

# Gene Expression of Antioxidant Enzymes in the Resected Intestine in Crohn's Disease

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## ABSTRACT

**Introduction:** The inflammatory process in Crohn's disease (CD) is closely associated with the formation of reactive oxygen species. Antioxidant enzymes can play an important role in the outcome of CD and may influence postoperative recurrence in these patients. The aim of our study was to evaluate gene expression of intracellular antioxidant enzymes in surgically resected intestinal specimens of patients with CD, both in macroscopically normal and in inflamed tissue.

**Methods:** A total of 28 patients referred for elective bowel resection were enrolled in the study. Full-thickness small intestinal specimens were investigated. Gene expression of antioxidant enzymes – superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GSR) – was evaluated both in macroscopically normal and inflamed samples.

**Results:** There were significantly lower levels of SOD1 mRNA ( $p = 0.007$ ) and GSR mRNA ( $p = 0.027$ ) in inflamed tissue compared to macroscopically normal areas. No significant differences were found between affected and non-affected intestinal segments in mRNA for SOD2, SOD3 and GPX.

**Conclusions:** Our pilot data clearly showed that the gene expression of major antioxidant enzymes is not a uniform mechanism in the pathogenesis of Crohn's disease. Topically decreased gene expression of SOD1 and GSR might facilitate the segmental tissue injury caused by reactive oxygen species.

## KEYWORDS

Crohn's disease; gene expression; intestine; antioxidant enzymes; superoxide dismutase; glutathione peroxidase; glutathione reductase

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## INTRODUCTION

Inflammatory bowel disease (IBD) is comprised of two entities: ulcerative colitis and Crohn's disease (CD). Ulcerative colitis affects the large bowel only, whereas CD can involve any part of the gastrointestinal tract (most commonly the ileum and proximal colon). These diseases have somewhat different pathologic and clinical characteristics, but with substantial overlap. CD is characterized by transmural inflammation and by segmental involvement of the bowel. The transmural inflammation may lead to fibrosis and strictures, and to obstructive clinical presentations, and may also result in sinus tracts, giving rise to microporations and fistula formation (1, 2).

The pathogenesis of IBD still remains poorly understood. Several studies both in humans and in animal models suggest that genetically determined factors contribute to susceptibility to CD. However, only about 15 percent of those with CD have family history of IBD. The pathogenesis of CD results from dysregulated immune responses to luminal bacteria (and/or their products) and from the impact of various environmental factors (e.g., dietary factors, xenobiotics, smoking, drugs, oral contraceptives in women, and others). The components of immune response must be properly balanced. Either too strong or inadequate immune response to microbes in the intestinal lumen can ultimately result in intestinal inflammation (3).

Immune response in CD is a complex process, comprising dysregulated all innate-immune-, cytokine-, adaptive-immune-, epithelial-barrier- and microbial-clearance pathways. Inflammatory reaction, among others mediated by polymorphonuclear neutrophils, is associated with production of several products that can cause endothelial dysfunction and further worsen the structural intestinal injury. Reactive oxygen species (ROS) are key signalling molecules that play an important role in this process. Oxidative stress produced by polymorphonuclears leads to the opening of inter-endothelial junctions and promotes the migration of inflammatory cells across the endothelial barrier (4).

Antioxidant enzymes are proteins involved in the catalytic transformation of free radicals and reactive oxygen species and their by-products into stable nontoxic molecules. They represent an important defence mechanism against oxidative stress-induced cell damage. These enzyme systems include superoxide dismutase, glutathione peroxidase, glutathione reductase, lipolic acids, peroxiredoxins and catalases (5).

Although advances in medical therapy have been associated with decreased need for bowel resection in CD, surgical intervention is often required in the setting of bowel obstruction, abscesses or fistulas, or refractory disease. The 10-year risk of surgical resection for CD is nearly 50 percent (6).

Antioxidant enzymes may play an important role in the outcome of CD and may influence the postoperative recurrence in these patients. The aim of our current study was to evaluate gene expression of intracellular antioxidant enzymes in surgically resected intestinal specimens of patients with CD, both in macroscopically uninvolved and involved tissue.

## METHODS

### PATIENTS

A total of 28 individuals referred for bowel resection were included in the study. The group consisted of 12 males (mean age  $37 \pm 13$ ; 6/12 were smokers) and 16 females (mean age  $38 \pm 13$ ; 8/16 smokers).

### SURGICAL PROCEDURE

All patients were indicated for intestinal resection according to the decision of a multidisciplinary team. All patients had L3B2 disease according to the Montreal classification (7). No preoperative bowel preparation was performed. A prophylactic dose of antibiotics was administered (amoxicillin/clavulanate 1.2 g i.v., metronidazole 500 mg i.v.). Patients underwent laparoscopic or open ileocecal resection or right hemicolectomy with a hand-sutured side-to-side or end-to-end anastomosis. For each patient, a full-thickness sample from small intestine was taken from the resected specimen by a surgeon from a macroscopically uninvolved and affected area. Samples were immediately stored in separate vials with RNAprotect Tissue Reagent (QIAGEN, Hilde, Germany) and were ready for further processing.

### ANTIOXIDANT ENZYMES

Analysis of mRNA expression of major intracellular antioxidant enzymes was performed, both in macroscopically normal and in involved tissue. Analyses were carried out of superoxide dismutase (SOD) and its three isoforms - cytoplasmatic (SOD1), mitochondrial (SOD2) and extracellular (SOD3). Gene expression of glutathione peroxidase (GPX) and glutathione reductase (GSR) was also investigated.

### RNA ISOLATION AND QPCR

Tissue samples were homogenized using a Precellys 24 homogenizer (Bertin Instruments, Bretonneux, France). Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed using a cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Gene expression was quantified with TaqMan Gene Expression Assays (POLR2A Hs00172187\_m1, SOD1 HS\_00916176\_m1, SOD2 Hs\_00167309\_m1, SOD3 Hs\_00162090\_m1, GSR HS\_00167317\_m1, GPX3 HS\_01078668\_m1). Gene expression was analysed using a QuantStudio 6 real-time PCR system (all purchased from Applied Biosystems, Foster City, CA, USA). Results were normalized to POLR2A RNA expression. mRNA levels were calculated using a comparative Ct method ( $\Delta\Delta C_t$  method) (8).

### STATISTICAL ANALYSIS

Data had non-normal distribution and were tested by Mann-Whitney test and Kruskal-Wallis test. All statistics was performed using GraphPad Prism 8.0.1.244 (San Diego, CA, USA).

## ETHICS

The project was carried out according to the Declaration of Helsinki and was approved by the Ethics Committee of University Hospital Hradec Králové (protocol number 201706S12P). All participants signed an informed consent.

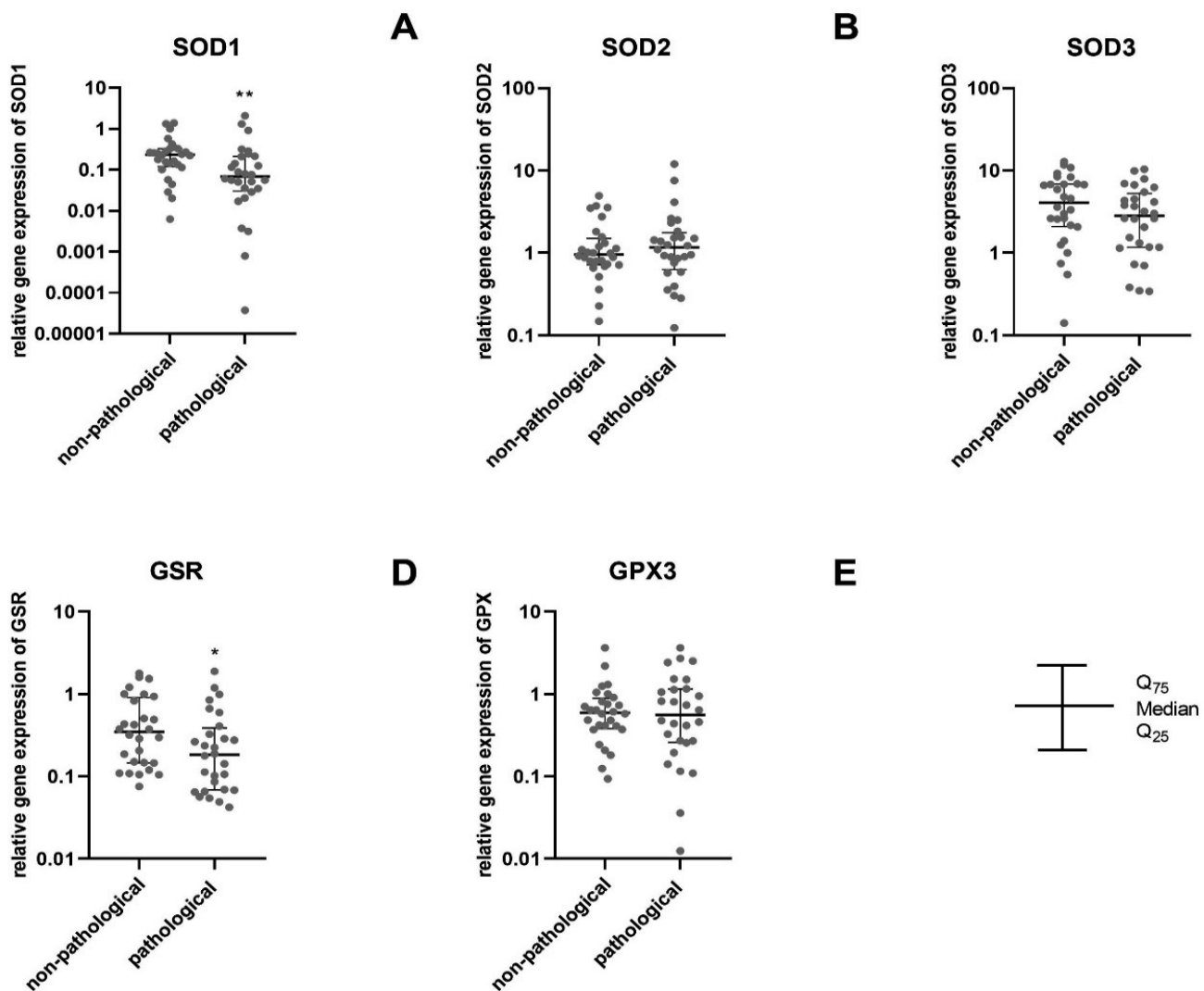
## RESULTS

There was a statistically significant difference between macroscopically non-pathological and pathological tissue in SOD1 mRNA level ( $p = 0.007$ ; Figure 1.A) and GSR mRNA level ( $p = 0.026$ ; Figure 1.D). No significant differences were found between macroscopically involved and non-involved intestinal samples in mRNA for SOD2 (Figure 1.B), SOD3 ( $p = 0.116$ ; type 2 error beta: 0.783; power of the performed test 0.217; Figure 1.C), or GPX (Figure 1.E).

The mRNA for each enzyme in the non-pathological and pathological tissue of the males group was compared to the mRNA for each enzyme in females group. No statistically significant differences in the mRNA levels were found between male and female non-pathological tissue

for SOD1 ( $p = 0.932$ ), SOD2 ( $p = 0.819$ ), SOD3 ( $p = 0.357$ ), GSR ( $p = 0.948$ ), or GPX ( $p = 0.765$ ). No statistical differences were found between male non-pathological and pathological tissue in mRNA for SOD1 ( $p = 0.981$ ), SOD2 ( $p = 0.999$ ), SOD3 ( $p = 0.995$ ), GSR ( $p = 0.893$ ), or GPX ( $p = 0.769$ ), nor were any differences identified between female non-pathological and pathological tissue in mRNA for SOD1 ( $p = 0.918$ ), SOD2 ( $p = 0.593$ ), SOD3 ( $p = 0.235$ ), GSR ( $p = 0.525$ ), or GPX ( $p = 0.340$ ).

The mRNA for each enzyme in the non-pathological and pathological tissue of the smokers was compared to the mRNA for each enzyme in non-smokers. No statistically significant differences in the mRNA levels were found in non-pathological tissue for SOD1 ( $p = 0.950$ ), SOD2 ( $p = 0.962$ ), SOD3 ( $p = 0.613$ ), GSR ( $p = 0.472$ ), GPX ( $p = 0.738$ ) between smokers and non-smokers. For non-pathological and pathological tissue in smokers, no statistical differences in mRNA levels for SOD1 ( $p = 0.999$ ), SOD2 ( $p = 0.944$ ), SOD3 ( $p = 0.240$ ), GSR ( $p = 0.995$ ), GPX ( $p = 0.993$ ) were identified, nor were there any differences between non-smokers non-pathological and pathological tissue in mRNA for SOD1 ( $p = 0.783$ ), SOD2 ( $p = 0.893$ ), SOD3 ( $p = 0.985$ ), GSR ( $p = 0.243$ ), GPX ( $p = 0.925$ ).



**Fig. 1** mRNA expression of SOD1 (A), SOD2 (B), SOD3 (C), GSR (D), GPX (E) in macroscopically non-pathological tissue ( $n = 28$ ) and macroscopically pathological tissue ( $n = 28$ ).

## DISCUSSION

Our study brought important insights into the role of gene expression of antioxidant enzymes in Crohn's disease (CD). From the analysis of pathologically altered and macroscopically uninvolved tissue samples, a statistically significant difference in mRNA level of SOD1 was found. A significantly reduced level of SOD1 was noted in the affected intestinal samples. Superoxide dismutase catalyses the disproportionation of superoxide radicals into  $H_2O_2$  and  $O_2$ . SOD1 binds copper and zinc ions and is one of three superoxide dismutases. SOD1 deficiency in the dextran-sodium sulphate (DSS)-induced mouse model of colitis resulted in severe oxidative stress with body weight loss, epithelial barrier disruption, and decreased antioxidant enzyme activities (9). In CD, lipid peroxidation was found to correlate positively with SOD1 (10). The remaining two isoforms of SOD in our study failed to show a statistically significant difference in mRNA levels. However, Kruidenier et al. (11) demonstrated increased SOD2 expression in their work. SOD2 transforms toxic superoxide to clear mitochondrial reactive oxygen species, thus protecting against cell death. Mulder et al. (12) measured the content of superoxide dismutase in intestinal resection specimens from patients with Crohn's disease and ulcerative colitis and compared the concentrations with those obtained in the normal mucosa of a control group of patients with colorectal cancer. The superoxide dismutase content was similar in control mucosa and non-inflamed mucosa from that in patients with inflammatory bowel disease but was decreased in inflamed mucosa. The decreased SOD3 protein level in inflamed tissue compared to non-inflamed has been described in other studies (11, 13).

Our study also demonstrated a statistically significant decrease in glutathione reductase (GSR) mRNA levels. This enzyme regenerates the glutathione (GSH). GSR plays a key role in protecting cells from oxidative damage, it catalyses the reduction of  $H_2O_2$  by GSH into  $H_2O$  and glutathione disulphide (GSSG). The inflamed ileum of patients with CD is characterized both by an increase of GSSG and decrease of GSH (14). The inflamed ileum in CD is not able to eliminate GSSG, probably due to a diminished GSR activity. Our results showed that such decrease is already evident at the gene level.

Glutathione peroxidase (GPX) was another enzyme whose gene expression was analysed in our study. GPX is involved in the protection of cells from damaging effects of reactive forms of oxygen. It follows the action of SOD. It catalyses the decomposition of  $H_2O_2$  into water and oxygen (15). Our study did not confirm differences between inflamed and non-inflamed tissue in gene expression of GPX.

We have also investigated the possible influence of gender and smoking on the gene expression of antioxidant enzymes. Sex hormones, e.g. oestrogen, are thought to be associated with risk of IBD as variations in disease severity occur during pregnancy, menopause, or oral contraceptive use (16, 17). Unfer et al. (18) showed that serum oestrogen and progesterone levels positively correlated with blood SOD1 and SOD3 activity. Strehlow et al. (19) revealed that expression as well as activity of SOD2 and SOD3 are enhanced by oestrogens by transcriptional pathways, while

GPX is not altered. In our study, we found no differences between the genders in gene expression of antioxidant enzymes.

Cigarette smoking in CD is put into context with accelerated disease, worse nutritional status, increased need for medical therapy (including biologics), and increased risk of recurrence following surgery, as well as a higher risk of postoperative complications (20–22). Contrarily, smoking has a protective effect for ulcerative colitis, and smokers are less likely to require colectomy (23, 24). However, our results showed no differences between smokers and non-smokers in CD, both in macroscopically normal and pathological tissue.

There is an increasing interest in miRNAs and exploring their possible role in the pathogenesis of CD. Current studies indicate that miRNA expression can be sensitive to the presence of intracellular  $H_2O_2$  levels (25–27). Epigenetic regulation at the DNA level is an important mechanism involved in  $H_2O_2$ -mediated expression changes of multiple genes, indicating that miRNA expression is very sensitive to  $H_2O_2$  stimulation. For example, in smooth muscle cells, cellular treatment with hydrogen peroxide resulted in an upregulation of microRNA-21 (10).

We are aware of possible limits of our study. We did not correlate our results with the histology of resected specimens (and tissue inflammatory grading). Due to the limited number of patients, we were not able to assess our data with respect to preoperative medical therapy. And last but not least, we did not evaluate other factors that can influence the gene expression of antioxidant enzymes and thus might create confounders. Nevertheless, our pilot data is an important basis for future research of this important topic.

## CONCLUSIONS

Our pilot data clearly showed that the gene expression of major antioxidant enzymes is not a uniform mechanism in the pathogenesis of Crohn's disease. Topically decreased gene expression of SOD1 and GSR might facilitate the segmental tissue injury caused by reactive oxygen species.

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