of PON

Tissue biopsies obtained at the time of colonoscopy were treated with RNA later (Qiagen, Valencia, CA) and stored at -80°C. RNA was extracted from these biopsies with RNeasy and treated with RNase-free DNase I set (Qiagen, Valencia, CA). Complementary DNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR was performed on an iCycler iQ real-time detection system (Bio-Rad Laboratories, Hercules, CA), using SYBR Green (iQ<sup>+</sup> SYBR<sup>+</sup> Green Supermix; Bio-Rad Laboratories, Hercules, CA) for real-time monitoring. For normalization purposes, GAPDH RNA levels were analyzed in parallel with PON 1, 2, and 3 [16]. The presence of a single specific PCR product was verified by melting curve analysis, presence of a single band on agarose gel electrophoresis, and confirmed by direct sequencing of the PCR products.

The primer pair for GAPDH consisted of the same primers used in our previous studies [17,18]. The primer pair for human PON 1 was

designed from one variant to have the forward and reverse primer annealing to exon 3 and exon 4 respectively. Human PON 2 was designed from two variants to have the forward and reverse primer annealing to exon 7 and exon 8 respectively in both variants. Human PON 3 was designed from one variant to have the forward and reverse primer annealing to exon 6 and exon 7 respectively. The replicon size for PON 1 is 72 bp, PON 2 is 149 bp, and PON 3 is 98 bp. The primers used for PON genes are as the followings:

PON 1 Forward primer: 5'tctgaagacttggagatactgccta3';

PON 1 Reverse primer: 5'aagctctttattccaggatacttta3'.

PON 2 Forward primer: 5'aacacactaatatgaatttaactcagt3';

PON 2 Reverse Primer: 5'aggattgttcgggtcatcacgaagag3'.

PON 3 Forward Primer: 5'ttttagtgccaatgggatcacagtc3';

PON 3 Reverse Primer: 5'tcatgttttccattatgtgaatgttc3'.

### Cell cultures and treatments

Human epithelial colorectal adenocarcinoma (CaCo-2) cell line (ATCC, Manassas, VA) was maintained in Dulbecco modified minimum essential medium (DMEM) ( Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) and 1% antibiotics. Cells were grown on 75 cm square flasks; media was changed every 3 days [19]. Upon reaching 70% confluency, cells were split using 0.05% trypsin-EDTA. Utilization was between passages 9 and 16.

In all experiments, Caco-2 cells were cultured at 37 degrees Celsius in a humidified air- 5% CO<sub>2</sub> atmosphere. In the first experiment for oxidative stress treatment, cells were incubated with 100 μM H<sub>2</sub>O<sub>2</sub> for 24 hours [20]. Untreated cells served as the control. In the second experiment for steroid treatment, Caco-2 cells were incubated with 0.01 μM dexamethasone for 24 hours. Untreated cells served as the control were untreated cells [13]. The third experiment involved testing the effect of dexamethasone with the presence of H<sub>2</sub>O<sub>2</sub>. Two groups were initially treated with 100 μM H<sub>2</sub>O<sub>2</sub>. After 24 hours the first group had 0.01 μM dexamethasone added for 24 hours, and the second group served as a control with no dexamethasone added. All experiments with dexamethasone involved dissolving dexamethasone powder in 1μl ethanol; therefore, same amount of ethanol was added to each control flask. For all treatments, four replicates were done. For each flask, RNA was extracted post treatment at 80-90% confluency (around 5 million cells) for real time qPCR analysis. Cell viability was checked after treatment using trypan blue stain and was found to be 95%.

### Statistical analysis

A 2 tailed t-test and ANOVA analysis were used to compare the gene expression in IBD patients to controls, and in treated CaCo2 cells vs. controls.

## Results

### Human study

A total of 35 patients were included in the study, 10 medication naïve CD, 10 medication naïve UC, 5 IBD patients on steroids (3 CD, 2 UC) and 10 control subjects. Demographic and clinical characteristics for the different groups, including anthropometric data

at the time of the procedure are presented in Table 1. There was no difference among the groups ( $p > 0.05$ ) with respect to age or BMI except the medication naïve patients with UC had a significantly lower BMI ( $p = 0.02$ ) when compared to controls. Clinical and histological

disease severity in patients varied from mild to severe disease, activity index scores and disease location are described in Table 1. Table 2 shows all concomitant medications taken with steroids at the time of the procedure.

	Control Subjects (N = 10)	CD Medication Naïve (N=10)	UC Medication Naïve (N=10)	CD On Steroids (N=3)	UC On Steroids (N=2)
Age in years (mean $\pm$ SD)	13.5 $\pm$ 3.9	13.4 $\pm$ 4.5	13.9 $\pm$ 4.3	15 $\pm$ 2	16 $\pm$ 0.0
Gender M	3	5	5	2	1
F	7	5	5	1	1
Height Z score (mean $\pm$ SD)	-0.4 $\pm$ 0.6	0.2 $\pm$ 1.5	-0.4 $\pm$ 1.2	0.5 $\pm$ 1.0	-0.7 $\pm$ 1.8
BMI Z score (mean $\pm$ SD )	1.0 $\pm$ 0.9	0.4 $\pm$ 1.3	-0.6 $\pm$ 0.7 *	-1.1 $\pm$ 2.3	-0.2 $\pm$ 0.7
Activity index score	N/A	37 $\pm$ 16 ‡	45 $\pm$ 17.3	41 $\pm$ 1.4 ‡	82.5 $\pm$ 2.5 †#
TI disease (%)	N/A	8 (80%)	0 (0%)	3 (100%)	0 (0%)
SC disease (%)	N/A	5 (50%)	3 (30%)	1 (33%)	2 (100%)

N: Number; M: Male; F: Female; SD: Standard Deviation; BMI: Body Mass Index; CD: Crohns Disease; UC: Ulcerative Colitis; TI: Terminal Ileum; SC: Sigmoid Colon  
 \*P value <0.05 when compared to control subjects  
 † P value <0.05 when compared to all other groups  
 ‡ Pediatric Crohns Disease Activity Index (PCDAI)  
 # Pediatric Ulcerative Colitis Activity Index (PUCAI)

**Table 1:** Demographics.

	Form of steroids	Dose of steroids	Concomitant medications
<b>Patient 1</b>	Prednisone	1 mg/kg/day	None
<b>Patient 2</b>	Solumedrol	2 mg/kg/day	5 ASA
<b>Patient 3</b>	Solumedrol	2 mg/kg/day	None
<b>Patient 4</b>	Solumedrol	2 mg/kg/day	None
<b>Patient 5</b>	Solumedrol	2 mg/kg/day	5 ASA, 6-MP

**Table 2:** IBD patients on steroids, the form and dose of steroids IBD patients were on at the time of procedure along with any concomitant medications.

Gene expression of paraoxonases is altered in some inflammatory diseases, but it is not clear if PON gene expression is affected in IBD. Therefore, we analyzed PON gene expression in TI and SC biopsies from medication naïve IBD patients, both inflamed and non-inflamed mucosa were included in the analysis. All PON genes were found to be expressed in the TI and SC of normal subjects. PON 2 was found to be the most highly expressed among the PON family.

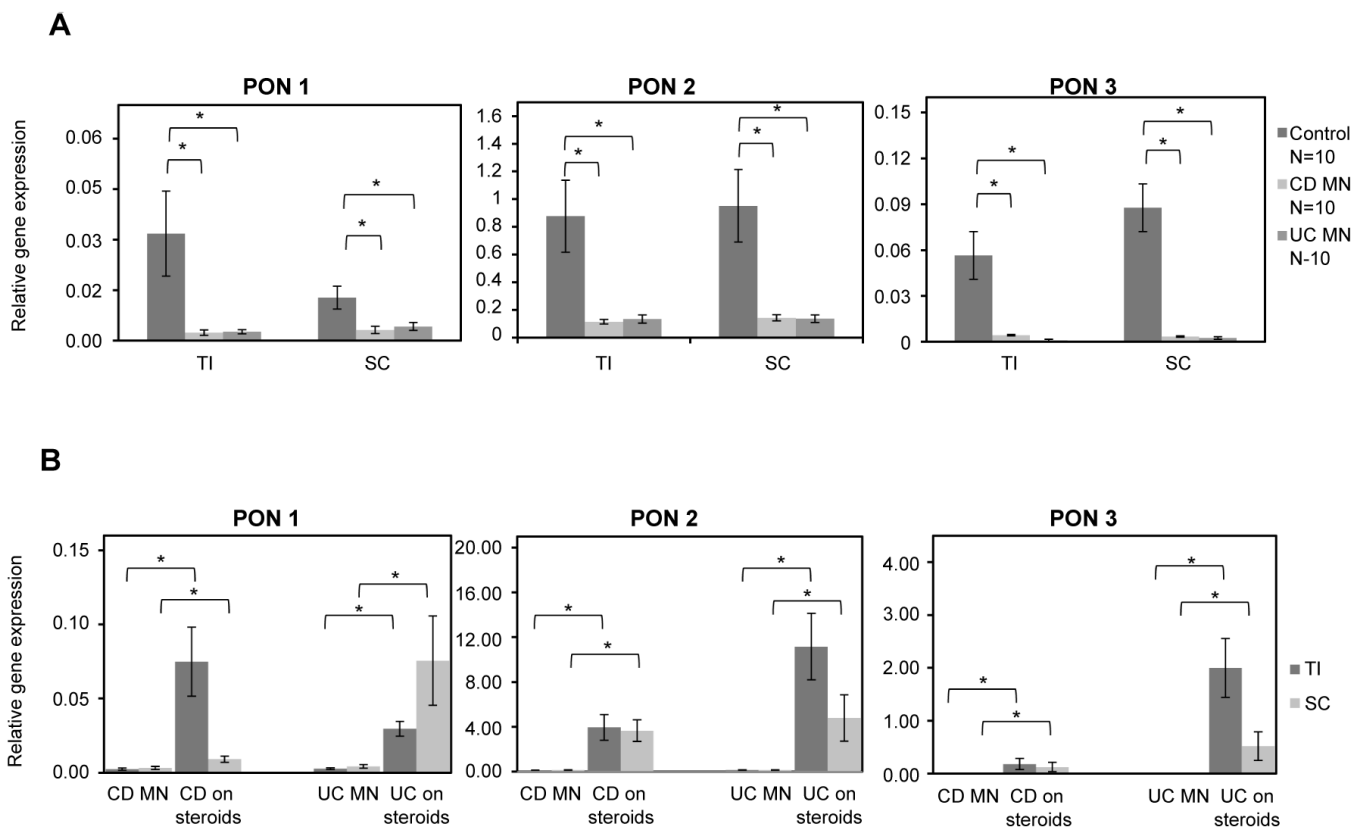
PON gene expression is significantly decreased in patients with IBD in both the TI and SC when compared to control group (Figure 1A).

We found that the expression of PON genes in IBD patients on steroids was up regulated when compared to medication naïve IBD ( $P < 0.05$ ). Steroids increased PON 1 gene expression by 92 and 40 % in

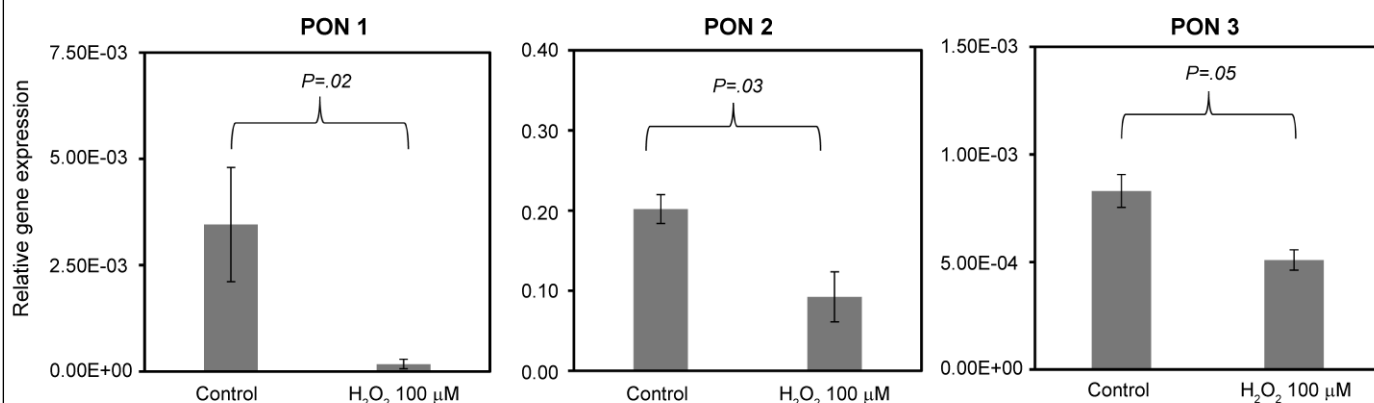
CD TI and SC respectively, 91 and 94 % in UC TI and SC respectively. PON 2 increased by 97 and 96 % in CD TI and SC respectively, 98 and 97 % in UC TI and SC respectively. PON 3 increased by 99% in both TI and SC of CD and UC (Figure 1B).

### CaCo-2 cell study

To test the hypothesis that oxidative stress caused the decreased PON expression in IBD, PON gene expression was examined in Caco-2 cells treated with  $H_2O_2$ . PON1, 2, and 3 gene expression is decreased in  $H_2O_2$  treated Caco-2 cells when compared to control cells by 95, 54, and 39% respectively ( $P < 0.05$ ) (Figure 2).

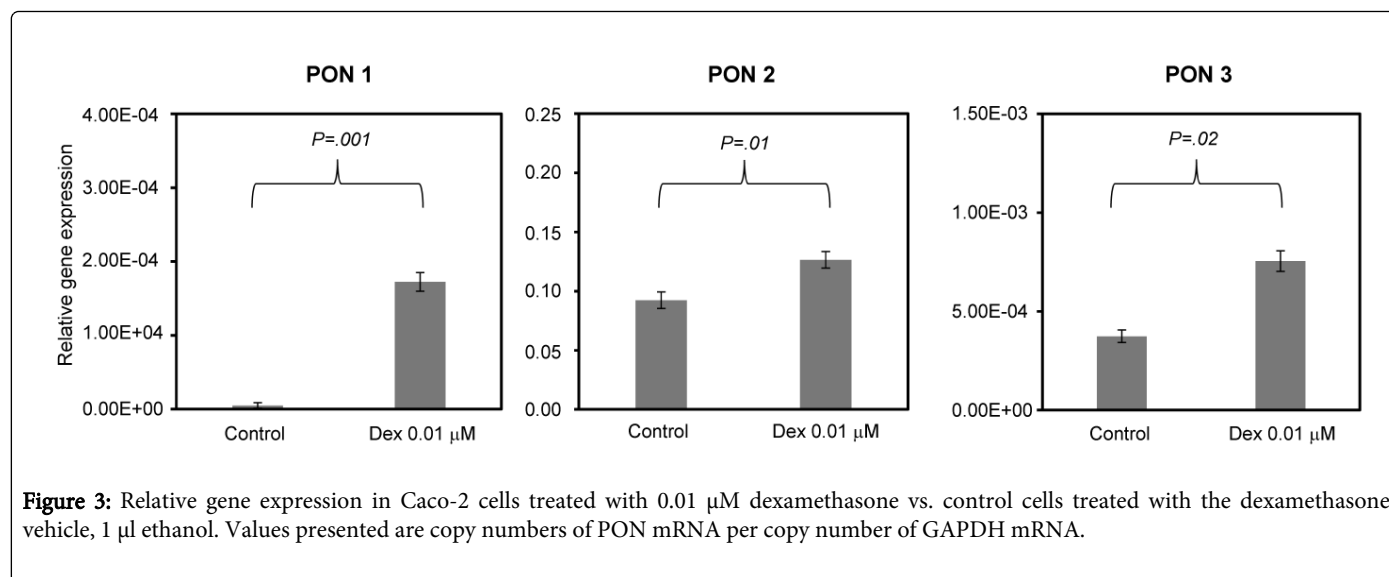


**Figure 1: A)** Relative expression of PON genes in medication naïve IBD patients when compared to controls. **B)** Relative expression of PON genes in IBD patients on steroids when compared to medication naïve IBD. Values presented are copy numbers of PON mRNA per copy number of GAPDH mRNA. \* Represents significant p value <0.05; MN: Medication Naïve; CD: Crohns Disease; UC: Ulcerative Colitis; TI: Terminal Ileum; SC: Sigmoid Colon



**Figure 2:** Relative expression of PON genes in Caco-2 cells treated with 100 µM H<sub>2</sub>O<sub>2</sub> vs. untreated cells. Values presented are copy numbers of PON mRNA per copy number of GAPDH mRNA.

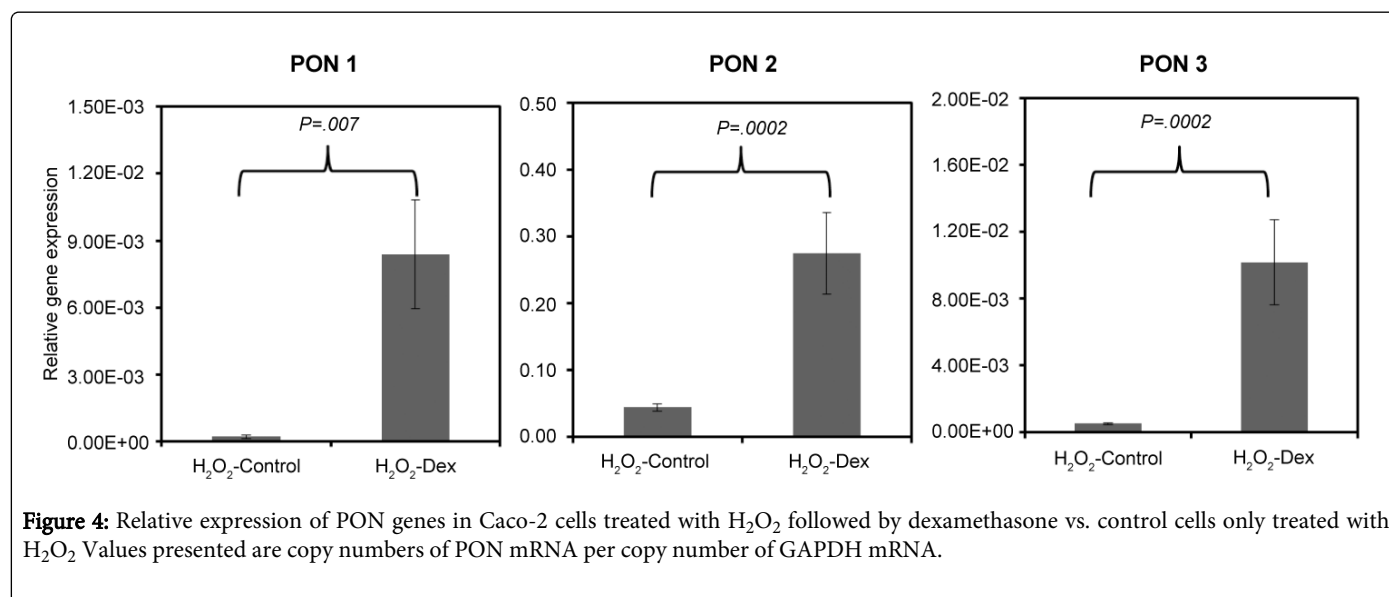
To confirm that steroids have a direct effect on PON gene expression, PON gene expression was examined in Caco-2 cells treated with dexamethasone. Similarly CaCo-2 cells incubated with 0.01 µM of dexamethasone had an increase in the relative gene expression of PON 1 by 97%, PON 2 by 27% and PON 3 by 50% when compared to untreated cells (Figure 3).



**Figure 3:** Relative gene expression in Caco-2 cells treated with 0.01 μM dexamethasone vs. control cells treated with the dexamethasone vehicle, 1 μl ethanol. Values presented are copy numbers of PON mRNA per copy number of GAPDH mRNA.

In order to mimic what happens in human intestinal tissue, the effect of steroids was studied in the presence of H<sub>2</sub>O<sub>2</sub> for 24 hours prior to adding the dexamethasone (HD). This experiment showed that dexamethasone increases PON 1, 2, and 3 gene expressions in

Caco-2 cells after being exposed to H<sub>2</sub>O<sub>2</sub> by 99, 66, and 96% respectively. Cells serving as controls with no dexamethasone added (HC) showed similar effects to adding H<sub>2</sub>O<sub>2</sub> alone for 24 hours (Figure 4).



**Figure 4:** Relative expression of PON genes in Caco-2 cells treated with H<sub>2</sub>O<sub>2</sub> followed by dexamethasone vs. control cells only treated with H<sub>2</sub>O<sub>2</sub>. Values presented are copy numbers of PON mRNA per copy number of GAPDH mRNA.

## Discussion

Our results showed that all PON genes are significantly down regulated in medication naïve IBD patients. Because PON genes encode for proteins that function as antioxidants, decreased PON expression might lead to a decrease in protection against oxidative stress, and thus play a role in the pathogenesis of IBD. Similarly, in a study of adult patients with IBD Boehm et al. found that PON1 protein activity was decreased in IBD patients when compared to controls [21]. Unopposed oxidative stress can lead to mucosal barrier damage which allows pathogens to invade the submucosa and initiate an immune cascade [8].

Unlike the adult study of Rothem et al., [22] which showed that only the TI in CD and colon in UC had decreased PON 1 and PON 3 gene expression and there was no difference on intestinal gene

expression of PON 2 in IBD when compared to controls, our results showed a significant decrease in the expression of all PON genes in medication naïve patients irrespective of the site of inflammation. This indicates that the decrease in gene expression is disease specific and not related to the presence of histological abnormalities.

A likely cause of the difference between gene expression in medication naïve IBD and normal controls is oxidative stress. As demonstrated with our CaCo-2 cells treated with H<sub>2</sub>O<sub>2</sub>, oxidative stress decreases the gene expression of all PON genes. Because the decrease in PON gene expression was observed in inflamed and non-inflamed tissue of IBD patients this may indicate that oxidative stress is an intrinsic characteristic of IBD, and may occur before any clinical or histological abnormalities.

We showed that after an oxidative stress, steroids can increase the expression of all PON genes *in vivo* and *in vitro*. Lim et al. showed that dexamethasone up regulates the expression of PON 2 mRNA in NIH3T3 cells [13]. Our study in children suggests that the expression of PON genes might contribute to the therapeutic effects of steroids. Further, targeting PON gene expression might lead to a novel treatment for IBD.

While two of our patients were on 5 ASA in addition to steroids, Boehm et al. [21] showed that 5 ASA had no effect on PON1 gene expression in IBD patients. However more studies are still warranted to look at the effect of other medications on PON gene expression in IBD.

Additional investigation is needed to elucidate the underlying mechanism of steroid up regulation for PON gene expression. One possibility is that PON genes are direct transcription targets that are up regulated by steroid binding to the glucocorticoid receptor (GR) [23].

A previous study showed variability among PON gene expression in normal intestinal epithelium [22]. Similarly we found a lower gene expression of PON 3 compared to other members of the PON family in intestinal epithelium from healthy children, yet all PON genes were expressed in all the intestinal tissues we obtained from the normal controls.

Although not investigated in this work, bacterial quorum sensing might be affected by PON genes. All PON genes encode for proteins that have lactonase activity [24,25], which acts to suppress bacterial quorum sensing. Quorum sensing is a signaling mechanism used by both gram positive and gram negative bacteria. It is required to control virulence gene expression in bacteria, influence the behavior of bacterial populations, and can also affect host responses to bacterial infections. IBD patients have decreased PON gene expression as we showed in this study, which suggests they lack an important protective mechanism against bacterial infection. This would allow bacteria (such as *E.coli*) to interact using quorum sensing mechanisms to develop more virulence and resistance to the host immune response [9,25,26].

A limitation of our study is the small number of patients, and that several factors can affect the PON gene expression. We focused on oxidative stress in the gastrointestinal tract. Future studies examine other factors such as infection or other inflammatory processes. Another future direction is to assess the protein levels and enzyme activity of PON genes in IBD patients and CaCo-2 cells and investigate their regulation by other medications used to treat IBD.

In conclusion, our data suggests that decreased PON expression in IBD patients is a consequence of oxidative stress which plays a role in the pathogenesis of IBD. Further, steroids counteract the effect of oxidative stress by up regulating PON gene expression. Interwoven in the pathways of oxidative stress, PON genes may be novel targets for the management of intestinal diseases like IBD.

## Acknowledgement

We thank Rajendra Rajnarayanan Ph.D. (SUNY Buffalo) for assisting in the dexamethasone experiment with Caco-2 cells.

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This article was originally published in a special issue, entitled: "**Inflammatory Bowel Disease**", Edited by Dr. Nancy Louis, Emory University, USA and Dr. Ostanin Dmitry Vladimirovich, Louisiana State University Health Sciences Center, USA