

Review

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Review

# Enzymatic Oxidants, Antioxidants, and Inflammatory Bowel Disease

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**Abstract:** The role of oxidants and antioxidants in inflammatory bowel disease (IBD) have been actively explored since the early 1980s, starting with the role of the respiratory burst of neutrophils and ischemia in bowel pathology. Since that time the enzymatic components contributing to the pool of reactive oxygen species including , superoxide,  $H_2O_2$  and lipid hydroperoxides and the counteracting antioxidants, catalase, glutathione peroxidases (GPX), peroxiredoxins (PRDX), superoxide dismutases and others have been fleshed out. My perspective on IBD is from the role of the balance or imbalance of enzymatic oxidant sources and enzymatic antioxidants in the inflammatory process. I will present evidence on the involvement of oxidant and antioxidant processes in IBD, based as much as possible, on my experiences with GPXs. This will be about both the immune system and local bowel oxidant and antioxidant systems. As GPXs are generally selenium-dependent, possible deficiencies in selenium uptake in active IBD and the impact on GPX expression is explored. The more recently introduced ferroptosis, an iron-dependent lipid peroxidation based pathological process, will be reviewed for its possible involvement in IBD.

**Keywords:** Inflammatory bowel disease; GPX1-4; PRDX; NOX1; DUOX2; ileum; colon; immune system

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## 1. Introduction

This discussion is not intended as an exhaustive foray into the area of oxidants, antioxidants, and inflammatory bowel diseases (IBD). With the exception of ferroptosis I am limiting it as much as possible to my experiences, which are largely constrained to studies of rodent tissues and human cancer-derived cell lines (GPX4 for this discussion), the consequences of eliminating oxidant and antioxidant enzyme expression, involving selenium-dependent glutathione peroxidases 1-4 (GPX), NADPH oxidases, NOX1 and DUOX2, and some limited work with superoxide dismutases (SOD)1 and 2 and catalase. Use of drug interventions in mice were intended to broaden the scope of our work to include lipoxygenases (ALOX), mitochondria, and xanthine oxidase (XO) as oxidant sources [1,2]. I will be not commenting on antioxidant supplements (selenium as an exception; trace elements copper, zinc and manganese will be briefly mentioned as components of superoxide dismutases), as these have consistently disappointed in large epidemiological and controlled studies and in some work, I have performed [1–5]. I am not a clinician nor an MD, so my knowledge of IBD comes from contact with co-workers who had IBD, what I have picked up from meetings where clinicians were present and from my reading. Briefly, Crohn's disease (CD) and ulcerative colitis (UC) are chronic idiopathic inflammatory bowel disorders with periods of active illness followed by periods of remission. UC is confined to the large intestine, generally limited to the mucosa, while CD can impact any region of the alimentary tract with involvement of all layers and occasionally manifesting as inflammation of skin, eyes and joints, liver, or bile ducts. I refer the readers to the following papers for a superior discussion of the clinical features of IBD then I can supply [6,7]. IBD is referred to as an autoimmune disorder and there is a demonstrable genetic predisposition leading to active disease (NOD2 in CD as a notable genetic component) with stressors found to contribute such as infections

or diet [8–11]. Gut microbe dysbiosis is thought to be a major component of IBD [12]. IBD cannot be cured, only managed, and the life span of sufferers may be slightly less than non-sufferers, with an increased risk for colon cancer due to the chronic inflammation [13,14]. In my limited contact with people who had IBD, I observed that they functioned productively, however, the condition could severely impact daily life, consistent with more general findings [15]. The enigma of IBD and a major concern is its increasing incidence in countries with lower socio-demographic indexes, where once it was negligible [16,17].

## 2. Enzymatic Oxidants, Antioxidants, and Inflammatory Bowel Disease

Suggestions for the involvement of oxidant and antioxidant enzymes in IBD and the use of antioxidant enzymes as therapy lagged 1–2 decades behind discovery of superoxide dismutase (SOD) and selenium-dependent glutathione peroxidase (GPX), gaining ground in the 1980's (catalase by 80 years; named in 1900) [18–26]. The first mention of GPX in reference to IBD involves selenium deficiency in CD subjects in 1984, following 12 years behind the discovery that GPX1 is a selenoprotein [27,28]. Selenium deficiency in CD was noted 1 year earlier (3 years, anecdotally), without relating it to GPX [29]. Likewise, trace elements involved in SOD function, copper and zinc, components of SOD1, and manganese, component of SOD2, have altered levels in IBD, zinc and manganese less, copper possibly elevated. This impact on trace element levels has been suggested to link IBD to antioxidant enzyme malfunction [19,27,30–37]. Iron deficiency is also part of this pattern and actions to treat this have been suggested to occasionally backfire, although most evidence for adverse effects come from animal studies with chemically induced colon pathology [38].

## 3. The Main Players

There seems to be a large gap until the roles of another major antioxidant family, peroxiredoxins (PRDX), are discussed for in IBD, possibly 2010 [39]. PRDXs are thioredoxin-dependent peroxidases (PRDX6 exception, GSH-dependent). The gap may be due to the strong pathology found in *Prdx1*-knockout (KO) mice, that “produced pathology of hemolytic anemia and several malignant cancers, including lymphomas, sarcomas, and carcinomas”, but no ileocolitis, and in *Prdx2*-KO mice yielded “hemolytic anemia, splenomegaly, Heinz body formation, and morphologically abnormal red blood cells”, but no ileocolitis [40]. At the cellular level, loss of either PRDX1 or PRDX2 is not consistently impactful, although simultaneous loss does yield a noticeable effect on cell line oxidant levels [41–44]. The lack of IBD in the single KOs is not conclusive for lack of a role and possible counter-balancing roles in the immune system, shared with *Gpx1*, seems to offset any deficiency in local antioxidant protection in the ileum and colon [45]. Single KOs of none of the named antioxidant enzyme families produces spontaneous IBD in rodents. *Sod2*-KO is a neonatal lethal condition in mice. We looked at intestine samples from long-lived *Sod2*-KO mice (mitochondrial isoenzyme; on some strain backgrounds mice lived to 19 days) and observed nothing [46]. There are no reports of IBD like issues by use of heterozygous mice or an intestine specific *Sod2*-KO [47,48]. By way of introduction, superoxide is a radical species, the one electron addition product of oxygen, generated in several processes, but largely by electron leakage from the electron transport system of mitochondria [49]. As such, there is a dedicated mitochondrial SOD, SOD2 with manganese in the active site [50,51]. SOD1 is the cytosolic, zinc/copper active site, version. Superoxide can be damaging, largely by reactions with iron [52]. Superoxide is converted into H<sub>2</sub>O<sub>2</sub>, which can act as an antimicrobial agent as is (a pathway usually downstream of NADPH oxidase, NOX2), based on its damaging properties, or after additional reactions with hypohalous acids or thiocyanate that yield more potent antimicrobial species [53]. It is also a signaling agent, able to react with free cysteine residues of proteins to induce activation, inactivation, or structural changes [54]. PRDXs are a family of widely expressed, generally abundant peroxidases, collectively found in all cell compartments and proposed to consume the bulk (90%) of hydroperoxides generated in cells. This is based on high rate constants and abundance [55]. While all are expressed in the ileum and colon, reports on KOs or rodents subject to knock-down of expression and chemically induced colitis largely showed less pathology, an effect often attributed to effects on the immune system (summarized in ref. 45) [45].

This greater impact on the immune system with chemically induced colitis was also found with a Gpx1-KO (and catalase-KO) mouse line and was also revealed (Gpx1-KO only) in a study of different responses of Gpx1-/Gpx2+/+ and Gpx1+/+Gpx2-/ mice to allergen-induced airway inflammation (OVA and aluminum hydroxide asthma) [56–58]. GPX2 protects the lung from this condition, with Gpx2-KO mice having worse asthma, while lack of GPX1 mitigated the allergic response by suppressing Th2 and Th17 cell development. *Gpx2* is not expressed in the immune system at significant levels.

One of the common suggestions dating back to 1984 is that impaired selenium uptake, associated with IBD, could constrain the levels of GPX1 and later GPX2, GPX3 and GPX4, all expressed in the ileum and colon [59]. The selenium-dependency of GPX1-4 and GPX6 is based on the co-translational incorporation of selenocysteine into the active site, giving the enzymes the capacity to operate efficiently at physiological pH (pKa ~5.2), although there are workarounds for cysteine (pKa > 8.4, thiolate anion is active form) centered peroxidases (PRDXs) that allow comparatively high ROOH rate constants (ROOH; H<sub>2</sub>O<sub>2</sub>, tert-butyl hydroperoxide, cumene hydroperoxide, linoleic acid hydroperoxide, etc.) [60]. Selenoprotein levels can reflect selenium intake when it is restricted, GPX1 more notably, but all are ultimately vulnerable [61–63]. This is noteworthy since the standard use of 10% serum in cell culture places most cell lines in the range of at least marginal selenium-depletion relative to tissues of selenium-sufficient subjects [61,63]. GPX4, the isoenzyme involved in iron, cystine, glutamine and lipid peroxidation-related cell death, ferroptosis, is as vulnerable and this might be a factor in studies using cell lines [64]. *Gpx2* is highly expressed in the epithelium of the mid-lower GI tract, and this is where we found its greatest impact in mice [46,65–67]. The study of Connie Eaves suggested that basal cell compartments, which would include the crypt/gland regions of the mid-to-lower GI tract, reverse the standard PRDX dominance (90% of ROOH reduction) in favor of GPX2 prominence for ROOH metabolism [55,68]. We found high levels of GPX2 more concentrated in the crypt/gland regions in rats [69]. GPXs1-4 are all expressed in the GI tract, with GPX2 confined to the epithelium, GPX1 and GPX4 in all layers and GPX3 in the mature absorptive cells and adherent to the basal lateral membrane [70,71].

### 3. Finding Overlapping Roles of GPX1 and GPX2 in the Intestine by Peeling Back the Layers of Antioxidants

Single KOs of Gpx1 and Gpx2 did not reveal obvious GI pathology. Our initial attempts to find a function for GPX2 using chemically induced colitis in wild type and Gpx2-KO mice did not turn up anything dramatic, so we turned to gamma-irradiation as a stress based on old, proposed links between GPX activity and prostaglandin levels and, at that time, a recent study showing the impact of Cox1 post-irradiation expression [72]. Whole body gamma-irradiation of mice resulted in an increase in the ileum epithelium GPX activity in wild-type mice, and GPX2 activity in Gpx1-/Gpx2+/+ mice, but not GPX1 activity (Gpx1+/+Gpx2-/ mice), and not in the jejunum [66]. The magnitude of the increase in the mRNA and the timing, post-irradiation, mimicked cyclooxygenase 1 (COX1; *PTGS1* gene) expression and PGE2 production in a prior study, upon which our study was modeled [72]. We were unable to tie PGE2 levels in the irradiated ileum epithelium to GPX activity levels with statistical significance, possibly by looking at Gpx1-/Gpx2+/+ vs. wild-type instead of Gpx1+/+Gpx2-/ mice. Later work would link GPX2 to expression with suppression of COX2 expression and older literature consistently associated COX activity with low GPX activity [73–76]. Finally, we did not identify any protective effect of GPX2.

Given the co-expression of *Gpx1* and *Gpx2* in the gut, it was natural to test the impact of combining the Gpx1-KO and Gpx2-KO constructs to look for effects. We were aware of PRDXs and their suggested roles, although at that time the rate constants were estimated to be fractional of GPXs and inactivation of PRDXs under ROS stress, actively built into eukaryotic PRDX structure, was still widely discussed [77,78]. We included *Prdx6* (*Aop2*) in our irradiation study. This knowledge and the still live debate over GPX and catalase for a greater role in protection of hemoglobin from oxidation meant that our estimation of the odds of finding an impact by combining the Gpx-KOs was 1 in 3 to 1 in 4 [79]. My experience with the main topic is largely limited to studies with mice lacking *Gpx1*

and *Gpx2*, in various combinations, *Gpx1+/+Gpx2-/-*, *Gpx1-/-Gpx2+/+*, *Gpx1+/-Gpx2-/-*, *Gpx1-/-Gpx2+/-* and *Gpx1-/-Gpx2-/-* [46]. The final combination, *Gpx1/2*-double knockout mice (*Gpx1/2*-DKO; mixed B6, 129 strain mice), resulted in spontaneous ileocolitis with high penetrance, the colitis onset prior to weaning and the ileitis peri- and post-weaning (21 days). This was also found at moderate penetrance in *GPX1+/-Gpx2-/-* mice and was largely suppressed in *GPX1-/-Gpx2+/-* mice. The spontaneous inflammation in *Gpx1/2*-DKO mice arises pretty much where *Gpx2* expression is detected at appreciable levels, beginning at the junction of the jejunum and ileum and continuing to the rectum, and later tumors arise in the ileum and colon from the chronic inflammation (microadenoma onset at 3 months, resulting in distress to some mice by 6 months) [46,80]. The shift in the handling of the oxidant load from peroxiredoxins to *GPX2* must occur at this point in the small intestine and continue into the colon. While we have conjectured this might be related to levels and types of microbes, it could also represent something related to the function of the ileum.

The condition is unique in that the pathology manifested as excess crypt/gland base (ileum/colon) apoptosis and the likely source of inflammatory pathology, crypt/gland base anoikis [1]. The apparent initiation of the pathology from within the crypt/gland epithelial cells coupled with lack of strong inflammatory infiltration in the mice confounded some pathologists we worked with, one of whom suggested on first examination of the histology that the pathology represented graft-versus-host disease. The scale of the apoptosis and the mixed nature of the abscesses (many exfoliated epithelial cells only, some mixed, few neutrophils only; IBD is dominated by neutrophils only and mixed) contributed to this impression. We eventually performed an MPO/cleaved caspase-3 differential analysis to confirm the epithelial cell only, mixed and neutrophil only calls from H&E, as recommended [81,82]. Anoikis or exfoliation/extrusion/shedding of intestinal cells occurs at the lumen as the terminal phase of enterocyte life [83]. It is a protracted process that is leaky to lumen components and excess anoikis has been linked to recurrence of Crohn's disease [84,85]. While we provided no formal evidence of this, the correlation of ileitis timing and severity with crypt anoikis was very strong. Apoptosis, in the absence of anoikis, as found in *Gpx1/2*-Duoxa-triple knockout mice, did not result promote formation of neutrophil only and mixed crypt abscesses, a characteristic inflammatory pathology of the *Gpx1/2*-DKO mice [1,46].

#### 4. Any application to IBD?

In a phrase, the outcome with *Gpx1/2*-DKO mice has little to do with IBD, although some suggestions do arise from the findings. *Gpx1/2*-DKO mice are not a model of anything based on the classical definition. They represent the fact of *GPX1/2* loss in the GI tract of mice and the simultaneous loss of *GPX1* activity in the immune system, which is not enough to turn the tide away from ileocolitis; loss of local protection trumps impact on immune function. As artificial as it was, this peeling back of multiple layers of antioxidant protection allowed unveiling of major sources of oxidants in the ileum and colon, showed that PRDXs do not dominate ROOH metabolism in all tissue compartments and allowed some estimation of the relative impact of *GPX1* and *GPX2*. Since this was eventually connected to the functioning of *NOX1* and *DUOX2* in the intestine, it would be a real demonstration of oxidant and antioxidant balance in the ileum and colon and an extreme example of the result of loss of that balance required for homeostasis. The one possible connection to IBD is the dramatically increased *DUOX2* expression levels [1]. However, use of other KOs results in uncovering other oxidant sources and other pathways to IBD [19,45,58]. The problem with *Gpx1/2*-DKO mice is that *GPX1* and *GPX2* levels are suppressed too much to be model of the impact of low selenium intake in IBD subjects and at the same time do not account for the simultaneous suppression of the other 23 (mice) or 24 (human) selenoproteins, *GPX4* in particular, with its link to ferroptosis [62]. The better model for this is dextran sodium sulfate (DSS) induced colitis after selenium deprivation of mice, although this yielded mixed outcomes in practice [30,31].

#### 5. Low Selenium Levels in IBD

The low selenium link in IBD remains a suggestion to me, as the degree to which impairment of selenium uptake would have to occur to approximate the GPX levels of *Gpx1/2*-KO mice and even

Gpx1-/Gpx2+/- mice with 1/4 the activity of wildtype and still completely protective, would be unapproachable. More telling, we selenium-depleted young Gpx1+/-Gpx2/- and Gpx1-/Gpx2+/- mice, so that the ileum epithelial GPX activity was 6-12% of the selenium sufficient controls, also Gpx1+/-Gpx2/- and Gpx1-/Gpx2+/- mice, finding the Gpx1+/-Gpx2/- mice developed pathology while the Gpx1-/Gpx2+/- mice did not [86]. One study shows GPX activity (upper colon?) in CD subjects to be about 1/2 the levels relative to healthy controls [87]. In line with this, plasma selenium levels in an IBD cohort were down to 75 ng/ml in one study and 60 ng/ml in two others, with a small number (3 of 54 subjects in one study and 1 subject of 30 in another) in the 40-50 ng/ml range after stratification into the worst clinical disease index category, relative to ~95 ng/ml as adequate [88-90]. Meta analysis found IBD subjects had between 61% and 100% the plasma selenium levels of controls [91]. Even the worst subjects had levels above that reported for the populations of China that experienced Keshan disease, an endemic cardiomyopathy (~20 ng/ml, resulting in blood plasma GPX activity 33- 43% of sufficient subjects [92,93]. IBD was not reported as a symptom of Keshan disease, recognizing that low selenium would not be a root cause of IBD only a potentially aggravating factor. In Keshan disease coxsackie virus is thought to be involved as a co-factor [94,95]. Anecdotal evidence for 5 pediatric subjects on parenteral nutrition for intestinal pathology during a shortage of selenious acid for 3 months showed no increased signs of pathology, although serum selenium levels dropped down to Keshan disease levels [96].

Notwithstanding, there is evidence from mouse models of DSS-induced colon injury that selenium supra-supplementation might be beneficial, 30 papers found using search terms, selenium, DSS, and mice, although lately the focus has shifted to GPX4 and ferroptosis. There is a study with DSS treated mice showing that selenium deficiency (0.01 ppm vs 0.1 adequate; torula yeast base; ref. AIN-76a, 0.11 ppm; AIN-93, 0.15 ppm) did not significantly worsen the pathology while supra-selenium supplementation (0.5 ppm) augmented colon GPX1/2 protein levels, and this possibly contributed to reduced pathology [97,98]. This study also found that COX2 was repressed and COX1 was elevated. A second study on selenium deprivation in mice with DSS (basal diet contained less than 0.01 mg Se per kg; Se-deficient; torula yeast base; the Se-sufficient diet was the same diet supplemented with 0.25 mg Se as sodium selenite per kg) did find a major worsening in pathology [30]. The discrepancy might be due to the second paper using a selenium level greater than standard for the selenium sufficient group. The blood plasma GPX activity levels were 10-fold lower in the selenium deficient group, more than difference in levels between selenium sufficient and deficient human subjects in Keshan disease studies. Another paper found that high selenium (oral gavage, daily) impacted T cells, fewer Th1/Th17 cells more Tregs, and lowered pathology levels [31]. Similar impact of selenium supplementation lessening DSS pathology levels is found in other papers [99,100].

Low zinc and manganese levels produced worse outcomes in the DSS model [36,101].

As a rule, selenium supplementation is most effective when the subjects are initially selenium-deficient, and risks may outweigh benefits by trying to increase levels in selenium adequate subjects [102]. The results from rodent studies indicate that supra-supplementation might be effective in IBD subjects balancing out the poor selenium uptake and even possibly yielding higher expression of GPXs than in the selenium adequate condition. Clinical trials are lacking or so limited in covering disease signs as to provide little useful information [103-105].

## 6. NADPH Oxidases and Pathology in Gpx1/2-DKO Mice and Normal Function in Wild Type Mice

The ileocolitis in Gpx1/2-DKO mice was linked to expression of both *Nox1* and *Duox2*, demonstrated in triple KO mice, Gpx1/2-Nox1-TKO, and Gpx1/2-Duoxa-TKO, where complete absence of disease was found in the former and the inflammation and anoikis were absent and the apoptosis was retained in the latter [1,82]. The NOX1 connection makes sense, as it is expressed in the crypt region and in human tissues shares high expression in colon tissues with GPX2 [106-109]. DUOX2 is normally found in the lumen region and this localization may be preserved in the Gpx1/2-DKO mice, no change in expression levels with pathology progression. Our examination of human IBD tissue showed that the range of DUOX2 protein expression was expanded into the gland base

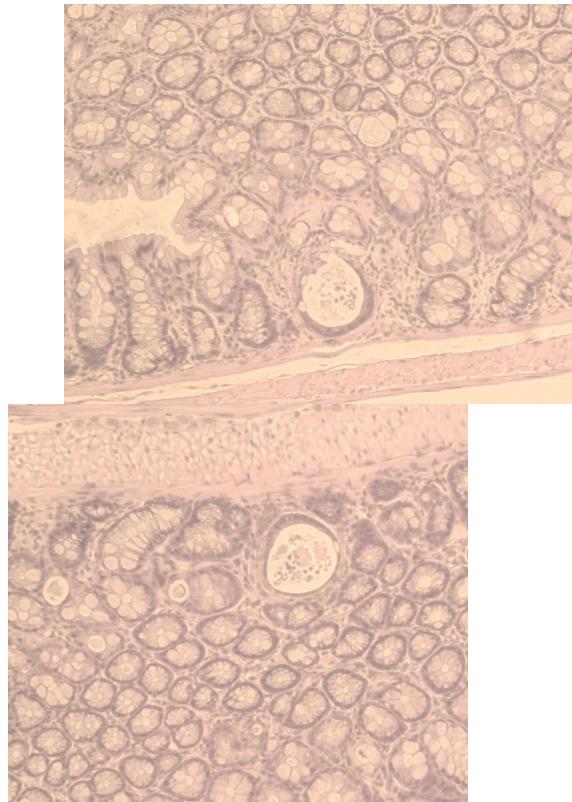
region [1]. This could explain part of the generally reported up-regulation of *DUOX2* mRNA expression in IBD samples [110,111]. A recent paper describes expansion of a *LCN2*, *NOS2*, and *DUOX2* expressing cell type (LND) in Crohn's disease (CD) that interacts with inflammatory cells [112]. Rare in non-IBD tissues, they are detected as almost 20% of total epithelial cells in active CD; this may be a partial replication our finding. The *LCN2* component is linked to ferroptosis by involvement with iron levels [113]. In mice, lumen constrained *DUOX2* could transmit oxidant signals to the crypt by way of a proposed cell-to-cell transmission mechanism; direct diffusion of H<sub>2</sub>O<sub>2</sub> from the lumen to the crypt/glands is considered unlikely [114]. The ability of GPX1 to limit ileocolitis in mice of the *Gpx1*+/−*Gpx2*−/− and more so in the *Gpx1*+/+*Gpx2*−/− genotypes, suggests some damping of the *DUOX2* oxidant signaling could occur via this proposed cell-to-cell transmission mechanism with *Gpx1* expression.

My take on this is very one sided, limited to what occurs when *DUOX2* and *NOX1* operate in the absence of the required antioxidant protection; there are enough oxidants generated to initiate apoptosis and the related anoikis. While overactive oxidant generation by *NOX*s, including *NOX2*, respiratory burst *NOX*, has been discussed as a factor in IBD, the flip side has gained traction, with chronic granulomatous disease and an associated IBD, caused by *NOX2* deficiency, the classic example [115,116]. The absence of a detected role for *NOX2* in *Gpx1*/2-DKO mice pathology (indicated by absence of pathology progression beyond excess apoptosis in *Gpx1*/2-*Duoxa*-TKO mice and general absence of pathology in *Gpx1*/2-*Nox1*-TKO mice) may be due to the timeframe over which we generally examined the pathology (birth- (colon) or ileum 21 days to 35-40 days of age) and milder early onset colon pathology in mixed strain and B6 mice. Here only NADPH oxidases operating in the pre-onset and peri-onset ileum pathology would be influencing the processes. Infiltration of possible *Nox2* expressing cells was not detected until 27-28 days of age in B6 mice (where we have an extensive timeline of events), rare neutrophil bearing abscesses observed at 28 days and higher levels of infiltration in the mucosa and crypt abscesses occurring at and after 30 days of age [1,2]. Neutrophil bearing abscesses, while a consistent feature, were never abundant in the B6 strain ileum. Direct testing in *Nox2*-KO mice showed mixed outcomes with DSS, worse outcomes in four studies vs. one no change and one less pathology [117,118]. A currently promoted view is that impairment of *NOX1* and *DUOX2* by congenital mutations are involved in very-early onset IBD and defects in *NOX2* mimicking CD [119,120]. Although, strong support for very-early onset IBD and *NOX1* was not backed up by another study [121]. *DUOX2* mutations are linked to congenital hypothyroidism. While the condition is rare (1/2000-4000), up to 40% of cases show mutation in the *DUOX2* gene [122]. The over-expression of *DUOX2* observed with later onset IBD would represent a possible opposing side of the impact [110,111]. However, with the association to the LND cell type, the impact in IBD is not clear. *NOX1* levels do not increase to the same degree as *DUOX2* in later onset IBD and the differences often do not approach statistical significance. The reverse pattern was found in *Gpx1*/2-DKO mice.

*NOX1* function in the lower GI may be related to induction of active cycling of quiescent stem cells and distribution of cells between the secretory and proliferative roles [123-125]. Under-expression seems to limit the ability to repopulated damaged areas of the mid-to -upper crypt or even higher areas [126,127]. This is somewhat at variance with our experience of mitigation of pathology and return to normalcy in the triple knockout, suggesting a more subtle role for *NOX1* that can almost escape notice or is somehow compensated by the *Gpx2*-KO condition. In *Gpx2*-KO mice, a similar subtle redistribution of proliferating cells seems to occur, observed as reduced levels of *Lgr5* [128]. Two studies, one very recent and both focusing on the ileum, suggest that lack of *NOX1* activity may limit the generation of peroxy nitrite, the product of superoxide and nitric oxide from nitric oxide synthase (NOS) [129,130]. Knaus et al note that the lack of peroxy nitrite seems to increase the exposure of the epithelium to bacterial antigens [130]. GPXs could protect from the direct tissue damage of this process by reacting with peroxy nitrite [131]. Absence of *NOX1* in the antimicrobial role in C57B6 strain mice may be unnoticed due to the functioning Paneth cells and for animal resource centers housed mice, absence of pathogens. We received *Nox1*-KO mouse colon tissue slides (H&E

stained) from another facility and noted some scattered focal epithelial cell loss and subsequent restitution (Fig. 1). Our animal facility would not accept the mice for presence of pathogens.

This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.



**Figure 1.** Nox1-KO mouse colon exhibiting some pathology.

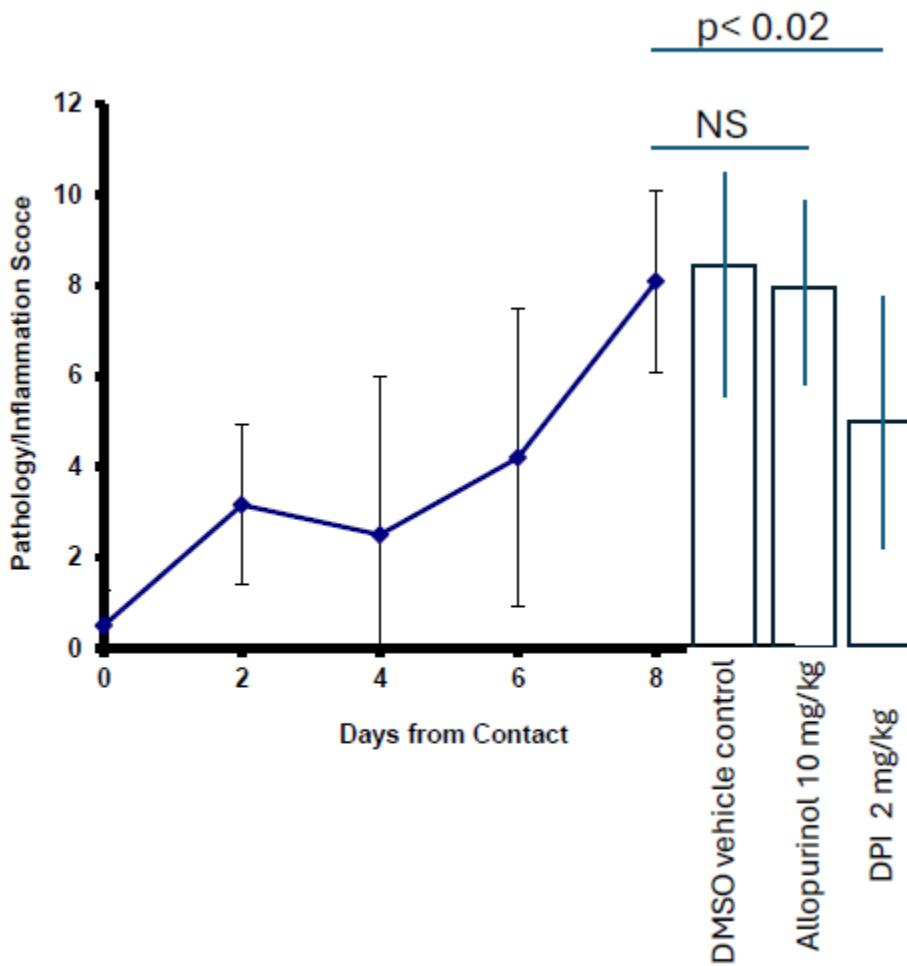
On the negative side, Dixon et al, suggest that NOX1 would participate in ferroptosis by reacting with Fe<sup>+++</sup> to yield Fe<sup>++</sup> that would be freed from storage to participate in lipid peroxidation [132]. It was clear from the study of Gpx1/2-Duoxa-TKO mice that cells were undergoing apoptosis under the stress of NOX1 function, by absence of crypt abscesses and absence of excess monocyte infiltration. Ferroptosis is inherently inflammatory. Another suggestion for the role of NOX1 is depletion of GSH by consuming NADPH [133]. While evidence of increased iron is shown in human IBD samples and DSS-colitis samples, the demonstration of GSH depletion was found in CACO2 cells with TNF- $\alpha$  and IL1- $\beta$  treatment. TNF- $\alpha$  and IL1- $\beta$  levels were substantially elevated in the colon of DSS treated mice. This markedly increases the level of NOX1 protein in the cells, which are at moderate to high levels to begin with in CACO2 relative to most COAD-derived cell lines [109]. The reduction in GSH levels was about 30%. While significant, it is not clear that this would compromise GPX4 and PRDX6 activity. Loss of viability in CACO2 cells seemed to require the added stimulation of hepcidin, which in the presence of TNF- $\alpha$  and IL1- $\beta$ , increased cellular iron stores and lipid peroxidation. Some controls are not presented so the actual impact of hepcidin cannot be fully evaluated. The link between NOX1 and GSH has been examined before, with the opposite outcome [134].

DUOX2's role would seem to be at least a moderate interaction with the microbiota. While H<sub>2</sub>O<sub>2</sub> alone could have some impact on the microbiota, it would be a substrate for lactoperoxidase, generating the more potent hypothiocyanite and other hypohalous acids [53]. Based on our take on the impact of knocking out Duox2 expression in Gpx1/2-DKO mice, we were more interested in the up to 25 or more-fold increase in DUOX2/DUOXA2 levels in IBD and the possible damaging impact on the tissue. The finding of an expanded population of LND cells as at least one reason for the indicated increase in DUOX2 expression in IBD complicates our simple notion, suggesting that the

increased expression is linked to a package of alterations to enterocytes (one proposed source of LND cells) which have an augmented interaction with immune cells. The specific role of DUOX2, if any, is unclear, and the authors of the LND paper make no comments about this other than to point out that under stress, ileum and colon cells tend to express genes of other lineages [111,112,135]. Grasberger and associates suggested that up-regulation of Duox2 in mice germ-free mice exposed to bacteria was a response to dysbiosis and expression did not sustainably alter the redox status of the mouse epithelium defined as no changes in antioxidant enzyme levels [136]. Thus, the normal presence of GPXs and PRDXs are adequate to buffer the H<sub>2</sub>O<sub>2</sub> released in dysbiosis. The intermediary between the microflora and DUOX2 was shown to be NOD2 with a strong history of linkage to Crohn's disease by way of GWAS studies and a sensor of muramyldipeptide, derived from bacterial cell walls [137,138]. As dysbiosis is one factor in IBD, there is the possibility of a vicious cycle of pathology driven by alterations in the microbiota that feed back to perpetuate the condition [139,140]. From the evidence presented, NOX1 and DUOX2 would be factors to prevent initiation of disease by limiting the exposure of the epithelium to the microbiota. Their proper function dependent on the fairly reliable presence of antioxidant enzymes.

## 7. Other Sources of Oxidants

As to other sources of oxidants, xanthine oxidase (XO), lipoxygenases (AloX), and mitochondria have been considered and as later discussed, monooxygenases of the P450 system. Gashler et al ruled mitochondria out as required for ferroptosis, a form of oxidant driven pathology that seems to be operative, at least, in experimental colitis [141]. Our own effort to examine this using MitoQ® (Coenzyme Q10 conjugated to the lipophilic triphenylphosphonium cation to accumulate in mitochondria), revealed a moderate impact that rivaled DTI (pan-NADPH oxidase inhibitor) but did not rise to the level of the Gpx1/2-Duoxa-TKO mice [1,142]. Kruidiner et al and Reynolds et al did not find increased XO protein in UC and CD subjects [143,144]. However, the XO inhibitor, allopurinol has been found to be somewhat effective in humans and more consistently effective in the DSS model of colitis with one dissenting outcome from 4 studies [145–151]. The minority finding suggests the other action of XO inhibition by allopurinol, purine salvage, was the cause of increased pathology [151]. Allopurinol has been a part of IBD therapy since 2005 when it was found to reduce toxicity related to the use of thiopurines, a cheaper means of therapy used in less developed countries [152]. While the work on XO does not address ferroptosis directly, the implication that DSS induced colitis involves ferroptosis is suggestive for such a role. Given the strong role of NOX1 and DUOX2 in the pathology of Gpx1/2-DKO mice, it is not surprising that allopurinol did not suppress pathology. We studied this in the context of exposing six to seven months-old Gpx1/2-DKO SPF mice (specific pathogen free; derived from a germ-free colony) to bedding from our original colony. We reported that young SPF exposed to the bedding tolerated the exposure with ramifications of cancer at 8 months of age [80]. At six-eight months ileum pathology was nearly completely resolved in the Gpx1/2-DKO SPF cohort [80]. Over the course of 8 days after exposure, the SPF mice showed a violent reaction often requiring euthanasia, with a commensurate increase in the ileum pathology score (N=11 at 8 days). Daily IP injection of allopurinol (10mg/kg, IP; standard dose for mice; n= 6) failed to prevent the increased pathology, while diphenyleneiodonium (DPI, 2mg/kg; n=11), a pan-NOX and DUOX inhibitor, provided some protection in this model and as reported in our later work using the less toxic derivative, di-2-thienyliodinium (6 mg/kg; DTI), on young DKO mice [1, 80 153-155 [80].



**Figure 2.** Effect of allopurinol and DPI in mitigation of pathology in ileum of SPF Gpx1/2-DKO mice over 8 days following exposure to soiled bedding from mice of the original colony. Day zero mice, n=8. DMSO control, n=4. Using DPI as model, N=6 statistical power is 0.85, p≤ 0.05.

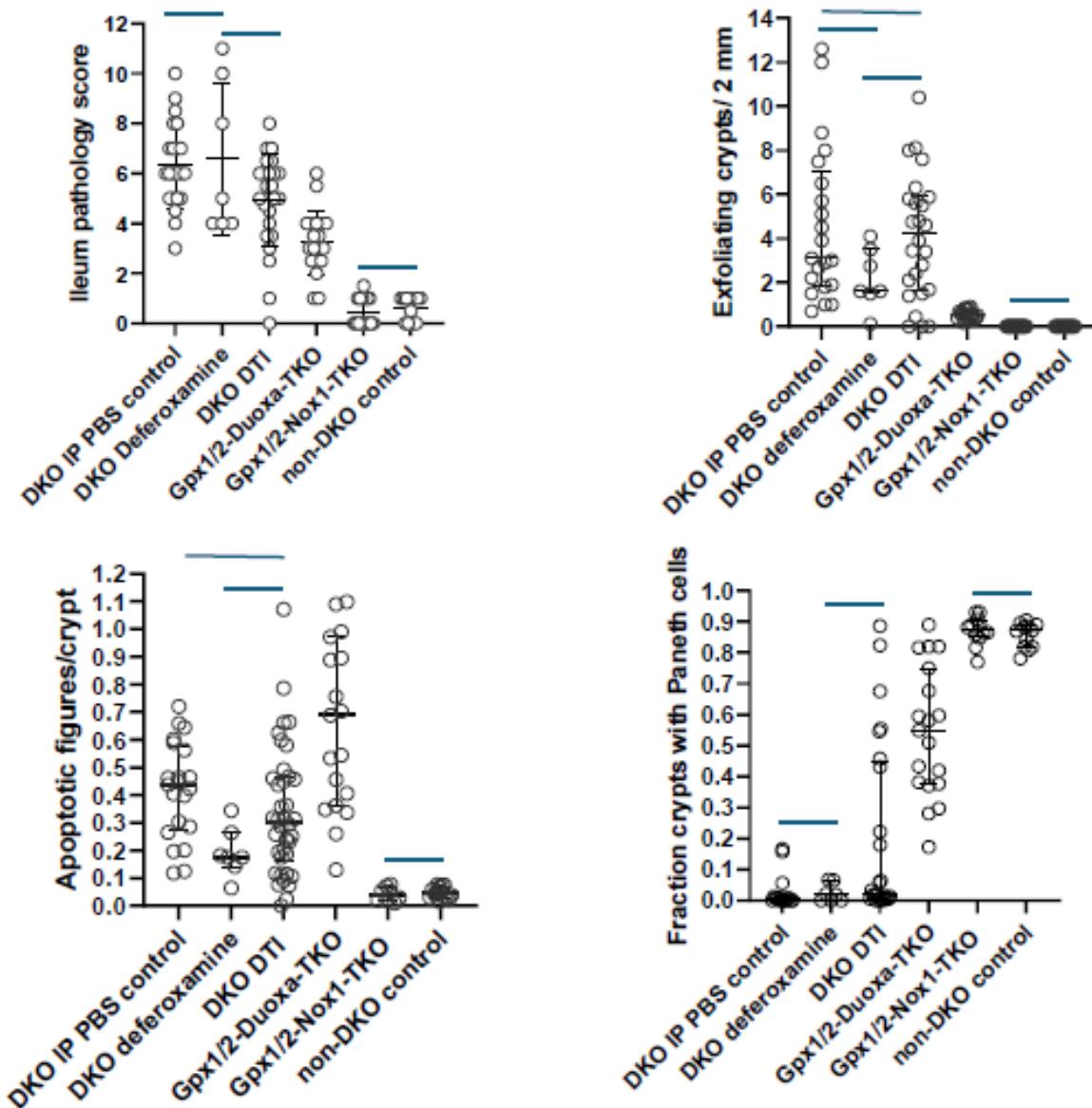
Similar results were found in the ileum treating mixed strain Gpx1/2-DKO mice with DTI from weaning to 40 days of age (untreated and mock PBS treated ileum pathology score,  $7.2 \pm 1.9$  (Std), n=19), with the toxic effect of DPI (obstruction at the gastroduodenal junction) as much a factor as its healing impact (N=7, with 5 others withdrawn for morbidity; ileum pathology score of  $5.6 \pm 2.7$ , p=0.12; statistical power of 0.9, p ≤ 0.05, using a semi-purified AIN-76A diet impact on mixed strain mice as the model positive effect, ileum pathology score  $4 \pm 3.5$ , p=0.002, n=26) [156]. The goal was to uncover pathways causing pathology in the mice by looking at broad spectrum of drugs in small groups of mice, effectively looking for miraculous impact. In this quick and dirty drug screen, we found that Trolox (12mg/kg, IP, to 40 days of age; 25mg/kg was not tolerated by the weanling mice; 7 mice, ileum pathology score  $7 \pm 1.7$ , p=0.9), a water-soluble form of vitamin E, did not impact pathology, unlike in DSS pathology with induction to promote cell death by hepcidin to tie the impact to ferroptosis (Trolox; 30mg/kg, adult mice, 3 days) [133,156,157]. Caffeic acid (20mg/kg IP; 9 mice, ileum score  $5.2 \pm 2.2$ , p=0.028) and DPI, and not allopurinol, seemed marginally effective with deferoxamine (n=5, ileum score  $6 \pm 1.6$ , p=0.18; statistical power= 0.75) coming in third as compounds not yielding miracles, but at least worth follow-up. A later follow up showed caffeic acid was ineffective in B6 Gpx1/2-DKO mice at dose of 60mg/kg (daily oral gavage) [1]. This could be due to the difference in the genetic background or route of delivery [156,158]. We generally lost interest in searching for effective drugs beyond NOX and DUOX inhibitors (DPI, DTI and others) when we

found that semi-purified AIN diets produced the strongest and most consistent moderation of pathology across all mouse backgrounds and sites and this appeared to involve alterations in the microflora [156,158].

Lipoxygenases (ALOXs) seem to have a role in ferroptosis, seemingly discovered before the term was coined, with some of the hallmark features of the phenomenon fleshed out in a 2008 paper [159]. This was found in the context of GPX4 suppression, a theme that is continued when discussing the roles of ALOXs and other factors contributing to ferroptosis. Lipoxygenases can peroxidize non-esterified polyunsaturated fatty acids (PUFA) downstream from the action of PLA2s and have a requirement for activation by ROOH to yield Fe<sup>+++</sup> in the active site [160]. As discussed below, the action of PLA2 may be anti-ferroptotic with later binding to fatty acid binding proteins producing ambiguous outcomes. This suggests that the most efficient way to the excess lipid peroxidation state of ferroptosis is within the cell membrane. The role of ALOXs was unclear, seemingly able to contribute, but not necessary; in some papers it is suggested is that ALOXs are initiators of the peroxidation [161]. Part of the problem might have stemmed from multiple mechanisms of suppression of ferroptosis by presumed ALOX inhibitors, with some action by direct lipid radical trapping [162]. For ALOX15 there is an apparent modification of its peroxidation potential in the presence of PEBP1, "scaffold protein inhibitor of protein kinase cascades" [163]. This allows ALOX15 to peroxidize esterified PUFAs, giving it a role in the more efficient membrane route of peroxidation. We looked at NDGA, a pan ALOX inhibitor, mentioned in the PEBP1 study, as part of our quick and dirty drug screen (N=5, 10mg/kg IP, ileum score 6.3±1.3, p=0.44, statistical power 0.75) [164]. Use of the drug yielded the marginal results generally found in this screen and it was not pursued. This mention of ALOXs leads into the final topic, ferroptosis.

## 8. Ferroptosis, the 800-Pound Gorilla in the Room

To my detriment, I basically ignored ferroptosis except to note the role of GPX4 and now the explosion of studies makes it difficult to perform more than a rudimentary summation (15,000 papers) [64]. I did not find ferroptosis to be novel. Lipid peroxidation initiation by Fenton chemistry was well documented by the time I entered the free radical research field as it was self-titled in 1987 (Society of Free Radical Biology and Medicine), and the initial work on lipid peroxidation can be dated to 1963 [165,166]. The essential role of cystine for cell culture and association with GSH and vitamin E was reported in 1977 [166]. My time spent collaborating with Ursini and Maiorino on the characterization of GPX4 in human cell lines and reading up on their discovery of the enzyme activity reinforced my knowledge this process and the roles of iron, GSH (cystine), GPX4 and PUFAs [168,169]. The ferroptosis inhibiting iron chelator, deferoxamine, was always stocked in the lab and was used in two studies of Gpx1/2-DKO mice (negative results mentioned but not shown in the final paper; B6 background), finding marginal impact on overall ileum pathology (250mg/kg, IP, daily; ileum pathology score=5.8±1.6, p=0.18, N=5 in the fast drug screen, statistical power 0.7, p≤0.05) as opposed to findings in the rodent colitis models, although the levels of apoptosis and anoikis were subdued but not eliminated in the samples (Fig. 3) [1,170–173].



**Figure 3.** Pathology scores of Gpx1/2-DKO (DKO; B6 background) mouse ileum (mean and STD), showing little impact of deferoxamine (150mg/kg, daily IP) by comparison to mock treated controls, DTI (6mg/kg, daily IP) and mice deficient in expression of Duox2 or Nox1. Non-DKO mice are a mix of Gpx1+/-Gpx2-/- and Gpx1-/Gpx2+/- mice, which on a B6 background have nearly normal histology. To simplify the display, statistically non-significant sets are shown by bars, all others significant by pairwise comparison (<0.02).

Deferoxamine failed to sustain Paneth cell numbers, which we showed were depleted by both the apoptosis and anoikis routes by direct evidence and inferred from the outcomes in Gpx1/2-Duoxa-TKO mice vs. DKO mice. In sets where exfoliation was found so were neutrophil only and mixed crypt abscesses; 10 of 21 mice in the DKO set, 2 of 7 mice in the deferoxamine set, 10 of 25 mice in the DTI set, 1 of 19 mice in the Gpx1/2-Duoxa-TKO set. The ileum and colon lengths were not restored with deferoxamine (ileum-30.74 cm deferoxamine (D) vs. 29.74 cm DKO and ~37.5 cm for non-DKO and both TKO sets; colon-8.14 cm D vs. 8.9 cm DKO, and ~10.5 cm for non-DKO and both TKO sets), nor with DTI (ileum-30.1 cm; colon 7.7 cm) [1]. This is contrary to results with the DSS model [174]. A role for labile iron is noted in apoptosis, so that some suppression of intrinsic apoptosis might be expected with chelation [175,176]. Unleashing my cynical side, if I were convinced that ferroptosis can be found almost everywhere (an impression from the literature), the apparent

depression of some marker levels in the Gpx1/2-KO mice by deferoxamine could be pushed as evidence.

My first impression of ferroptosis was that it is a manufactured mode of cell death. That is, you had to add multiple unnatural stresses to cells to observe it, deplete GPX4 (possible with low selenium levels in IBD and which might the standard condition of cell lines in culture), starve cells of cystine, overdose them with glutamine and iron, etc., so that was unlikely to be found naturally [180,181]. The value of the revelation was by way of providing more pharmacological (Stockwell, team lead for introduction of ferroptosis, and team members goal) and genetic interventions to reduce pathologies or attack cancers. The only original observation suggesting it might arise in a useful setting was RAS mutations were linked to increased free iron content in cancer-derived cells in the initial papers on erastin [180,181]. As related by Stockwell, the RAS mutated Bej cell line (skin derived fibroblast line) was engineered to be tumorigenic and follow up analysis on tumor-derived lines bearing RAS mutation did not find consistent ferroptosis sensitivity, reinforcing my impression of the contrived nature of ferroptosis [182].

My point of contact is the isolation of the cDNA for human GPX4 and finding the mitochondrial insertion sequence [183]. I worked on the initial characterization of GPX4 in human cancer-derived cell lines with Ursini and Maiorino, and collaborated with A.W. Girotti, who has extensive experience with GPX4 and was a co-author on the 2014 paper that suggested GPX4 was a key factor in ferroptosis [169,184,185]. The 2014 paper also used a cell line, COHBR-1, that I helped characterize for GPX protein expression, showing COHBR-1 cells and 2 other cell lines did not express GPX4 [64,186]. The latter findings, lack of any need to invoke ferroptosis as a source of pathology in Gpx1/2-DKO mice (Fig. 3) and a lack of impact of silencing GPX1 (GPX2 surrogate; GPX2 not expressed) on BJeLR cells, while silencing GPX4 was lethal, are the reasons I generally ignored ferroptosis [64]. One side note is that silencing GPX7 and GPX8 did have some impact on viability of BJeLR cells. These are not selenoproteins; they are ER residents and thought to be involved in protein folding [187]. The absence of an impact by silencing of GPX1, in hindsight, is not as convincing now as then. PRDXs tend to dominate for ROOH metabolism, as indicated. I did not hear of Winterbourn's upward revised estimates of PRDX rate constants (PRDX1/2, comparable to GPX1; PRDXs more abundant) indicating PRDX dominance until 2012-2013 (Society for FRBM meeting), and probably resisted the implication that my work to date had been with enzymes that generally seem to have a minor role [41,55]. Inactivation of PRDXs under ROS stress, actively built into eukaryotic PRDX structure, was still widely discussed leaving a way GPX1/2 could be important as we found in Gpx1/2-DKO mice [77,188].

There is now a small body of literature suggesting that ferroptosis is a factor in IBD, publications starting in 2020-2021 (10, excluding reviews and mouse models; 85 including rodent models, with some of these mixing human sample derived information, and many using only bioinformatics) and the finding that sulfasalazine, a mainstay of IBD therapy inhibits ferroptosis (from the 2012 ferroptosis paper), which I missed [189]. I still regard ferroptosis, in most instances, as one of the branching end points of severe pathology, a variant of necrosis (essentially failed apoptosis), and dependent on too many processes failing [190]. Alternatively, it could be viewed as an outcome of autophagy responding to cystine deprivation and consequently degrading ferritin [191]. Most refer to ferroptosis as a form of regulated cell death, which is true from the standpoint that the genes involved are, of course, subject to regulation and importantly pharmacological or genetic inhibition or augmentation. A few persist in calling it program cell death, which I reject. For me to be convinced of that ferroptosis has that status in IBD, I would have to see evidence of advanced priming of some cell types for a ferrotopic response or the opposite, enhanced resistance (strong expression bias of genes involved), followed with experimental evidence to show how this would function in a natural setting or logically as a response to stress. As shown below, there is possibly some evidence of this, while there are more signs not of advanced priming to specially promote cell death or resistance but of predilections in metabolism and/or mutations rendering a few cell types susceptible, some by input of common environmental stresses, such as a high fat diet, iron supplementation for IBD subjects or glutamate usage as found in the nervous system [192-194]. The

metabolic predilections seem to serve positive functions of the cells rather than being intentional precursors for ferroptosis activation [195,196]. The two more studied environmental factors both in humans and rodent models are high fat or western diets and iron supplementation, the latter used as IBD subjects are often depleted of iron as with selenium. Searching for DSS and high fat diets turned up 31 papers, only 2 on ferroptosis, one of the two using DSS/AOM [197,198]. Of these 27 suggest a negative impact while 4 claim a beneficial impact. Impact on the microbiota is the most reported finding with the immune system second and two mentions of impact on epithelial cells. Searching for iron supplementation or dietary iron (to weed out papers only mentioning iron or only measuring tissue iron levels) and DSS yielded 19 papers, four on ferroptosis. The outcomes are mixed, with two finding that both low and excess iron are bad in this model and one finding both an initially damaging effect of iron and a subsequent healing regenerative effect by way of stimulating stem cells [199–201]. The impact of iron in these studies is attributed to the epithelium, neutrophils and largely to the microbiota composition [202–208].

My second issue with ferroptosis is the fact that went from being almost nowhere in 2012 to being almost everywhere in a few years (PubMed; 2015 being arguably the real launching point). It seems too easy to demonstrate and this flies in the face of redundant controls on oxidative stress, iron, and cell membrane lipid metabolism [208–210]. Dating back to my attendance of Gordon conferences in the early 1990s, the question was, where was the labile iron? [211]. The ease of inducing ferroptosis in cell lines may involve use of the common protocol of applying ferroptosis inducing agents on unintentionally GPX4 depleted cell lines (10% serum) in a high oxygen environment to detect influences on the process [212]. While I entertained the notion that low selenium levels in cell culture would render many cell lines vulnerable to ferroptosis in my LinkedIn account posts, a recent paper found this to be factual [63]. Also, a newly published pre-print finds that standard adherent 2D cell culture primes cell lines for ferroptosis with low levels of stearoyl-CoA desaturase (SCD) resulting in high levels of cell membrane PUFAs relative to 3D culture [213]. SCD was also indicated to be an activity that could compensate for GPX4 and PRDX6 by *in silico* analysis [214]. This paper also suggests that PRDX6 might augment GPX4 levels by simulating the supply of selenocysteine, found in other work. The effects of ferroptosis markers, indicated from human samples, may be amplified to the level of apparent significant effects in cell lines under these conditions, regardless of their impact in tissues. None the less, Stockwell recently reviewed evidence that ferroptosis has been detected in natural processes like red blood cell maturation and the pathophysiology of anti-viral immunity and tumor suppression [215–217].

As shown below, experiments largely based on the DSS mouse model, likened to ulcerative colitis (UC), suggest ferroptosis is directly involved in death of epithelial cells and strongly influenced by death or resistance to death in several immune cell types, although adaptive immune cell action in the DSS model is not absolutely required and the microbiota can be depleted [218–222]. The involvement of oxidative stress in IBD through the immune system is strongly suggested in the Prdx- and Gpx1-KOs as shown above. This could be linked to ferroptosis both in the immune cells and epithelial cells of the gut by control of non-esterified lipid peroxides.

GPX4 has its impact on esterified lipid hydroperoxides, showing propagation of lipid peroxidation in cell membranes is a unifying feature of ferroptosis [223]. Several studies using different methods suggest that action on non-esterified lipid hydroperoxides is important and allow for an effect of ROOH metabolizing enzymes, like GPX1/2 and PRDX1-5, with PRDX6 in a crossover role [224–231]. The intermediaries for generation of non-esterified lipid hydroperoxides are phospholipase A2 activities (PLA2). The PLA2 activities could suppress ferroptosis by two means: first cleavage of esterified lipid hydroperoxides and release from the membrane for reduction by GPXs or PRDXs with less membrane distortion consequently and, second, upon activation by the presence of lipid hydroperoxides and protein kinase C, exhibiting a somewhat selective removal of arachidonic acid (AA), the main peroxidizable fatty acid constituent of cell membranes from the sites of lipid peroxidation propagation [232–234]. PLA2s have been shown to confer resistance to ferroptosis [235,236]. However, in the CNS PLA2 activities are noted for increasing lipid peroxidation [237].

A combination of PLA2 activity and reduction of non-esterified lipid peroxides is found with PRDX6. PRDX6 has a standard hydroperoxidase activity and GPX4 activity, which might be comparably high, and a PLA2 activity at acid pH [238,239]. The relative roles of GPX4 and PRDX6 would depend on expression levels and the importance of the independent PLA2 activity of PRDX6. PRDX6 overexpression could not compensate for GPX4 loss in Pfa1 cells (fibroblasts), while FSP1 did (FSP1 catalyzes the regeneration of CoQ<sub>10</sub> using NADPH) [240–242]. PRDX6 did inhibit ferroptosis in GPX4 intact cells by increasing the supply of selenium delivery in cells and therefore GPX4 levels [243,244]. A study in lung presents a different story, with the 5-fold higher protein levels of PRDX6 over GPX4 conferring the major resistance to ferroptosis, induced *in vitro*. This involves action of both the ROOH/GPX4 activity domain and the PLA2 activity domain. The peroxidase and PLA2 activities are in separate protein domains and can be individually impaired by mutation and were studied, independently [245]. PRDX6 also has a lysophosphatidylcholine acyl transferase activity that may also explain its ability to remodel cell membrane composition and impact ferroptosis susceptibility [246,247].

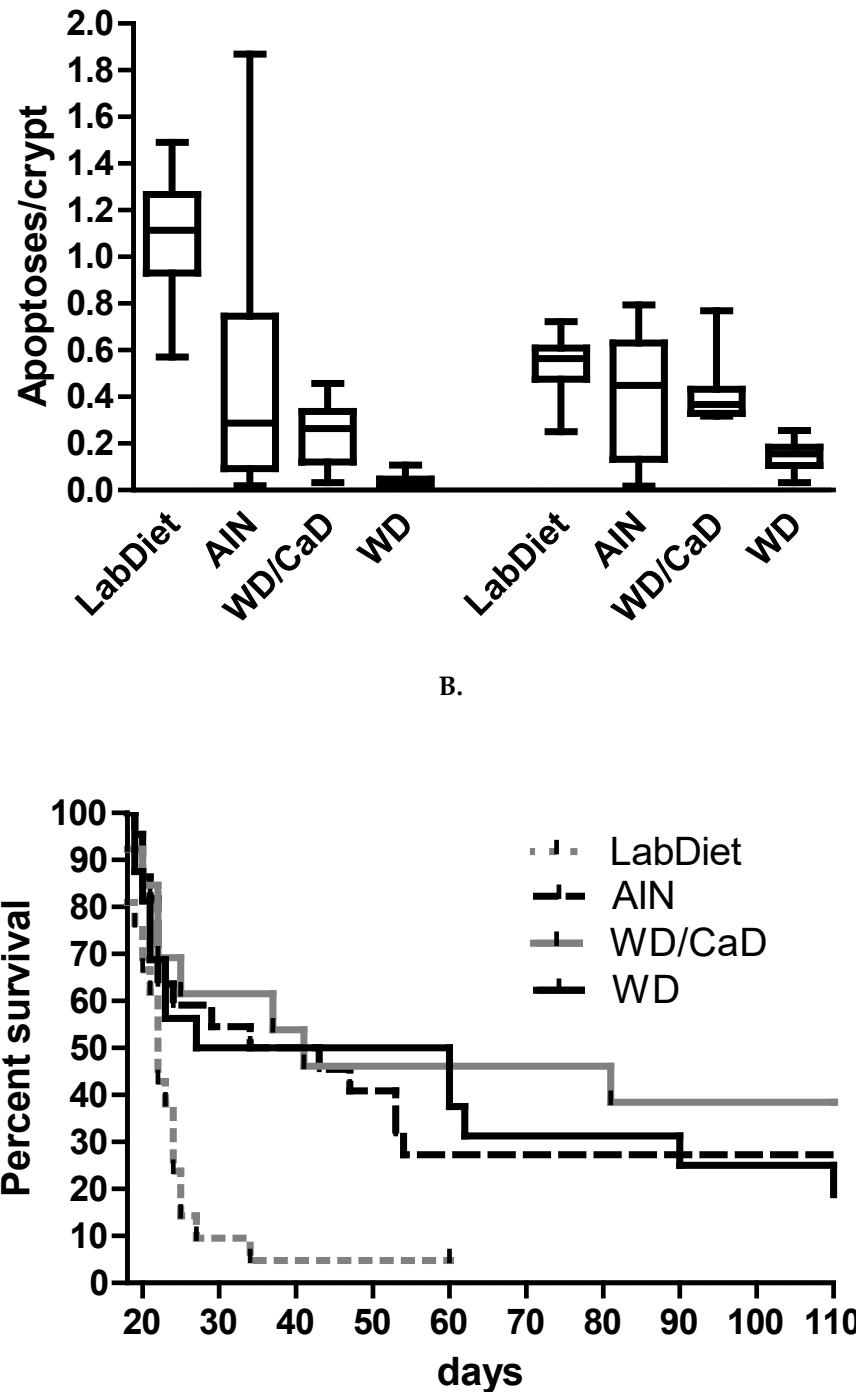
After release, the fatty acids and oxidized derivatives are subject to binding by fatty acid binding proteins (FABP). I was curious about how this would impact reduction of lipid peroxides by GPX3, using defatted serum albumin, the major fatty acid binding protein in blood plasma and linoleic acid hydroperoxide as a model lipid ROOH. Serum albumin inhibited the action of GPX3, presumably through steric hindrance and/or less diffusion and I cautioned against using high levels of serum albumin as stabilizing agent in the GPX assay with substrates like linoleic acid [248]. The action of FABPs could simplistically have a dual impact, beneficially sequestering membrane distorting fatty acid hydroperoxides and peroxidable AA and detrimentally hindering reduction of non-esterified lipid hydroperoxides by GPX1/2 and PRDXs, limiting this route of lipid hydroperoxide detoxification. Serum albumin is not a good model for the action of cytosolic FABPs due to its high K<sub>d</sub> (10<sup>-6</sup>M) while cytosolic FABPs have a K<sub>d</sub> ranging from 3 nM to 1000 nM [249–251]. On the flip side, lipid peroxidation can be either promoted or inhibited by FABPs with variety of mechanisms proposed for each, some of which stray far beyond simple sequestration and steric processes [249–255]. Generation of non-esterified free fatty acids by the action of PLA2 activity may result in an overall protective effect based on reduction of membrane distortion that might favor less membrane peroxidation and loss of peroxidable AA. The ambiguous actions of FABPs provide no clear role in reducing ferroptosis vulnerability by binding the non-esterified products generated by PLA2 action. While indicated in some work, the actions of GPX1/2 and PRDX1-5 on non-esterified fatty acid hydroperoxides could be minor relative to GPX4 with PRDX6 being a sole exception, perhaps by its tripartite capacity as a PLA2, with action both on non-esterified fatty acid hydroperoxides and esterified fatty acid hydroperoxides. More papers suggest inhibition of ferroptosis by PRDX6 than the opposite, with a split over whether this action is more reliant on the peroxidase activity or the PLA2 activity with one paper suggesting both contribute, equally (search terms: PRDX6 and ferroptosis and not filtering for IBD; 16 papers) [245].

Ferroptosis could have three major points of impact in IBD, directly in the epithelial cells, the immune system and by influence on the microbiota or feedback from the microbiota. Claims for all have been made in the limited number of studies available. The study closest to my experience suggested that Western diets (fish oils added to mouse diet to increase peroxidizable arachidonic acid content) might increase the oxidizable lipid content of the epithelium and render it susceptible to ferroptosis [256]. This parallels findings in a paper to be discussed later, where this effect is assigned to Treg cells [257]. First, they show that the GPX4 protein levels in CD ileum samples and not colon samples were down by 50%, a possible consequence of low selenium levels, as discussed, although odd for the specific impact in ileum. Another paper does find colon GPX4 mRNA to be down by almost 2-fold in IBD colon (this second paper seems to be the first linking ferroptosis and IBD)[258]. Second, they assayed GPX activity, calling it a GPX4 assay. However, the substrate was cumene hydroperoxide. GPXs1-4 are all active with this substrate, GPX4 with a low rate constant relative to the rest [248]. So, this has little meaning for GPX4 and suggests a general reduction in GPX1/2 activity. GPX assays present a problem in the Yang et al (2014) paper as well [64]. As far as I can tell, the assay

shown in figure 2F, suggesting inhibition of GPXs by erastin, omitted GSH (tert-butyl hydroperoxide; later it was included for the Phosphatidyl choline hydroperoxide and cholesterol hydroperoxide assays), and so is another assay of the GSH content of the samples and not GPX activity. Modern literature measuring tissue GPX activities is full of such errors [248]. An examination of mice heterozygous for GPX4 expression showed iron or arachidonic acid feeding produced pathology in the ileum, marked by neutrophil infiltration. Vitamin E protected against the arachidonic acid effect. Vitamin E is a chain-breaking antioxidant for lipid peroxidation and known for its interaction with selenium in pathology [259]. One major function would be to convert lipid hydroperoxyl radicals to hydroperoxides allowing GPX4 and PRDX6 to act. We found that feeding Gpx1/2-DKO mice a cholegenic/lithogenic diet with high cholesterol, high fat and cholate (AIN-76A base) induced colitis beginning 4 weeks after introduction of the diet, however, cholate seemed to be the active agent, yielding high levels of deoxycholate in fecal pellets of Gpx1/2-DKO mice and the wild-type control; omission significantly reduced the morbidity [260]. These diets mark the single exception to the AIN base being preventative for Gpx1/2-DKO mouse colitis. We attributed the colitis to a disturbance of the unfold protein response, not looking for ferroptosis (this was 2010), reminiscent of the modest impact of silencing GPX7 and GPX8 in the Yang et al study, thought to be a factor in IBD from GWAS and other studies and, linked to autophagy, another factor in IBD [64,261–263]. The original colitis/ferroptosis paper mentioned above actually linked ferroptosis to ER stress [258]. That paper presents a limited marker analysis of human IBD samples, pointing to signs of ferroptosis sensitivity. Mice were subjected to DSS treatment. One important finding was that FER1, a ferroptosis inhibitor, reduced the levels of pathology in the mice, suggesting ferroptosis involvement [264]. Second, they showed signs of an ER stress response along the lines we did in the atherogenic diet study and showed that the PERK inhibitor, GSK2606414, lowered the pathology levels and Fe<sup>++</sup> content of epithelial cells. Another paper using wild-type mice and DSS to induce colon pathology found that deoxycholate enemas produced conditions favoring ferroptosis in enterocytes, elevated Fe<sup>++</sup>, and ACSL4 protein and mRNA, lowered levels of GSH and GPX4 protein and mRNA, associated with increased pathology [265]. While not showing direct signs of ferroptosis, they found that apoptosis (TUNEL) and pyroptosis (marker analysis) were not elevated leaving, in their opinion, only ferroptosis as the factor producing the increased pathology. Preceding this demonstration, they showed that high fat diets (60% fat vs. 16% fat) aggravated DSS-induced pathology and found high levels of deoxycholate in the mouse sera. Finally, they linked Western-style, high fat content diet intake levels by correlation to ulcerative colitis severity in human subjects and to ferroptosis by measure of the markers, GPX4 (IHC, mRNA), DMT1(IHC) and HIF-2alpha (IHC), all showing modest but significant alterations favoring ferroptosis [266,267]. While not looking at ferroptosis, another group found that a deoxycholate enema following DSS administration increased IL-1 $\beta$  production in macrophages [268]. The Western-styled, and so-called pro-hyperplasia inducing Newmark diet (20% corn oil; AIN-76A; WD and WDCaD are Newmark diet with low vitamin D and calcium and with vit. D and Ca at AIN-76a levels) being a derivative of AIN-76A and lacking yeast, other components of LabDiet® mouse chows and cholate, nearly cured the ileum of Gpx1/2-DKO B6 strain mice shown as by the impact on apoptosis in the ileum and colon (Fig. 4a) and reduced the morbidity of the Gpx1/2-DKO 129 strain mice (N10) (Fig. 4b) [156,260,269].

A.





**Figure 4.** Impact of two Western diet formulations and semi-purified AIN-76A diet (AIN) on Gpx1/2-DKO mouse (129 N10) morbidity in comparison with standard LabDiet® mouse chow.

The apparent vitamin D/Ca effect was attributed to vitamin D3 inducing NOX1, based on the literature, and we did not confirm that [270]. Note, the 129 N10 Gpx1/2-DKO cohort (B6, 129 mixed strain mice backcrossed to 129) was difficult to manage due to extreme morbidity. The preventative impact of AIN diets made it possible to maintain and study this cohort. This differed from the N5 cohort, with moderate morbidity [156,158]. The difference was mapped to a locus on chromosome 2 containing the Duox2 gene, the sequence of which differs between B6 and 129, yielding alter enzyme activity [1,158]. Although we had indications from the use of DPI, it was that finding this led us to explore the roles of DUOX2 and NOX1 in Gpx1/2-DKO mouse pathology, over and above NOX2. The

2012 Dixon et al paper indicates that NOXs play some role in ferroptosis as a source of superoxide, however the specific suggestion that NOX1 is a prime candidate is not supported by the expression profile in the studied Calu-1 cells due to the similarly high expression of NOX2 which is a more potent source of superoxide [132]. Use of the NOX1/NOX4 inhibitor, GKT137831, provides some support for the claim [271]. In colon tissues, NOX1 could be a major source of superoxide before the onset of inflammation [109]. There is one paper suggesting high-fats diets reduce ferroptosis levels in DSS stressed mouse colon by increase uptake of cystine through the SLC7A11transporter (xCT) [272]. This group also reported that high fat diet increased pathology with DSS/AOM and ferroptosis marker levels, while showing, invitro in cancer-derived cells that high lipid exposure lowered ferroptosis by reducing CHAC1 levels, linked to less GSH degradation and less ER stress [273–275]. Chac1 was identified by us as one of ten top candidates, alongside Duox2, as an expression level variant with SNPs of unknown effect impacting pathology levels in the Gpx1/2-DKO mouse N5 vs. N10 study [276]. An additional paper on ferroptosis in human samples and DSS-induced colitis also reported the ER stress in the epithelial cells involving the PERK pathway was a major factor [258]. This study does not examine dietary fat. FER1 and deferoxamine were found to demonstrate an impact on the pathology level related to ferroptosis and the PERK inhibitor, GSK2606414, to show the effect of ER stress in epithelial cells in the DSS model. The study jumps to use of cell lines to examine this in detail [258]. Finally, the role NF- $\kappa$ Bp65 was examined as an intermediary between ER stress and ferroptosis, finding that an IEC specific NF- $\kappa$ Bp65-KO (floxed gene driven by Villin-cre) resulted in increased colon damage in the DSS model. The final assessment was that phosphorylated NF- $\kappa$ Bp65 suppressed ER stress by interacting with eIF2 $\alpha$ , component of the integrated stress response, which is linked to autophagy [275].

The remaining papers on ferroptosis in the epithelium largely nominate other factors for impact on GPX4 expression, iron uptake, cystine, glutamine, and lipid levels, suggest possible alternative therapies (the bulk promoting traditional medicines) or prognosis based on ferroptosis marker signature sets, a few of the latter mentioning GPX2. A limited number of the roughly 50 mouse/DSS studies (some with AOM) on ferroptosis employ inhibitors or inducing agent in the animals to advance the claim of a role in IBD [277–281]. The use of ferroptosis inhibitors is often limited to parallel studies on cell lines, with CACO2 being the most used. In many, the association is based on marker analysis with the assumption that ferroptosis is a fact in DSS-induced pathology and trusting that the change in marker levels indicate significant effects of the agents or pathways under study show an effect through ferroptosis. In summary, there is some evidence of ferroptosis in IBD, possibly confined to the active stages, where marginal to severe selenium deficiency might be present to impair GPX expression and possibly PRDXs by way of thioredoxin reductase (selenoprotein), the epithelium is under stress, ER stress and autophagy being candidates, and of course the effect of the immune infiltrate. High fat in diets and iron supplementation may be environmental factors that would push the stressed colon toward ferroptosis. Activation of NOXs and presence of DUOX2 outside of its usual boundaries could supply oxidants to fuel the lipid peroxidation.

Ferroptosis could impact the immune system with as great or greater effects on IBD outcome as anything in the epithelium. Limiting search terms to ferroptosis, immune system and inflammatory bowel disease, 9 papers emerged, with 4 being reviews; a few of the mouse studies look at immune function. The fact that the impact of some knockouts of PRDXs and GPX1 seem to operate largely on the immune response (exceptions will be noted below), despite expression in the colon, further suggests their minor contribution to crypt/gland base antioxidant protection, relative to the colon gland based confined GPX2, particularly in the context of lack of GPX1 [282]. There are opposing papers for PRDX4. One paper on PRDX4 shows a local colon effect with DSS treatment of PRDX4- $/y$  mice and linked the pathology to ER stress, the ER being one of the sites for PRDX4 localization (recall GPX7 and GPX8). GPX1 and GPX2 levels were low with DSS in both wild-type and PRDX4- $/y$  mice, attributed to iNOS expression, inactivation of GPXs by nitrosylation and possible destruction [283,284]. One difference from the PRDX4-KO/DSS/AOM cancer paper in addition to use of AOM is the background strain, FVB/N for the immune system DSS/AOM cancer effect and B6 for the local DSS colitis effect. A PRDX6-KO was also found to be protective from DSS-induced pathology; the

effect suggested to be based on compensatory up-regulation of other antioxidants in epithelial cells of the gut by activation of NRF2 signaling [285]. Another study found an opposite effect of PRDX6 in association with ferroptosis [286]. Neither study examined possible immune involvement.

As indicated, ferroptosis in the immune compartments could impact IBD and this seems to be the case in DSS- and other mouse colitis models. The second paper under consideration for the impact of Western-style high fat diets, suggests a detrimental effect could be mediated through ferroptosis in the Treg population [257]. A key logical consideration in the study was that fats are absorbed in the upper small intestine, so that high-fat diets should not directly impact the colon. This is like my complaint that papers invoke iron uptake in the gut as a factor in IBD associated ferroptosis, when the absorption occurs in the duodenum and jejunum [287]. Supplemental iron, given to some IBD sufferers, is known to aggravate IBD and one case-control study found even high-end levels of normal intake affected IBD [288]. In two experimental studies, however, the effects of dietary iron were attributed to an impact on neutrophils (DSS) and the intestinal microbiota (TNF $\Delta$ ARE mice) [202,289]. The Treg lamina propria population numbers were less upon feeding high fat diet (60% calories fat) to mice, down to one-third the normal levels. *In vitro*, Tregs took up arachidonic acids more readily than Tconv cells, that made its way into the membrane phospholipids. The GPX4 levels were marginally lower with high fat diet, but similar to that in the Tconv population. The subsequent elevated cell death was reversible by ferrostatin-1. To make the link to a ferroptosis role in IBD, they made a mouse line with a Treg specific GPX4 conditional KO (Fox3 YFP-Cre, GPX4 F/F). The KO mice and the colon tissues were fine on standard diet by in several analyses and developed severe colitis and other problems on high fat diet. Treg populations were depleted. This could be replicated on normal fat diet with vitamin E depletion. High levels of vitamin E countered the high fat diet effect. Here there is an example of metabolic priming favoring ferroptosis, based on a preference of the cells for arachidonic acid for cell membrane phospholipids. I would not declare that the consequent high fat diet effect is an example of advanced priming of Treg cells specifically for a ferrotopic response as it leads to a greater level of pathology. While the demonstration of ferroptosis is based on the inherent preference of the T-cells for AA, it was dependent not only on a high fat diet but also forced, reduced expression of GPX4.

A second case involving alter AA usage in immune cells and ferroptosis, studies M2 macrophages [290]. The paper is prefaced with disappointment over the inability of 5-ASA to control IBD. It then jumps to ferroptosis as having been demonstrated in enough cases to justify studying FER1 as combination therapy with 5-ASA in UC. This study is an instance of not tipping the balance in favor of ferroptosis from the beginning by suppressing expression of one or more pathways involved before screening for an impact of another entity. DSS is used to induce colon pathology with the finding that products of lipid peroxidation, like 4-HNE, were not impacted by 5-ASA, nor were ferroptosis suppressing factors like GPX4 and FPS1 elevated. FER1 addition aided in reversing this trend, suggesting ferroptosis was occurring unaided. On the assumption that M1/M2 polarization is a key factor in IBD, they found that combination of FER1 and 5-ASA enhanced the numbers of M2 macrophages in association with lessening of pathology. Finally, they do prime the macrophages with Erastin treatment to examine the relative sensitivity of M1 and M2 population, finding M2 macrophages to be more sensitive to ferroptosis. They suggest that the combination of the action of the PLA2, Pla2g4a, and Ascl4 (acyl-CoA synthetase long-chain family 4), act to remodel the cell membrane in favor of ferroptosis by increasing levels of AA [291]. These findings reveal a possible negative impact of PLA2 activities in ferroptosis in conjunction with ASCL4.

A third paper suggests that observed up-regulation of GPX4 levels in IBD, act to suppress ferroptosis in the ILC3 population of innate lymphoid cells, found in the mucosa [292,293]. Again, this paper operates on the premise that ferroptosis is a fact in induced rodent models of colitis, some based on indirect evidence in prior work (iron chelator effects as discussed and marker analysis) and one direct application of FER1 in the TNBS model following marker analysis to suggest involvement of ferroptosis [257,294–296]. Using *Citrobacter rodentum* to induce inflammation, they show that activated ILC3 cells are resistant to ferroptosis in a GPX4-dependant manner (IEC specific Gpx4-KO),

like findings in human samples, although the primary factor may be LCN2. The net effect of resistance to ferroptosis was a lessening of the pathology.

A final paper suggest intraepithelial lymphocytes (IEL) are subject to ferroptosis, due to expression of another source of oxidants, CYP1A1 (cytochrome P450 family monooxygenase), regulated by the aryl-hydrocarbon receptor (AHR) [297]. AHR is heavily involved in shaping the immune system of the intestine [298]. One action of CYP1A1 is generation of 19-HETE from AA [299]. However, the catalytic cycle of CYP1A1 can be disrupted leading to production of superoxide and H<sub>2</sub>O<sub>2</sub> [300]. This demonstration of ferroptosis was dependent on eliminating Ahrr, a repressor of AHR in mice. This resulted in a 2-fold increase in CYP1A1 activity (3-fold mRNA) in IELs, increases in lipid peroxidation being the sole direct link to ferroptosis. The net effect was fewer IELs in the intestine, which renders the KO mice more susceptible to DSS [301]. Providing the Ahrr-KO mice with selenium or vitamin E lessened the lipid peroxidation in the IELs and restored IEL numbers. The link to ferroptosis is plausible but weak and was dependent on Ahrr status. The authors say their findings might reveal a vulnerability of the IELs in association with AHR function, which includes CYP1A1 up-regulation and gain of function mutations associated with IBD [302,303]. AHR is also a link to the microflora by being a receptor for microbial generated ligands, largely tryptophan derivatives like L-kynurenine [304].

Additionally, other studies have found evidence of ferroptosis in neutrophils and NK cells in other pathologies, like systemic lupus erythematosus and gastric cancer [305–307]. One NK study links L-kynurenine production in the cancer to ferroptosis by way of GPX4 suppression rather than CYP1A1, while a paper on neutrophils shows INF $\alpha$  promotes transcriptional repressor CREM $\alpha$  recruitment to the GPX4 gene. Collectively, a host of immune cell types and epithelial cells are subject to modulation by ferroptosis based on work to date. To the extent that the studies involve cell culture, the results might be taken with a grain of salt, as suggested earlier. Some papers depended on compromising the target cell type before ferroptosis could be demonstrated, then suggested ferroptosis would be a major factor in their regulation [307]. This would not necessarily indicate a natural mechanism of regulation, as sometimes suggested, but indicate pharmacological means to exploit for elimination of the cell types. This could be nuanced, depending on the ferrotopic Achilles' heel of the cell types, AA uptake, GPX4 levels, cystine and iron metabolism.

The impact of experimentally altering or eliminating the microbiota of Gpx1/2-DKO mice dramatically affecting the levels of ileum and or colon pathology is just one example of many studies demonstrating the impact of the microbiota composition, with ours running counter to the list of usual suspects [80,308]. Papers on high fat diet and supplemental iron, generally with DSS, often report on alteration of the microbiota (over 31 papers on high fat diet and 19 papers on iron, with 2 high fat diet papers and 6 iron papers on ferroptosis) [174,309–312]. This is not the only significant finding in these papers, with adipose tissue leptin found to inhibit DSS induced colitis/ ferroptosis by impact on apoptosis pathways [313]. Supplemental iron was found to activate NF-kappaB to promote DSS-colitis [202]. Also, bucking the trend for a worse outcome from high fat, one group found an inhibiting effect on Slc7a11 (cystine/glutamate transporter, Xc-), resulting from high fat diets and DSS blunting ferroptosis [197]. Associations with the microbiota and DSS colitis extent to bacterial metabolites, such as butyrate and as previously mentioned, L-kynurenine. Low butyrate levels in DSS were found to be low and supplemented DSS treated mice with Na butyrate relieved the colitis and ferroptosis based on GPX4 as a marker [314]. High fat diet and resultant  $\omega$ -6 PUFAs are thought to aggravate CD [315]. A variation on this theme is a more direct impact of bacteria on epithelial lipid peroxidation. Adherent-invasive *E. coli* is linked to IBD [316]. One group showed that adherent-invasive *E. coli* given to DSS treated mice stimulated lipid peroxidation by lowering GPX4 and ferritin heavy chain levels, yielding 4-hydroxynonenal as a marker of ferroptosis [317]. In conjunction with AA feeding (surrogate high fat diet condition) the pathology was worse. FER1 lowered the levels of pathology suggesting another link to ferroptosis.

## 9. Use of Markers for Identifying Ferroptosis

One question is, how much papers based largely on marker analysis suggesting a tendency for ferroptosis can be held as evidence? Ferroptosis has been detected in IBD, although sometimes by rather weak marker analysis, operating by way of the epithelial cells, components of the immune system and by participation of the microbiota. Following as many papers as I can, leads me the notion that ferroptosis is not everywhere but certainly pervasive. The skeptic in me does question the breath of these claims. If ferroptosis is occurring in so many components of IBD, could they somehow cancel each other out, not necessarily in DSS-induced pathology but the human counterpart? I wonder whether there is a middle ground of more controlled lipid peroxidation, involving ALOX/PEBP1, that could serve a signaling purpose and be misclassified as ferroptosis. Signaling pathways, including those derived from esterified lipids have been long proposed, along with the idea that not all lipid peroxidation would contribute equally to ferroptosis because of different impact of products on cell membrane integrity [318,319]. Iron chelators might have action by inhibition of ALOXs whether full blown ferroptosis is involved or not [320–322].

There is some reliance on 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) as markers of ferroptosis. 4-HNE production is not universally bad and has been reported to confer resistance to ferroptosis at low levels [323–328]. MDA is notoriously unreliable as a marker of oxidative stress or lipid peroxidation [329]. It has been reported that LiperFluo is superior to the more widely used C11-BODIPY (10-times more usage) as a marker of lipid peroxidation in cell membranes [330]. Other markers, based on mRNA or protein levels, are shown to be increased or decreased in levels with the suggestion of altered susceptibility to ferroptosis. As mentioned, the confirmation of the findings is often relegated to cell line work, where standard culture conditions may promote the tendency for ferroptopic responses [63,213]. There is some room for doubt that ferroptosis is occurring in all the reported instances or would occur without pre-manipulation.

One use of mRNA level marker analysis resulted what I consider a very odd result [331]. Sun et al. decided to evaluate UC subject clustering based on a possible bifurcation into ferroptosis and immune driven (neutrophil infiltration) types. It is not clear how they came to this idea. They reference their earlier paper, finding signs of ferroptosis in UC [332]. One side point in this paper is the exaggeration of ferroptosis marker level differences in DSS pathology relative to UC vs. healthy controls with another paper showing this for GPX4 [333]. Using machine learning tools, the group examined Geo datasets of UC subjects to find that the marker analysis produced 3 groupings, one for ferroptosis, another for infiltration with a third set of combined markers, close to 1/3<sup>rd</sup> of subjects each, with another set labelled quiescent. The ferroptosis set was low for immune infiltration, NK cells one exception (> mixed and neutrophil groups) and CD8+ T cells (= neutrophil group; low vs. mixed) and tended to resemble the quiescent set. Based on the proposed roles of NK cells and granzymes in IBD they may have inadvertently found 3 UC subtypes; not based directly on ferroptosis, rather the degree and type of immune involvement in UC in 3 phases (transition from active to quiescent and the reverse via mixed and ferroptosis like phases?) or patterns of the disease. The proposed importance was the ability to predict success of infliximab treatment. NK cell involvement in IBD has been examined with similar findings for golimumab or ustekinumab and for granzyme B (NK and CD8+ T cells) with infliximab without invoking ferroptosis [334,335]. Granzyme A (NK and CD8+ T cells) is reported to suppress ferroptosis [336]. However, IFN $\gamma$  secretion by CD8+ T-cells or NK cells may shift epithelial cells in the direction of ferroptosis sensitivity [337–339]. Aside from the mix of possible outcomes of NK cell action, there are various findings, some contrary to others, for levels and types of immune cell infiltration in IBD using marker analysis.

## 10. Concluding Remarks: Ferroptosis in IBD

If the goal is to genuinely find new ways to impact diseases, the study of ferroptosis is of value. Neurodegenerative diseases studies may benefit [340]. Cynically, the explosion of papers suggests to me that many studies are based on a low bar for credibility fueled by hamstringing cell lines or other samples at the outset, so the results are skewed in favor of finding ferroptosis with an extra nudge. This is in addition to standard methodologies in cell line culture also favoring ferroptosis [63,213]. Is ferroptosis a thing in IBD? The conditions of full blown IBD could certainly yield factors favoring

ferroptosis, low selenium intake, and increases in tissue macrophages and other infiltrating immune cells could favor alterations to iron distribution and other metabolic changes that could promote ferroptosis, operating like the imposed experimental conditions used by many to enforce ferroptotic outcomes [341–344]. An increased impact of ferroptosis in IBD might result from high fat diets and iron supplementation and this might also act on the immune system. So, yes, it may be real factor in IBD. The problem is sorting through papers with such claims for overly enthusiastic tinkering of the sample materials to skew the outcomes. Any prior manipulations must be consistent with real trends that might be occurring in IBD and because of dietary habits or supplementary iron for any specific cell type. Knocking down GPX4 expression or suppressing cystine uptake, for example, must be justified and the extent employed matched to actual effects. Our use of the Gpx1/2-DKO construct in mice is clearly going too far to demonstrate the impact of selenium in IBD and misses the mark both in the extreme reduction of GPX1/2 based on what is possible in humans and the failure to affect levels of the other selenoproteins, notably, but not exclusive to GPX4. This places real limits on the expanse of things I can say about IBD from the evidence of Gpx1/2-DKO mice. Claims for ferroptosis may be often exceeding the limits.

## 11. Concluding Remarks: Oxidative Stress in IBD

As to oxidants and antioxidants in IBD, the evidence from Gpx1/2-DKO mice shows that NOX1 and DUOX2 are generating enough oxidants to require the presence of at least some GPX2 (Gpx1-/Gpx2+/- mice, even with low selenium levels). However, the possible surplus of GPX1/2 (wild type mice) seems more than capable of handling the load from the summed sources of oxidants even with low selenium intake (perhaps PRDXs have a role here; or the canceling of effects of immune system and the gut epithelium). This seems to be independent of GPX4 and possibly ferroptosis. The available GPX4 and PRDX6 seemed capable of limiting cell membrane lipid peroxidation to tolerable levels even with the low selenium intake in our study with Gpx1-/Gpx2+/- mice [86]. Historically, NOX2 and XO were implicated in oxidative stress by way of studies of ischemia-reflow and mitochondria have been discussed for as long as I can recall [345–347]. Now the focus is on NOX1/NOX4, DUOX2, and ER stress. Since ROOH signaling can globally impact cellular processes, oxidant composition and levels are expected to affect IBD with roles in control of the microbiota another major another factor. Currently the exploration is based on under-performance of oxidant sources as much as overabundance of oxidants.

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