Review

Enzymatic Oxidants, Antioxidants, and Inflammatory Bowel Disease

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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; ferroptosis

1. Introduction

This discussion is not intended as an exhaustive foray into the area of oxidant, antioxidant involvement in 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 coverage will include 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]. However, due to the generally negative findings, only the study of mitochondria was published [1]. There will be only limited commentary 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 [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. The readers are referred to the following papers for a superior discussion of the clinical features of IBD [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

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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]. The goal of this review is to identify major enzymatic oxidants and antioxidants that might contribute to IBD and examine the likelihood that they would be involved as precusors to active disease or aggravating factors. Use of supplemental selenium, in various forms, will be discussed as a possible remedy for IBD.

2. History of Oxidants and Antioxidant Involvement in Inflammatory Bowel Disease

Neutrophil infiltration is one of the hallmarks of active IBD [18]. The connection of oxidants to IBD begins around 1973 with studies of the antimicrobial neutrophil oxidative/respiratory burst revealing that superoxide (radical, one-electron addition product of oxygen) and H_2O_2 (reactive oxygen species, ROS) were products, and later that there were damaging impacts on adjacent tissues [19,20]. 5-aminosalicylic acid (5-ASA), a therapeutic agent for IBD since the 1940's, was found to affect neutrophil migration in 1979 [21]. Soon after, it was suggested that 5-ASA interacted with oxygen free radicals as one mode of its therapeutic action [22,23].

At roughly the same time it was recognized in the model system of cat intestine that superoxide could be generated by xanthine oxidase (XO) in the phenomena of ischemia, yielding microvascular injury [24]. Almost immediately, SOD, which produces H_2O_2 from superoxide, was proposed as a possible therapy for intestine related pathology with catalase (purified in 1900) sometimes included in formulations to eliminate the H_2O_2 [25–35]. Efforts to employ SOD as IBD therapy continue to the present and are highlighted in two studies by the use of purified bacterial SOD or SOD containing spores introduced by oral gavage into mice, both wild type and SOD1-knockout (KO) mice treated with dextran-sodiun salt (DSS) to induce colitis. In another study, *E. coli* harboring an extra catalase gene were similarly gavaged into wild type mice during DSS exposure [36–38]. One of the two SOD papers and the catalase paper show benefits from the extra antioxidants in wild-type mice. The other SOD study makes the case that a SOD1-KO mouse line suffers greater pathology than wild-type mice in DSS-induced colitis and the introduction of bacterial SOD or spores returns the SOD1-KO pathology levels to that of wild-type mice.

The first mention of GPX in connection with IBD involves a study of selenium deficiency in CD subjects in 1984. This was 12 years after the discovery that GPX1 is a selenoprotein [39,40]. Selenium deficiency in CD was noted 1 year earlier (3 years, anecdotally), without relating it to GPX [41]. Like catalase, GPX1-3 reduce H_2O_2, although using the glutathione tripeptide (GSH) as a subtrate. 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 [39,42–49]. 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 [50].

In this early interval of oxidants and IBD studies, oxidative stress markers were found to be elevated in active disease samples from IBD subjects. GAPDH is known to be a target of sulfhydral oxidation with loss of activity. Much higher oxidation of GAPDH was found in inflamed vs. non-inflamed samples from CD and UC subjects, while GSH and GSH + GSSG levels were lower in UC but not CD [51,52]. Higher levels of 8-hydroxy-2'deoxyguanine (product of DNA oxidation) were found in colon mucosal biopsy samples from CD subjects vs.controls and not UC; luminol detection of reactive oxidant intermediates showed high levels in both CD and UC vs. controls, along with protein carbonyl content (measure of protein oxidation) [53]. Breath alkanes, a measure of lipid

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peroxidation, were elevated to a level commensurate with smokers in IBD subjects over controls [54]. Etheno-DNA adducts (indirect measure of 4-hydroxynonenal, a breakdown product of lipid peroxidation) were elevated in CD and UC [55]. Malondialdhyde, also a product of lipid peroxidation, was elevated in UC samples from active disease (4-fold) vs. quiescent (1.4-fold) or in subjects with other bowel issues (1.0-reference) [56]. These studies served to confirmed the major premise at the time that oxidants derived from infiltrating immune cells were damaging the tissues.

3. The Enzymatic Antioxidant Families

There seems to be a large gap until the roles of another major antioxidant family, peroxiredoxins (PRDX), are discussed for IBD, possibly 2010 [57]. 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 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 [58]. 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 [59–62]. Single KOs of none of the named antioxidant enzyme families produces spontaneous IBD in rodents. The lack of IBD in the single KOs is not conclusive for lack of a role and possible counter-balancing effects in the immune system, shared with *Gpx1*, seem to offset any deficiency in local antioxidant protection in the ileum and colon uncovered in the DSS colitis model [63]. Superoxide is generated in several processes, by NADPH oxidases (NOX; NOX2 in the oxidative burst), by XO, but largely by electron leakage from the electron transport system of mitochondria [64]. As such, there is a dedicated mitochondrial SOD, SOD2 with manganese in the active site [65,66]. SOD1 is the cytosolic, zinc/copper active site version. Sod2-KO is a neonatal lethal condition in mice. We looked at intestine samples from long-lived Sod2-KO mice (on some strain backgrounds mice lived to 19 days) and observed nothing [67]. There are no reports of IBD like issues by use of heterozygous mice or an intestine specific Sod2-KO [68,69]. Superoxide can be damaging, largely by reactions with iron and also spontaneously reacts with nitric oxide (NO, a radical species produced by nitric oxide synthases, NOS) to produce very reactive and damaging peroxynitrite [70,71]. Superoxide is converted into H_2O_2, which can act as an antimicrobial agent as is (downstream of NOX2 or alternatively the direct product of DUOX2) based on its damaging properties, or after additional reactions with hypohalous acids or thiocyanate that yield more potent antimicrobial species [72]. In the presence of iron (Fe++), it can generate the damaging hydroxyl radical (Fenton reaction), which can be one precusor to lipid peroxidation [73]. It is also a signaling agent, able to oxidize free cysteine residues of proteins to disulfides or sulfenic acid (both easily reversed) to induce activation, inactivation, or structural changes [74]. In this role, H_2O_2 can have a broad impact in IBD beyond acting as a damaging agent, although oxidation of protein sulfhydral groups (excessive levels and/or oxidation to sulfinic acid, reversable by sulfiredoxin/ATP and sulfonic acid, not reversable) is sometimes used as a marker of H_2O_2 damage [75–77]. 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 at the expense of thioredoxin. This is based on high rate constants and abundance [78]. 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 impact on the immune system (summarized in ref. 63) [63]. This greater impact on the immune system with chemically induced colitis was also found with a combined Gpx1-KO and catalase-KO mouse line and was also revealed in a study of different responses of Gpx1-/-Gpx2+/+ and Gpx1+/+Gpx2-/- mice to allergen-induced airway inflammation (OVA and aluminum hydroxide asthma) [79–81]. 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.

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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 [82]. 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). Cysteine (pKa> 8.4, thiolate anion is active form) centered peroxidases (PRDXs) have basic amino acid residues around the active site that effectively lower the pKa resulting in comparatively high ROOH rate constants (ROOH; H_2O_2, tert-butyl hydroperoxide, cumene hydroperoxide, linoleic acid hydroperoxide, etc.) [78,83]. Selenoprotein levels can reflect selenium intake when it is restricted, GPX1 more notably, but all are ultimately vulnerable in the long-term [84–88]. This is noteworthy since the standard use of 10% serum in cell culture places many cell lines in the range of at least marginal selenium-depletion relative to tissues of selenium-sufficient subjects [86–90]. GPX4, the isoenzyme involved in iron, cystine, glutamine and lipid peroxidation-related cell death, ferroptosis, has also been shown to have low levels in cell culture with standard 10% serum relative to culture media with selenium supplementation [86]. *Gpx2* is highly expressed in the epithelium of the mid-lower GI tract, and this is where we found its greatest impact in mice [67,91]. The study of Connie Eaves suggested that basal cell compartments, which would include the crypt/gland regions of the mid-to lower GI tract have high levels of GPX2 protein, exceeding those of the PRDXs [91,92]. GPX2 is more concentrated in the crypt/gland regions in rodents and humans [93–95]. GPX1-4 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 [96,97].

4. 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. 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 post-irradiation Cox1 expression [98]. 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 [91]. 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 [98]. 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 [99–102]. Finally, no protective effect of GPX2 was found.

Since *Gpx1* and *Gpx2* are both expressed in the gut and share almost identical substrate specificity, it seemed logical to test the impact of combining the Gpx1-KO and Gpx2-KO constructs. 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 [103,104]. *Prdx6* (*Aop2*) was included in our irradiation study [91]. 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 [105]. 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-/- [67]. The final combination, Gpx1/2-double knockout mice (Gpx1/2-DKO; mixed B6, 129 strain mice), resulted in spontaneous ileocolitis with high penetrance (94% with some pathology; 67% with colitis), the colitis onset prior to weaning and the ileitis peri- and post-weaning (21 days). In the original study, 60% of Gpx1+/-

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Gpx2-/- mice exhibited a colon pathology of elevated apoptosis and goblet cell depletion, with 10% showing actual colitis. Pathology was largely suppressed in Gpx1-/-Gpx2+/- mice, 20% with elevated apoptosis and some goblet cell depletion. The spontaneous inflammation in Gpx1/2-DKO mice arises 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) [91,106]. The shift in the handling of the oxidant load from peroxiredoxins to GPX2 is presumed to 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 [107,108]. Anoikis or exfoliation/extrusion/shedding of intestinal cells normally occurs at the lumen as the terminal phase of enterocyte life [109]. It is a protracted process that is leaky to lumen components and excess anoikis has been linked to recurrence of Crohn's disease [110,111]. 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, found in Gpx1/2-Duoxa-triple knockout mice, did not result promote formation of crypt abscesses, a characteristic inflammatory pathology of the Gpx1/2-DKO mice [1,67].

5. Any Application to IBD?

The findings from studies of Gpx1/2-DKO mice have little to do with IBD. 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. B6 Gpx1/2-DKO mice have different responses from other mice in another important way. Strains of *Lactobacillus* act a probiotics in experimental colitis. Production of H_2O_2 by *Lactobacillus* may serve two functions; first, to suppress potential pathogens, and second to promote restitution [112]. In B6 Gpx1/2-DKO mice, we found that *Lactobacillus* abundance in the ileum positively correlated with pathology levels and using antibiotics that promoted *E. coli*. overgrowth at the expense of *Lactobacillus* reduced the pathology [113]. In common with other mice, B6 Gpx1/2-DKO mice responded to DSS the same way, although tolerating it only at lower concentrations (1.5% DSS for DKO mice was equivalent to 2.5-3% DSS for wild-type mice). One percent DSS barely induced any gross pathology while 1.5% DSS induced SAA3 mRNA levels, an acute-phase protein, highly expressed during inflammation [72]. SAA3 was not detected in B6 GPX1/2-DKO mouse colon, although it was in 129 mice where there was lethal pathology [72,114].

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 superoxide generating NOX1 and H_2O_2 generating 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 [1,108]. The one possible connection to IBD is the dramatically increased *DUOX2* expression levels (up to 25-fold), the consequences of which might be paralleled by the

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failure to reduce DUOX2 generated H_2O_2 in the Gpx1/2-DKO mice [1]. However, use of other KOs results in uncovering other pathways to IBD such as p38-MAPK/NF-κB in a SOD1-KO with DSS treatment [29,63,81]. The problem with Gpx1/2-DKO mice is that GPX1 and GPX2 levels are suppressed too much to be a 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 22 (mice) or 23 (human) selenoproteins, GPX4 in particular, with its link to ferroptosis [115,116]. Recently, the roles of selenoproteins SelK, SelS and SelW in IBD are being explored, with impact already suggested for SelS and SelW involving macrophage polarization/epithelial cell necrosis and T cell differentiation, respectively [117–119]. And, global suppression of selenoprotein expression in macrophages might produce effects in colitis that outweigh impact elsewhere in the body [120]. The better model for this is DSS induced colitis after selenium deprivation of mice, although yielding mixed outcomes in practice [42,43,120]

6. Low Selenium Levels in IBD and Supra-Supplemention

Low selenium levels as a causative factor for IBD is unlikely, 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 ¼ 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 Gpx1+/-Gpx2-/- mice developed pathology while Gpx1-/-Gpx2+/- mice did not [121]. One study shows GPX activity (upper colon?) in CD subjects to be about ½ the levels relative to healthy controls [122]. 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 [123–125]. Meta analysis found IBD subjects had between 61% and 100% the plasma selenium levels of controls [126]. Even the worst subjects had levels above that reported for the populations of China that experienced Keshan disease, an endemic cardiomyopathy (levels down to 20 ng/ml), resulting in blood plasma GPX activity 33-43% of sufficient subjects [127–129]. 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 [129,130]. There are at least 10 case reports of cardiomyopathy in subjects on parenteral nutrition related to selenium deficiency, some without IBD and mention of intestinal complications in one case [131]. 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 [132]. In livestock, there is a selenium/vitamin E deficiency syndrome, white muscle disease, without any reported co-factors. Impact on the alimentary tract is limited to effects of muscle weakness [133].

There is evidence from the mouse model of DSS-induced colon injury that selenium supra-supplementation might be beneficial, 39 papers found using search terms, selenium, DSS, and mice, although lately the focus has shifted to GPX4 and ferroptosis (many of these are covered in the translational research section). One study with DSS treated mice showed that selenium deficiency (0.01 ppm vs 0.1 adequate; torula yeast base; reference selenium levels in standard diets AIN-76a, 0.11 ppm; AIN-93, 0.15 ppm) did not significantly worsen the pathology while supra-selenium supplementation (0.5 ppm, sodium selenite) augmented colon GPX1/2 protein levels, and this possibly contributed to reduced pathology [134,135]. 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 = 0.25ppm) did find a major worsening in pathology [42]. The discrepancy might be due to the second paper using a selenium

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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 the difference in levels between selenium sufficient and deficient human subjects in Keshan disease studies. A third paper looked at DSS induced pathology with four levels of dietary selenium, torula yeast as above (<0.01ppm; deficient), 0.08ppm (sodium selenite; adequate, although lower than AIN standards), 0.4ppm (supplemented) and 1ppm (high), provided for 10-12 weeks; 10 mice per group [120]. The motivation was to examine the role of selenium in macrophage prostaglandin metabolism and the effect of elimination of all selenoprotein expression in macrophages for response to DSS colitis pathology. The response to DSS in the selenium deficient and adequate groups were significantly worse than the other two groups. Parameters in the deficient and adequate groups were not statistically different, however, the body weights and histology scores showed some separation, deficient for the worse. There are two additional important points from this paper. First, the supplemented and high selenium groups had DSS-treated colon GPX2 levels elevated over the adequate group, while GPX2 protein was still detectable after 10-12 weeks on the selenium deficient diet and treatment with DSS. Second, the positive effect of supplemental selenium with DSS treatment was negated by elimination of selenoprotein expression in macrophages. This suggest that GPX2 elevation in the supplemented group had little impact on DSS colitis responses, when compared to the impact of selenoprotein negation in macrophages. A SelS-KO promoted M1 macrophage polarization and increased oxidant production in colon epithelial cells resulting in slightly increased but significiant DSS-induced pathology [118]. Supplementation with sodium selenite and several other selenium formulations (κ-selenocarrageenan, selenomethionine, and nano-selenium; not described) to an effective dose of selenium at 2mg/kg/day, yielded a marginal beneficial impact on wild-type mice, with sodium selenite performing worst and nanoselenium the best. Sodium selenite was unable to impact colon GPX activity levels in wild type mice while nano-selenium doubled the activity with the other supplements falling in between. Another study found sodium selenite supplemention at 1mg/kg/day over that supplied in diet for 14 days reduced pathology with DSS. Plasma selenium levels were partially sustained by the supplementation. DSS treatment lowered plasma selenium content by 20%. GPX4 protein levels were not elevated in the selenium supplementation group, but were partially restored to control levels relative to a 45% reduction in the unsupplemented group [123]. A similar impact of sodium selenite supplementation (1mg/kg/day) on GPX4 levels and mitagation of DSS pathology was found by another group [136]. Using supplementation of sodium selenite at 2mg/kg/day (oral gavage) over that from standard diet (0.1mg/kg/day), another group found increased plasma selenium levels, 375.9 ± 13.8 to 421.6 ± 17.9 μg/L, in control mice with partial restoration of plasma levels in the DSS arm of the study. The selenium content of the colon followed suit. This yielded a modest impact on the DSS pathology [43]. This same group later found that high selenium (2mg/kg/day oral gavage) impacted T cells, fewer Th1/Th17 cells more Tregs, and lowered pathology levels after multiple DSS cycles [137]. Similar impact of selenium supplementation lessening DSS pathology levels is found in other papers and attributed to increased selenoprotein expression (GPX1/2 or GPX4), effects on microbiota composition, preservation of barrier function or impact on cytokine profiles [138]. Most of these deal with modified selenium delivery, incorperation into organic compounds and nanoparticles or supplied as selenium-enriched probiotic bacteria. All report at least a moderate beneficial effect on chemically induced pathology. As an aside, low zinc and manganese levels aggravated DSS pathology [48,139].

As a rule, selenium supplementation is most effective when the subjects are initially selenium deficient (DSS effect, IBD), and risks may outweigh benefits by trying to increase levels in selenium adequate subjects [140]. 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 or minimally partial restoration to normal levels.

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7. Clinical Trials of Selenium Impact in IBD

There are a limited number of actual clinical trials on selenium supplementation and IBD outcomes with relatively small numbers of subjects. The bulk of studies only document the extent of selenium and other vitamin and trace element deficiencies in IBD [126]. There is some indication that lower selenium levels are associated with worse disease [141]. Of these the most interesting study found 6.5% of IBD patients (13 of ~195 subjects; documentation on the selenium arm of this study is deficient) had low plasma selenium levels (<0.77μmol/L, ~95ng/ml; not clearly stated) and this predicted clinical flares in the UC group. There were other deficiencies noted (folate, zinc, Mg, iron as ferritin, vitamins A, B12, C, D and E), and the overlap with low selenium was not described [142]. Studies where selenium was supplemented, or intake augmented often did this in combination with vitamins and/or other trace elements or with lipid modifying agents (fish oil with vitamins and selenium) or as part of gross dietary alterations (Mediterranean vs. Western diet) [143–145].

Three trials tested selenium supplementation with very small study populations of 9, 20 and 16 subjects. The first provided selenium (600μg/day) with β-carotene, ascorbic acid, and α-tocopherol for 8 weeks [146]. The form of selenium is not specified. Plasma selenium levels increased from 69 to 105 ng/ml, and this was accompanied by improvement in bowel movements from 6 per day to 1.5 per day. The second trial looked only at selenium supplementation to CD patients with serum selenium levels below 80μg/L, supplying 360μg/day sodium selenite for 8-12 weeks to 13 subjects (8 weeks applies to 3 subjects with mild symptoms who volunteered to waive treatment, possibly starting treatment at the end of 8 weeks; all subjects show reduced pathology at the end point) and leaving 7 others unsupplemented [147]. The context for the trial was the finding that high expression of SelW seemed to limit differentiation of Th1 cells and reduce CD pathology. Ten of the 13 subjects receiving selenium and all seven controls continued to receive treatment and this is reflected in a large reduction in CD clinical parameters from the beginning to the end of the trial. While all clinical parameters were low at the end point, there was a significant difference between the supplemented and unsupplemented groups, the supplemented group with more improvement. The third trial administered selenium at 200μg/day in combination with curcumin and green tea extract for 8 weeks [148]. Each tablet contained curcumin (500 mg), green tea (250 mg) and selenium (100 μg). Selenium appeared to be in the form of selenomethionine. Plasma selenium levels were not reported. As with the trial with 9 subjects, there appeared to be general improvement, significantly fewer bowel movement per day, less blood, and reduced clinical activity index, C-reactive protein and erythrocyte sedimentation rate. Mice treated with the same combination of ingredients, showed improvement in DSS induced colitis. Of note, curcumin produced a similar level of pathology reduction as selenium treatment and the combination further reduced pathology. Green tea extract did nothing, alone or in combination. However, the specifics of mouse dosing are lacking.

The only randomized, double blind, placebo-controlled study had 100 IBD subjects with mild to moderate UC using oral selenomethionine (200μg/ day) for 10 weeks on 50 subject and placebo on the other 50 [149]. Improvement in the simple clinical colitis activity index by 3 levels of 19 total with the levels starting at 5 to 12 (mild to moderate) was found in 38% of the selenium group and 6% of the placebo group ($p=0.001$) and clinical remission, simple clinical colitis activity index ≤2, in 20% with selenium supplementation and 4% placebo ($p=0.014$). This corresponded with increasing plasma selenium levels from 74μg/L to 117μg/L (selenium group) and no change in the placebo group (75μg/L to 76μg/L). Note that Rayman et al found 200μg/day administered for 5 years did not impact all cause morbidity, however, 300μg/day increased all cause morbidity by 11% at the 10 years follow up point [140]. It is specified that treatment of any kind less than or 1 month prior was a basis for exclusion. However, any treatments during the trial are not explicitly addressed. Basically, this is only a single well designed and controlled study testing the impact of selenium supplementation on IBD. The results seem consistent with other at least two other reports that selenium levels correlated with IBD disease activity [150,151]. Sousa et al provide more details of each of these studies, lamenting the lack of large well designed and controlled studies [145].

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8. Translational Research on Selenium Supplementation

Translational research using selenium in rodent colitis models is trending toward studies of nanoparticle formulations with complex compositions and properties with some involving selenium modification of organic agents (17 papers found from PubMed; terms, transitional research, selenium, IBD and selenium, nanoparticles, IBD; an additional 5 found using, selenium, DSS and colitis). Rather than itemizing all the findings, an overview is presented. While the potential benefits of supplementing rodent selenium intake above levels recommended for standard diets are acknowledged in such papers, the base nanoparticles or selenium modified components are often selected for potential therapeutic properties on their own; e.g. carrageen, antioxidant (not a nanoparticle study) [152]; Prussian blue, antioxidant [153]; silymarin, antioxidant [154]; tripterine (celestrol) NOX inhibitor, antioxidant [155]; Celecoxib, COX2 inhibitor (not a nanoparticle study) [156]; tungstate, modulates bacterial molybdenum-cofactor-dependent microbial respiratory pathways [157] (see references in papers for the separately mentioned properties of the co-incorporated nanoparticle agents or other components). Another feature of some formulations is the ability to target the GI tract. An alginate and chitosan hydrogel formulation enhanced nanoparticle uptake by intestinal epithelial cells [158]. Coating particles with hyaluronic acid allowed binding to CD44 expressed on epithelial cells [159]. There are a few papers in which selenium is the focus and the nanoparticle is simply a carrier, with another option being selenium used as a coat to provide stabilization to the particles without regard to any impact on supplementation of levels Radical scavenging by the nanoparticles, in vitro, is commonly reported along with SOD and catalase activities. In some cases, there is insufficient or no dosing information specifically for selenium, occasionally compensated by reporting on plasma selenium levels or impact on selenoprotein levels. There is a lack of consistent reporting on the impact on selenium plasma levels and GPX levels, and sometimes the reports conflate the potential GPX activities of the nanoparticles with possible alterations in GPX protein levels. The latter is often confined to showing partial or complete restoration of selenoprotein levels to normal relative to depletion in colitis. Another feature of much of the work is overdosing of selenium (up to 5mg/kg/day with 0.5-1.5mg/kg/day the most common dosing levels; reference ~0.02-0.030mg/kg/day for mice from AIN76 or AIN96, possibly up to 0.05 mg/kg/day) in the rodent models, possible without adverse effects due to the short duration of the studies or the attenuation of release due to incorporation into nanoparticles or with other agents. In one study, the plasma selenium levels reached 3.2µg/ml vs. ~0.18µg/ml for untreated rats, which is in the range reported in other work (0.14µg/ml-0.375µg/ml, mice and 0.17µg/ml-0.47µg/ml, rats) [118,137,158,160–163]. This was from an estimated 0.5 to 1mg/kg/day selenium equivalent dose of selenocoxib (2 forms) for 1 week [164]. While gross parameters (body weight, colon length) and colon histological parameters are reported in common, a few incorporate an examination of the microflora and impact on immunity [118,152–159,164–178]. All these publications report positive outcomes in the DSS and/or 2,4,6-trinitrobenzene sulfonic acid (TNBS; CD model) models, occasionally with the reference effect of 5-ASA.

9. NADPH Oxidases, 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.* This was demonstrated in triple KO mice, Gpx1/2-Nox1-TKO, and Gpx1/2-Duoxa-TKO. Complete absence of disease was found in the Gpx1/2-Nox1-TKO mice and the inflammation and anoikis were absent in the Gpx1/2-Duoxa-TKO while the elevated levels of apoptosis remained [1,108]. 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 [179–183]. We presumed that DUOX2 was normally confined to the lumen, based on work in other species. However, a recent paper on mice shows that while a lumen localization is the case for colon, in the ileum DUOX2 may reside in both the upper villus and

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the upper crypt [184]. This may differ from mice with DSS treatment where one study showed high expression of *Duox2* mRNA in abscessed colon crypts with a retreat to the lumen upon recovery [185]. 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 [186,187]. A recent paper describes expansion of a *LCN2*, *NOS2*, and *DUOX2* expressing cell type (LND) in ileum and colon of CD subjects that interacts with inflammatory cells via "antigen processing and presentation, Th17 cell differentiation, Th1 and Th2 cell differentiation, HIF-1 signaling pathway, and TNF signaling pathway" [188]. 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 [189]. A second paper described a DUOX2/DUOX2A/NOS2 (DN) subset in CD, that similarly interacts with T cells by the CXCL16-CXCR6 ligand/receptor pair in the ileum and monocytes via the SAA1-FPR2 ligand-receptor interaction in the colon [190]. In this case, DUOX2 was localized to the lumen. Lumen constrained DUOX2 could still transmit oxidant signals to the crypt by way of a proposed cell-to-cell transmission mechanism; direct diffusion of H_2O_2 from the lumen to the crypt/glands is considered unlikely [191]. 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. The papers defer to work by Sommer and Grasberger for a discussion of DUOX2 function [184,192].

It appears that when DUOX2 and NOX1 operate in the absence of the required antioxidant protection in mice, there are enough oxidants generated to initiate apoptosis and the related anoikis. Overactive oxidant generation by NOXs, including NOX2, respiratory burst NOX, has been long discussed as a factor in IBD. 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 [193]. Use of the NOX1/NOX4 inhibitor, GKT137831, provides some support for the claim [194]. The opposite view, deficiencies in oxidant production, has gained traction. Chronic granulomatous disease and an associated IBD, caused by *NOX2* deficiency, is the classic example [195,196]. 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 for 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 were observed at 28 days and higher levels of infiltration in the mucosa and crypt abscesses occurred 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 of *Nox2*-KO mice showed mixed outcomes with DSS, worse outcomes in four studies vs. one no change and one less pathology [197,198]. A currently promoted view is that impairment of *NOX1* and *DUOX2* by congenital mutations are involved in very-early onset IBD (about 20 documented cases) and defects in *NOX2* mimicking CD [199,200]. Although, strong support for very-early onset IBD and *NOX1* was not backed up by another study [201]. *DUOX2* mutations are also linked to congenital hypothyroidism (CH). While the condition is rare (1/2000-4000), up to 40% of cases show mutation in the *DUOX2* gene [202]. Subjects with CH had higher risk for IBD; transient CH (OR 2.39 (95% CI 1.77-3.23), permanent CH (OR 1.69 (95% CI 1.31-2.18) [203]. The over-expression of *DUOX2* observed with later onset IBD would represent a possible opposing side of the impact [186,187]. However, with the association to the LND or DN cell type that interacts with immune cells, the impact in IBD is not clear. *NOX1* levels do not

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show consistent elevation in IBD samples and when elevated are on the order of 2-to 4-fold in later onset IBD. Even when elevated, the differences often fail to approach statistical significance *DUOX2* levels are noted to increase as much as 25-fold in later onset IBD. We examined *Duox2* mRNA levels in mouse colon samples only to find that they were very low in B6 mice compared to levels in the ileum and that the Gpx1/2-DKO mice levels were not different from the wild-type levels [108]. For *Nox1*, colon levels were quite high, but again did not differ between the DKO and wild-type mice. In Gpx1/2-DKO mouse ileum, *Nox1* levels are elevated by up to 10-fold over wildtype mice with increasing levels as mice mature and the pathology increases [1]. Ileum *Duox2* levels remained at their weaning values throughout, which were lower than in wild-type mice [2].

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 [204–206]. Under-expression seems to limit the ability to repopulated damaged areas of the mid-to -upper crypt or even higher areas [180,207,208]. 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* [210]. Two studies, one very recent and both focusing on the ileum, suggest that lack of NOX1 activity may limit the generation of peroxynitrite, the product of superoxide and nitric oxide from nitric oxide synthase (NOS) [211,212]. Knaus et al note that the lack of peroxynitrite seems to increase the exposure of the epithelium to bacterial antigens and two other studies suggest that NOX1 and NOS2 help shape the colon microbiota [212,213]. GPXs could protect from the direct tissue damage of this process by reacting with peroxynitrite [214]. Absence of NOX1 in the antimicrobial role in C57B6 strain mice may be unnoted due to the functioning Paneth cells and for animal resource centers housed mice, absence of pathogens. On the negative side, Dixon et al, suggest that NOX1 would participate in ferroptosis by reacting with Fe+++ to yield Fe++ that would be freed from storage to participate in lipid peroxidation [193]. 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 [215]. 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-α and IL1-β treatment. TNF-α and IL1-β 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 [182]. The reduction in GSH levels was about 30%. While significant, it is not clear that this would compromise GPX4 and PRDX6 activity (see comments on this in the ferroptosis section). Loss of viability in CACO2 cells seemed to require the added stimulation of hepcidin, which in the presence of TNF-α and IL1-β, 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 [216].

DUOX2's role would seem to be at least a moderate interaction with the microbiota [184,192]. H_2O_2 alone could have some impact on the microbiotia, it would be a substrate for lactoperoxidase, generating the more potent hypothiocyanite and other hypohalous acids [72]. Based on our findings 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 presence 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 makes few comments about this other than to point out that under stress, ileum and colon cells tend to express genes of other lineages and refer to other

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work for possible meaning [189,190,217]. 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 substantially alter the redox status of the mouse epithelium defined as no changes in antioxidant enzyme levels [192]. Thus, the normal presence of GPXs and PRDXs are adequate to buffer the H_2O_2 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 [218,219]. 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 [220,221]. 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 in this regard dependent on the fairly reliable presence of antioxidant enzymes. NOX1 might promote healing after episodes of active inflammation. Until the role of LND/DN cells is sorted out, the function of DUOX2 in active disease remains unclear.

10. 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 [222]. 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 [1,223]. Kruidiner et al and Reynolds et al did not find increased XO protein in UC and CD subjects [224,225]. 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 [226–232]. The minority finding suggests the other action of XO inhibition by allopurinol, purine salvage, was the cause of increased pathology [232]. 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 [233–236]. While the work on XO does not address ferroptosis directly, the implication that DSS induced colitis involves ferroptosis is suggestive for such a role. While we explored XO involvement in Gpx1/2-DKO mouse pathology in unpublished studies; the effect of allopurinol was weak and along with other agents, like Trolox, a water-soluble analog of vitamin E, paled in comparison to the effect of rearing mice on semi-purified AIN diets [237,238]. Using a semi-purified AIN-76A diet had a beneficial impact on all of the mouse backgrounds and sites of pathology, the effect attributed to alterations in the microflora [237].

Lipoxygenases (ALOXs) seem to have a role in ferroptosis, seemly discovered before the term was coined, with some of the hallmark features of the phenomenon fleshed out in a 2008 paper [239]. This was found in the context of GPX4 suppression, providing a model for the basis of future ferroptosis research. Lipoxygenases can peroxidize non-esterified polyunsaturated fatty acids (PUFA) downstream from the action of phospholipase A2 activities (PLA2) and have a requirement for activation by ROOH to yield Fe+++ in the active site [240]. As discussed below, the action of PLA2 may be anti-ferroptopic 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 [241]. 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 [242]. For ALOX15 there is an apparent modification of its peroxidation potential in the presence of PEBP1, "scaffold protein inhibitor of protein kinase cascades" [243,244]. This allows ALOX15 to peroxidize esterified PUFAs, giving it a role in the more efficient membrane route of peroxidation. This mention of ALOXs leads into the final topic, ferroptosis.

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11. Lethal Lipid Peroxidation, AKA, Ferroptosis

11.1. Historical Perspective

To my detriment, I basically ignored the ferroptosis field except to note the role of GPX4 and now the explosion of studies makes it difficult to perform more than a rudimentary summation (16,000 papers) [245]. Ferroptosis, the concept, not the term, is not entirely novel since it is lipid peroxidation and the iron dependency was long since documented. The conceptual leap is that lipid peroxidation can be pushed to a lethal end by multiple mechanisms. Lipid peroxidation initiation by Fenton chemistry was widely discussed in the free radical research field as it was self-titled in 1987 (Society of Free Radical Biology and Medicine) with important work on lipid peroxidation dating to 1963 [246,247]. The essential role of cystine for cell culture and association with GSH and vitamin E is from work reported in 1977 and cell culture requirements for selenium and support of cell line GPX activity were reported at the same time [248–252] A key point was the discovery and characterization of GPX4 in 1982 by Ursini, Maiorino and their associates, tying control of iron initiated lipid peroxidation to cystine (GSH), selenium (GPX4) and, vitamin E, and showing GPX4 was unique among GPXs with its ability to reduce esterified lipid hydroperoxides [253]. Zheng and Conrad (Conrad, arguably, the first to demonstrate ferroptosis as it is currently perceived), and Hirschhorn and Stockwell (Stockwell, team lead for the conceptualization of ferroptosis and originator of the term) provide histories of the related and relevant findings that date back as far as 1935 [254,255]. I invite the readers to examine these reviews for a more complete survey on ferroptosis than can be supplied here. Zheng and Conrad, and Hirschhorn and Stockwell suggest that inducing ferroptosis might be thought of as "metabolic sabotage", the endpoint of the perturbation of many pathways that an investigator could disrupt, lacking a central set of active executioners as in apoptosis or necroptosis. These diverse pathways converge on iron regulation, cell membrane lipid composition, GSH levels, GPX4 synthesis and stability, coenzyme Q/vitamin K levels, and glutamine levels not narrowing the selection of possible pathways by much. The value of studies is that they provide a myriad of possibile ways to attack pathologies, like cancer, afresh. The initial goal of Stockwell was to find new pharmacogical agents to treat cancer, erastin (inhibitor of VDAC2 and cystine uptake) being one discovered early on and RSL3 (inhibitor of GPX4) later [256,257].

My first impression of ferroptosis was that it is a manufactured mode of cell death. That is, cells had to be inflicted with multiple unnatural stresses to observe it, deplete GPX4 (possible with low selenium levels in IBD and which might be 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 [254,255]. The role for ALOX in ferroptosis was advanced in the context of GPX4 suppression, a theme that is continued when exploring the roles of many other potentially contributing factors [239]. 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 [256,257]. 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 [254]. Stockwell and Conrad 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 and, may have been a product of evolution [258–260]. While there is some evidence of this, 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 in IBD subjects or glutamate usage as found in the nervous system [261–263]. The metabolic predilections seem to serve positive functions of the cells rather than being intentional precursors for ferroptosis activation [264,265]. Either way, liabilities are presented that can be used for therapeutic goals.

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11.2. Limitations of Cell Line Based Studies and Reliance on DSS and TNBS Animal Models

Both Stockwell and Conrad point out limitations of relying on cell culture to demonstrate that any particular pathway is promoting or inhibiting ferroptosis. It seems too easy to demonstrate and this flies in the face of redundant controls on oxidative stress, iron, and cell membrane lipid metabolism [266–269]. The study of ferroptopic agents in IBD is often limited to parallel work on cell lines, with CACO2 being the most used (noted while reading papers for this review; a systematic search, CACO2, IBD and ferroptosis returned 18 papers). The ease of inducing ferroptosis in cell lines may involve use of the common protocol of applying ferroptosis inducing agents to unintentionally GPX4 depleted cell Iines (10% serum) in a high oxygen environment to detect other influences on the process [246,270]. Low selenium levels in standard cell culture can render cell lines more vulnerable to ferroptosis, defined as increased dose dependent sensitivity to erastin and RSL3, which can be overcome by selenium supplemention [86]. Selenium-supplemented cell line GPX activity is often comparable to parent tissue samples from selenium-sufficient subjects [89,90]. Given this possiblity in culture, the use of additional ferroptosis inducing agents to detect new pathways may render lines too sensitive for the findings to be of translational value. At the very least, selenium supplementation in cell culture would serve to make results more reproducible given variations in the selenium levels of different batches of bovine serum [86–88]. One issue with high oxygen levels relates to the conversion of cysteine to cystine in culture, forcing cells to use the x_{c^-} system for uptake, in addition to other stresses inflicted on the cells [270]. Mouse embryonic fibroblasts (MEFs) isolated from an apparently healthy Slc7a11-KO mouse line (cystine/glutamine transporter, x_{c^-}) were unable stay alive in culture unless mercaptoethanol was supplied [271]. It was suggested that high oxygen might promote formation of hydroxyl radical and lipid breakdown products [246]. However, Conrad did not find oxygen levels to be a factor in the survival of GPX4 null MEFs, although there was a dependance on α-tocopherol, a lipid radical quencher [239]. The conclusion was that the peroxidation process was intrinsic to the cell membranes, supported by showing that increased cytosolic oxidant levels lagged behind lipid peroxidation intiation, one indication of the possible separation of "oxidant stress" as measured by the DCFDA assay and lipid peroxidation. Cell density and 2D cell culture, itself, can alter sensitivity to ferroptosis, producing low levels of stearoyl-CoA desaturase (SCD) resulting in high levels of cell membrane PUFAs relative to 3D culture [272]. This was also associated with responses downstream of YAP (including SCD) that alter cell membrane lipid composition [273]. GPX4 null cell survival in culture was dependent on high cell density (possibly linked to YAP via Merlin) [239]. 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.

Another issue that arose by studies looking at, but not confined to cell lines, has to do with measuring GPX activities and GSH levels with extrapolation to GPX function [274]. The assay shown in Figure 2F of Yang et al (2014), 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 strictly speaking GPX activity; directly measured total GSH levels after erastin or BSO treatment, 15 and 25% of original, while the estimated activity is ~13 and ~23% from the assay traces [245]. The conclusion is drawn from the mistake that GPX1-4 function in cells would certainly be responsive to changes in GSH levels. The standard, coupled GPX assay is set up so that there is relative saturation of the enzyme with ROOH (25-100μM, supraphysiological) and a dependency on GSH levels (not saturable with the high ROOH, being confined to levels found in cells, 1-5mM). Given that GSH is regenerated with NADPH via glutathione reductase, a steady rate of ROOH consumption (pseudo-saturation relative to the GSH levels; ping-pong mechanism) for seconds to minutes is achieved that reflects enzyme levels. This may not model what is going on in cells where the opposite conditions generally exist, [ROOH]<0.1 μM (commonly measured in the 10-100 nanomolar range in cells) and [GSH] remains in the mM range. Here, the enzyme is saturated with GSH and the activity is responsive to ROOH levels. Moderate changes in the GSH levels should not affect reaction rates, while less enzyme

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obviously will. This was empirically reframed as true under the condition that ROOH levels were less than GPX levels (0.2-6μM) [275]. The presence of PRDXs should enforce this condition, with PRDX6 able to reduce esterified lipid peroxides, partially covering GPX4 (some gap between performance of PRDX6 and GPX4). If the ROOH levels exceed the GPX levels, then a GSH concentration dependency may exist across the physiological range; actually the study used [GSH]<1mM. Generally, these parameters are not specified in studies, so the impact of marginally lower GSH levels (30% in one study [215]) and 80% in the Yang et al study is not clear [215,245]. GPX4 kinetics seem to be similar, so that the constraint that [GPX4]>[ROOH] for GPX4 to be somewhat independant of GSH levels would apply. However, the kinetic parameters were worked out largely using phosphatidyl choline hydroperoxide in Triton X-100 micelles, which may might disperse the substrate and orient the hydroperoxide so that the reactions are not dissimilar to the assay of GPX1/2/3 with H_2O_2 or linoleic acid hydroperoxide [253]. Some reaction kinetics with lipid peroxides in membranes and GSH are known [276,277]. The reaction is limited by the initial requirement for binding to the membrane, although composition dictates the rates, which can be accelerated by cardiolipin. Of greater interest is that under low GSH conditions, GPX4 might be "glued" to the membrane after a partial reaction cycle. This could create a circumstance where available [GPX4]<[ROOH] and GSH dependancy is heightened. This was demonstrated under very low GSH levels (BSO and DEM treated cells) and testing of partial depletion was not done. The levels of lipid peroxides that constitute the onset of ferroptosis are unclear and would be very context dependent. For example, brain is super sensitive, requiring seleno-GPX4, while mice have some tolerance for selenocysteine →cysteine substituted GPX4 (100-fold lower specific activity; construct made with brain protected) that depends on the mouse strain background [239,278]. Activation of PLA2 at 5 mol% ROOH content in vesicles was was determined in vitro [279]. Such activation might define incipient ferroptosis, being an emergency action where esterified lipid hydroperoxides are dispersed so that GPX1/2 and PRDXs can deal with them. The selenocysteine →cysteine substituted GPX4 ability to provide some ferroptosis resistance in mice, also conferred by selenium supplementation in cell culture, suggest that there is some reserve capacity built into normal GPX4 expression as we found for GPX2 in Gpx1-/-Gpx2+/- mice under selenium-deficient conditions. So, how did erastin treatment and the resulting 80% lower GSH levels promote ferroptosis in the Yang et al study? First, VDAC2 and 3 were targets of erastin and inhibition was found to have some effect while not essential. Second, the positive effect was confined to HRAS over expressing cell lines, which were indicated to have elevated labile iron pools [257,280]. Third, the work was performed with standard 10% serum, so that the GPX4 levels might have been somewhat low, but we have no way to determine this from the paper. The condition that [GPX4]<[ROOH] and so confering a GSH dependency was more likely in this case with the increased free iron levels contributing to elevated [ROOH] as esterified lipid peroxides. Here is the common formula for a demonstration of ferroptosis in cell lines involving multiple inputs, some intended and possibly some not, SCD and PUFA composition or Slc7a11 levels in the face of greater cystine dependence. As indicated, the findings from the study of engineered HRAS expressing BJeLR cells had less application in other RAS mutated cells lines.

A second issue is the reliance on 3 animal models in ferroptosis studies, DSS and 2,4,6-Trinitrobenzene sulfonic acid induced injury and, occasionally Citrobacter rodentum induced inflammation. This was discussed for the more general circumstance of selenium supplementation in translational research. Some discrepancies beween the conditions that apply to DSS induced inflammation and those of IBD will be indicated. Mouse and rat models may put research on a bit more solid footing with standardized diets that are sufficient in trace element and vitamin composition. However, there are major differences between mouse (rats are a little better) and human physiology that may preclude direct translation of results from mice to humans.

11.3. Oxidative Stress as Distinct from Ferroptosis

As pointed out by Stockwell and Conrad, oxidative stress and ferroptosis can be distinct processes and iron chelators may not be suitable in making a case for ferroptosis in live samples

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[254,259,270]. Ferroptosis might have some role in GPX1/2-DKO mouse pathology, but probably not in the B6 line and perhaps limited to the few extreme cases in the B6.129 mixed strain mice. The ferroptosis inhibiting iron chelator, deferoxamine, produced weak and mixed effects in a study on B6 Gpx1/2-DKO mice as opposed to findings in the rodent colitis models (data not shown in reference 1) [1,281–285]. A role for labile iron is noted in apoptosis, so that some suppression of intrinsic apoptosis might be expected with chelation [286,287]. For B6 and the mixed strain mice, we see no need to invoke ferroptosis. As to 129SV mice, there is some room for ferroptosis. The colon pathology was extreme in these mice yielding high SAA3 mRNA levels, while in Gpx1/2-DKO B6 mouse colon pathology was minimal and use of DSS (1.5%) was required to induce SAA3 [72]. The 129 N10 Gpx1/2-DKO cohort (B6, 129 mixed strain mice backcrossed to 129) was difficult to manage due to extreme morbidity. We found in unrelated work that semi-purified AIN diet (lacking yeast found in LabDiet® mouse chows; yeast was indicated to be one aggravating factor) could reduce the level of pathology in Gpx1/2-DKO mice and we were able to maintain the 129 colony [237]. The diet effect was partially attributed to microflora. This differed from the N5 cohort with moderate morbidity [114]. The difference was mapped to a locus on chromosome 2 containing the Duox2 gene, the sequence of which differs between B6 and 129, yielding altered enzyme activity [1,288]. 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. Chac1 was identified 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 [288]. CHAC1 degrades GSH and is implicated in this role as a regulator of ferroptosis [289]. Wild type 129 mice had about 2-fold higher Chac1 expression than wild type B6 mice. The levels in 129 Gpx1/-DKO mice harboring the B6 Chac1 gene and the those harboring the 129 gene were 5- and 7-fold greater than the wild type 129 and B6 mice, respectively. It seems possible that this level of increase and the greater final levels in the more impacted Gpx1/-DKO 129 Chac1 line could be part of triggering phenotype for ferroptosis. As bad as pathology was in the Gpx1/2-DKO 129 mice, a single Gpx2 wild type allele could effectively shut down disease signs and morbidity with mice of the Gpx1+/-Gpx2-/- genotype having low morbidity. We depended on this to breed the mice. Since Chac1 levels were responsive to the pathology, it is likely GSH was spared in the heterozygous mice.

11.4. GPX1/2 and PRDX1-5 Roles in Ferroptosis

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 [290–294]. 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 or by elimination of H_2O_2 and ROOH, preventing the Fenton reactionband lipid equivalent. GPX4 has its impact on esterified lipid hydroperoxides, showing propagation of lipid peroxidation in cell membranes is a unifying feature of ferroptosis [295]. 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 [296–303]. The intermediaries for generation of non-esterified lipid hydroperoxides are PLA2s. 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 [304–306]. This effect may be triggered by a critical level of lipid peroxides in the membrane. PLA2s have been shown to confer resistance to ferroptosis [307,308]. However, in the CNS, PLA2 activities are noted for increasing lipid peroxidation [309].

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A lack of impact of silencing GPX1 (GPX2 surrogate; GPX2 not expressed) on BJeLR cells was found, while silencing GPX4 was lethal [245]. The absence of an impact by silencing of GPX1, in hindsight, is not as convincing now as then. PRDX1-4 tend to dominate for ROOH metabolism, as indicated, while PRDX6 often does not act as an adequate backup for GPX4. 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 [310]. In one paper, GPX4 expression was knocked out in HepG2 cells [296]. Uptake and high levels of reduction of exogenous phosphatidylcholine hydroperoxide were detected without the authors investigating the sources of the reducing activity. HepG2 cells have average cell line levels of PRDX6 (DepMap). GPX4 is not absolutely required by cell lines for viability under standard culture conditions. We found 3 cell lines absent expression of GPX4, one of which was used heavily by A.W. Girotti in his studies of photodynamic therapy [311].

There are several papers showing that PRDXs, other than PRDX6 and, GPX1 impact ferroptosis. In some cases for PRDX, this involves indirect effects by virtue of binding to and modifying properties of other proteins such as Cullin 3 [298]. A few papers identify mitochondrial PRDX3 as a target for hyperoxidation or oxidation initiated destruction as part of or leading to ferroptosis [302,303]. Overoxidized PRDX3 was found in erastin and FIN56 treated cells. The FIN56 result (GPX4 degradation) suggests the effect initiated with esterified lipid peroxidation and the impact on PRDX3 was secondary to PLA2 action. The PRDX3 then vacated the mitochondria and blocked cystine uptake a the cell membrane. Ferroptosis required the active participation of PRDX3, presumably by the blockade of cystine uptake [303]. Protoporphyrin IX mediated cell death was found to involve ferroptosis and hyperoxidation of PRDX3 [312]. PRDX3-knockdown enhanced ferroptosis. Additonal work shows the antioxidant activities of PRDX1, PRDX2 and even GSTMu3 have protective roles in ferroptosis [300–302,313,314].

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 [315,316]. 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_{10} using NADPH) [317–319]. PRDX6 did inhibit ferroptosis in GPX4 intact cells by increasing the supply of selenium delivery in cells and therefore GPX4 levels [320,321]. 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 [322]. PRDX6 also has a lysophosphatidylcholine acyl transferase activity that may also explain its ability to remodel cell membrane composition and impact ferroptosis susceptibility [323,324].

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 [274]. 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 Kd (10^{-6}M) while cytosolic FABPs have a Kd ranging from 3 nM to 1000 nM [325–327]. 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 [325–331]. Generation

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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 and one paper suggesting both contribute, equally (search terms: PRDX6 and ferroptosis and not filtering for IBD; 16 papers) [322].

11.5. Ferroptosis in IBD

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 [193,332]. Ferroptosis is one of the branching end points of severe pathology, a variant of necroptosis, and seemingly dependent on multiple metabolic perturbations [333]. Alternatively, it could be viewed as an outcome of autophagy responding to cystine deprivation and consequently degrading ferritin [334]. 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 [335,336]. 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 [337–339]. The impact of iron in these studies is attributed to the epithelium, neutrophils and largely to the microbiota composition [340–346]. Claims for ferroptosis in intestinal epithelial cells, the immune system and by influence on the microbiota or feedback from the microbiota have all been made in the limited number of studies available.

11.6. Ferroptosis in the Epithelium/Diet

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 [347]. This parallels findings in a paper to be discussed later, where this effect is assigned to Treg cells [348]. 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)[349]. Second, they assayed GPX activity, calling it a GPX4 assay. However, the substrate was cumene hydroperoxide. GPX1-4 are all active with this substrate, GPX4 with a low rate constant relative to the rest [288]. So, this has little meaning for GPX4 and suggests a general reduction in GPX1/2 activity. 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 [350]. Another study found an opposite effect of PRDX6 in association with ferroptosis [351].

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

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against the arachidonic acid effect. Vitamin E is a chain-breaking antioxidant for lipid peroxidation and known for its interaction with selenium in pathology [352]. 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 chologenic/lithogenic diet with high cholesterol, high fat (15% coco butter) 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 [353]. These diets mark the single exception to the AIN base being preventative for Gpx1/2-DKO mouse colitis [237]. The basal diet with high fat and lacking cholesterol and cholate yielded the same low ileum and colon pathology scores found with other AIN diets [237,353]. We attributed the colitis to a disturbance of the unfolded 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. The unfolded protein response is thought to be a factor in IBD from GWAS and other studies and, linked to autophagy another factor in IBD [245,354–356]. The original colitis/ferroptosis paper mentioned above actually linked ferroptosis to ER stress [349]. 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 [357]. 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++ 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++, and ACSL4 protein and mRNA, lowered levels of GSH and GPX4 protein and mRNA, associated with increased pathology [358]. 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 [359,360]. While not looking at ferroptosis, another group found that a deoxycholate enema following DSS administration increased IL-1β production in macrophages [361]. Western-styled, high fat and low vitamin D diets were also indicated to produce hyperplasia in rat and mouse colon (20% corn oil; AIN-76A base; low vitamin D and calcium) [362]. We observed no such effects in Gpx1/2-DKO mice on a somewhat similar high fat AIN-76 diet [353].

These findings seem to further distinguish Gpx1/2-DKO mice from the DSS model with one exception. There is one paper suggesting high-fats diets reduce ferroptosis levels in DSS stressed mouse colon by increase uptake of cystine through the SLC7A11 transporter (xCT) [335]. 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 [363–365]. 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 [349]. 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 [349]. Finally, the role NF-κBp65 was examined as an intermediary between ER stress and ferroptosis, finding that an IEC specific NF-κBp65-KO (floxed gene driven by Villin-cre) resulted in increased colon damage in the DSS model. The final assessment was that phosphorylated NF-κBp65 suppressed ER stress by interacting with eIF2α, component of the integrated stress response, which is linked to autophagy [349,365]. The remaining papers on ferroptosis in the epithelium largely nominate other factors for

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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 [366–370]. 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.

11.7. Ferroptosis in the Immune System

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 [93–95]. One study suggested that macrophages from a cohort of CD subjects had low GPX1 levels while retaining GPX4 expression and, the subjects showing signs of selenium adequacy. The macrophages were of interest, as in culture the CD derived cells showed low viability during replating. H_2O_2 treatment induced ferroptosis based on lack of apoptosis, pyroptosis or necrosis markers and responses to RSL3 and liproxstatin. Cell death was shown to be GPX1 dependent by knockout. The absence of any detectable protection by PRDXs might be due to using 500μM H_2O_2, which may have overoxidized them. Overoxidation of a cysteine substituted GPX4 was also found in studies by Conrad and associates on ferroptosis [371].

There are opposing papers for PRDX4. While neither explicitly mentions ferroptosis, the use of DSS makes this possible and one of the papers measured and found lipid peroxidation in PRDX4-KO colon tumor samples. 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 [372,373]. The other paper using DSS/AOM found an effect of a PRDX4-KO producing smaller sized colon tumors that was partially mediated by impact on the immune system with expression in macrophages and reduced infiltration into the colon emphasized [63]. There was second impact found in the tumors that involved lipid peroxidation possibly promoting cell death. One difference from the 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.

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 [348]. 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

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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 [374]. In two experimental studies, however, the effects of dietary iron were attributed to an impact on neutrophils (DSS) and the intestinal microbiota (TNFΔARE mice) [340,375]. 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. While the demonstration of ferroptosis in 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 altered AA usage in immune cells and ferroptosis, studies M2 macrophages [376]. 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 [377]. 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 [378,379]. 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 [348,380–382]. 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) [383]. AHR is heavily involved in shaping the immune system of the intestine [384]. One action of CYP1A1 is generation of 19-HETE from AA [385]. However, the catalytic cycle of CYP1A1 can be disrupted leading to production of superoxide and H_2O_2 [386]. 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 [387]. Providing the Ahrr-KO mice with

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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 [388,389]. AHR is also a link to the microflora by being a receptor for microbial generated ligands, largely tryptophan derivatives like l-kynurenine [390].

Additionally, other studies have found evidence of ferroptosis in neutrophils and NK cells in other pathologies, like systemic lupus erythematosus and gastric cancer [391–393]. 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 INFα promotes transcriptional repressor CREMα 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 [393]. 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 ferroptopic Achilles' heel of the cell types, AA uptake, GPX4 levels, cystine and iron metabolism.

11.8. Ferroptosis and the Microbiota

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 [113]. 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) [285,394–397]. 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 [398]. Supplemental iron was found to activate NF-kappaB to promote DSS-colitis [340]. 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 [335]. Associations with the microbiota and DSS colitis extent to bacterial metabolites, such as butyrate and as previously mentioned, L-kynurenine. Butyrate levels in DSS were found to be low and supplementing DSS treated mice with Na butyrate relieved the colitis and ferroptosis based on GPX4 as a marker [399]. High fat diet and resultant ω-6 PUFAs are thought to aggravate CD [400]. A variation on this theme is a more direct impact of bacteria on epithelial lipid peroxidation. Adherent-invasive E. coli is linked to IBD [401]. 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 [402]. 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.

12. Use of Markers for Identifying Ferroptosis

One question is, how much papers suggesting a tendency for ferroptosis based largely on marker analysis 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. Ferroptosis is not found everywhere but certainly seems pervasive. There may be 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

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of products on cell membrane integrity [403,404]. Iron chelators might have action by inhibition of ALOXs whether full blown ferroptosis is involved or not [405–407].

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 [408–413]. MDA measured as TBARS is notoriously unreliable as a marker of oxidative stress or lipid peroxidation [414]. 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 [415]. Lower GSH levels are not certain indicators of compromised GPX function. 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 [86,272]. 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 in either a very odd or possibly very interesting outcome [416]. 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 [417]. 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 [418]. 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 1/3rd 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 [419,420]. Granzyme A (NK and CD8+ T cells) is reported to suppress ferroptosis [421]. However, IFNγ secretion by CD8+ T-cells or NK cells may shift epithelial cells in the direction of ferroptosis sensitivity [422–424]. 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.

13. 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 [425]. Some work is directed at finding IBD therapies by repressing ferroptosis. 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 experimental conditions used by many to find ferroptopic outcomes [426–429]. 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. The problem is sorting through papers with such goals for manipulations that are inconsistent with real trends and extent of those trends that might be occurring in IBD or because of dietary habits or supplementary iron for any specific cell type. On the other hand, induction of ferroptosis to reduce infiltration of selected immune cells may be possible, again with the same caveats. One of the interesting ideas along these lines are iron containing nanoparticles that can induce ferroptosis. This could be coupled with targeting coatings that could be used to modify selected components of the immune system [430].

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14. 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 [121]. 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 [431–433]. Now the focus is on NOX1/NOX4, DUOX2, ER stress and autophagy. 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 factor. Currently the exploration is based on under-performance of oxidant sources as much as overabundance of oxidants.

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