

THE EFFECT OF DOCOSAHEXAENOIC ACID ON DISEASE PROGRESSION,  
INFLAMMATORY MEDIATOR PRODUCTION, AND PROTEIN SYNTHESIS IN  
GUINEA PIGS WITH DEXTRAN SULPHATE-INDUCED COLITIS

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## **ABSTRACT**

A guinea pig model of dextran sulphate-induced colitis was developed to investigate nutritional strategies in improving gastrointestinal inflammation. The present study aimed to determine if a diet rich in docosahexaenoic acid (DHA) improves clinical and physical symptoms of inflammatory bowel disease (IBD) and whether DHA alters inflammatory lipid mediator production, and subsequent systemic effects on the acute phase response (APR). DHA improved symptoms of IBD, including; fecal blood, diarrhea, lethargy, survival, and dietary intake. Feeding of DHA increased the percent of DHA in livers (1.2% vs 0.3%), tended to decrease inflammatory mediator production, but did not decrease intestinal damage or alter protein kinetics. These results demonstrate that DHA improves clinical and physical symptoms associated with IBD, possibly involving decreased lipid mediator production. The mechanism of action does not appear to involve a blunting of the APR nor decreases in intestinal damage.

## RÉSUMÉ

Un modèle de cochon-dinde injecté de dextran sulfate colite a été développé pour investiguer les stratégies nutritionnelles afin d'amenuiser les symptômes d'inflammation gastro-intestinale (IBD). La présente étude visait à déterminer si une diète riche en acide docosahexaénoïque (DHA) améliore les symptômes cliniques ainsi que physiques de la maladie inflammatoire de l'intestin, si le DHA altère la production de lipides inflammatoires médiateurs et, les effets systémiques subséquents sur la phase de réponse aiguë (APR). Le DHA a amélioré les symptômes de l'IBD, incluant; sang fécal, diarrhée, léthargie, survie et l'apport diététique. L'apport de DHA a augmenté le pourcentage de DHA dans le foie (1.2% vs 0.3%), a tendu à diminuer la production de médiateur d'inflammation mais n'a pas diminué le dommage intestinal ou altéré la cinétique des protéines. Ces résultats démontrent que le DHA améliore les symptômes cliniques et physiques associés avec l'IBD, possiblement à travers la diminution la production de lipides médiateurs. La mécanique ne semble pas provoquer un émoussement de l'APR ou diminuer le dommage intestinal.

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## LIST OF ABBREVIATIONS

**AA:** arachidonic acid  
**ANOVA:** analysis of variance  
**APR:** acute phase response  
**ASR:** absolute synthesis rate  
**BHT:** butylated hydroxytoluene  
**CD:** Crohn's disease  
**COX:** cyclooxygenase  
**DAI:** disease activity index  
**DHA:** docahexaenoic acid  
**DSS:** dextran sodium sulphate  
**DS:** dextran sulphate  
**DXA:** dual X-ray absorptiometry  
**EER:** estimated energy requirements  
**EPA:** eicosapentaenoic acid  
**FSR:** fractional synthesis rate  
**GI:** gastrointestinal  
**IBD:** inflammatory bowel disease  
**IL-1 $\beta$ :** interleukin-1 $\beta$   
**IL-2:** interleukin-2  
**IL-6:** interleukin-6  
**IL-8:** interleukin-8  
**LOX:** lipoxygenase  
**LTA<sub>4</sub>:** leukotriene A<sub>4</sub>  
**LTB<sub>4</sub>:** leukotriene B<sub>4</sub>  
**LTB<sub>5</sub>:** leukotriene B<sub>5</sub>  
**LTC<sub>4</sub>:** leukotriene C<sub>4</sub>  
**LTD<sub>4</sub>:** leukotriene D<sub>4</sub>  
**LTE<sub>4</sub>:** leukotriene E<sub>4</sub>  
**PBMNC:** peripheral blood mononuclear cells  
**PEM:** protein energy malnutrition  
**PGE<sub>2</sub>:** prostaglandin E<sub>2</sub>  
**PGE<sub>3</sub>:** prostaglandin E<sub>3</sub>  
**PUFA:** polyunsaturated fatty acid  
**SCID:** severe combined immunodeficient  
**TCA:** trichloroacetic acid  
**TER:** transcapillary escape rate  
**TNBS:** trinitrobenzene sulphonic acid  
**TNF $\alpha$ :** tumour necrosis factor  $\alpha$   
**TXB<sub>2</sub>:** thromboxane B<sub>2</sub>  
**UAER:** urinary albumin excretion rate  
**UC:** ulcerative colitis

## CONTRIBUTION OF AUTHORS

A manuscript entitled “*Docosahexaenoic acid improves clinical and physical symptoms of intestinal inflammation in a guinea pig model of dextran sulphate-induced colitis*” is included as a part of this thesis. The following is the contribution of authors to this manuscript.

As primary author I wrote the manuscript, including all figures and tables. My responsibilities in the study included; daily maintenance of the guinea pigs, blood sampling, DXA, treatment administration, and partook in the administration of the flooding dose, as well as sample collection. I completed all sample preparation and GCMS analysis, except for the preparation of total protein in plasma and liver tissue, which was prepared by Evan Nitschmann of McGill University. Histological slide preparation and concentrations of total and albumin protein in serum was conducted at the Centre for Bone and Periodontal Research at McGill University. Dr. Marilene Paquet at McGill University subsequently analysed the slides. I was responsible for all of the statistical analysis of all of the data presented in the manuscript. Dr. Weiler provided advice on the manuscript contents, as well as technical advice on utilizing her GC and DXA, which she provided for the liver fatty acid and body composition analyses. Furthermore, she supplied this project with most of the guinea pigs, as well as all of the diets.

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## 1. INTRODUCTION AND OVERVIEW

Inflammatory bowel disease (IBD) refers to chronic inflammatory diseases of the bowel, most notably Crohn's disease and Ulcerative Colitis, which are of unknown etiology (Marteau 1999). One of the consequences of this chronic inflammation is a sustained acute phase response (APR), which results in such complications as weight loss, increased catabolism of body protein, and hypoalbuminemia (Bistrain 1999). Specifically, immune cells are activated following inflammation and release inflammatory mediators, including cytokines and products of eicosanoid metabolism. The cytokines interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumour necrosis factor alpha (TNF $\alpha$ ) mediate changes associated with the systemic phase of the acute phase response, which results in the breakdown of muscle protein for amino acid use in the liver (Blok 1996; Gruys 2005). The characteristic result of this APR on plasma protein concentrations is an increase in positive acute phase proteins, such as fibrinogen and C-reactive protein, and a decrease in negative acute-phase proteins, such as albumin (Gauldie 1994; Gruys 2005).

One of the principal pharmaceutical therapies to treat IBD is steroids. Unfortunately the various potential risks associated with long-term use of steroids illustrate the need for alternate therapies. Despite the decreased side-effects associated with current nutritional therapies such as enteral diets, there still remain unpleasant limitations, for example, the frequent use of feeding tubes, which render this therapy unappealing. Novel nutritional therapies will need to overcome these limitations, while still striving to be comparably effective in relation to steroid treatment (Jeejeebhoy 1999). Fish oil, which is rich in the omega-3 polyunsaturated fatty acids eicosapentaenoic acid

(EPA) and docosahexaenoic acid (DHA), has been shown to ameliorate the complications of IBD in both animal studies and in some clinical trials (Calder 2002). This effect of fish oil has largely been attributed to the decrease of the amount of arachidonic acid-derived pro-inflammatory lipid mediators such as leukotriene B<sub>4</sub> as well as an increase in the anti-inflammatory lipid mediators derived from EPA and DHA, termed the resolvins and protectins (Simopoulos P. 2002) (Hong Song 2003).

Studies have demonstrated that EPA and DHA, although both precursors to anti-inflammatory lipid mediators, have different downstream effects in response to inflammation (Kew 2004). These studies have illustrated the need for further research investigating the discrete anti-inflammatory effects of EPA and DHA. Despite the fact that DHA has been shown to be anti-inflammatory in the absence of EPA (Kew 2004) (Kelley 1999), there currently remains no information on the effects of DHA, in the absence of EPA, in an intestinal inflammation model. We have refined a less severe and more consistent guinea pig model of dextran sulphate-induced colitis for the purpose of studying nutritional interventions and protein kinetics. Furthermore, we have adapted the flooding dose technique for evaluating protein kinetics in this model. This model was characterized by extensive cecum crypt damage and inflammatory cell infiltrate with moderate colon involvement. This model was then used to determine the effects of a DHA rich diet on clinical and physical symptoms of intestinal inflammation, as well as its effects on the acute phase protein response via inflammatory mediator production. The principal aim was to determine if the anti-inflammatory omega-3 polyunsaturated fatty acid (PUFA) DHA is successful in ameliorating clinical and pathological outcomes of IBD.

## **2. LITERATURE REVIEW**

### **2.1 Inflammatory Bowel Disease (IBD)**

Inflammatory bowel disease (IBD) refers to inflammatory diseases of the bowel such as Crohn's disease (CD) and ulcerative colitis (UC). Although the etiology of IBD remains unknown, there is an increased understanding of the immune response associated with this disease (Dionne 1999) and of the consequences on nutritional status (Bistrrian 1999). Chronic inflammation, such as that present in IBD, stimulates a sustained acute phase response, which results in altered cytokine and inflammatory mediator production by immune cells (Bistrrian 1999; Gruys 2005). Some complications arising from this include increased catabolism of body protein (Flores 1989; Gruys 2005), increased resting energy expenditure (Azcue 1997), hypoalbuminemia and weight loss (Levine 1994; O'Sullivan 2006).

#### **2.1.1 Characteristics of Inflammation of Crohn's Disease and Ulcerative Colitis**

Chronic intestinal inflammation is characterized by the infiltration of inflammatory cells including macrophages and lymphocytes into gut tissues. Consequently, inflammatory mediators such as eicosanoids, growth factors, and cytokines are released from these inflammatory cells and initiate, synchronize and augment the inflammatory response (Collins 1994). The inflammation that occurs in both CD and UC results in the infiltration of leukocytes, which causes severe alterations of normal gut structure. The pathological result of this is edema, epithelial cell damage, increased vascular permeability, erosions and ulceration (Yamada 1994).

CD and UC are distinguishable by the location of inflammation as well as the nature of gut tissue involvement (Collins 1994). The inflammation that occurs in UC is restricted to the mucosa of the colon and is pathologically characterized by distorted crypt structure (Baumgart 2007) and continuous lesions (Marteau 1999). Macroscopic lesion characteristics include edema, erythema, and ulceration, which can penetrate further into the colonic wall in severe forms of UC (Marteau 1999). Microscopically, lesions are characterized by neutrophil infiltration, crypt abscesses, vascular congestion, and crypt branching (Marteau 1999).

Alternatively, CD inflammation is transmural and discontinuous and can occur at any section of the gastrointestinal (GI) tract. Furthermore, CD inflammation is characterized pathologically by granulomas, fissures and strictures (Baumgart 2007). The lesions present in CD may not be limited to the mucosa and can therefore penetrate into the deeper muscular, and serosa layers of the GI tract. The transmural properties of CD lesions cause bowel wall thickening and lumen narrowing (Marteau 1999).

### **2.1.2 The Acute Phase Protein Response and Inflammatory Bowel Disease**

While the acute phase response has a protective role, an overwhelming response resulting from chronic stimulation, as in inflammatory bowel disease, can have damaging effects such as destruction of tissue integrity, loss of function of tissues (Gauldie 1994), and alterations in protein metabolism (Gruys 2005). More specifically, immune cells such as neutrophils and monocytes are activated in response to inflammation and release inflammatory mediators such as proteinases, lysosomal enzymes, products of eicosanoid metabolism, including prostaglandin E<sub>2</sub> and leukotriene B<sub>4</sub>, and cytokines such as TNF $\alpha$ ,

IL-6, and interleukin-8 (IL-8). The cytokines IL-6, IL-1 $\beta$  and TNF $\alpha$  mediate changes associated with the systemic phase of the acute phase response, which results in the breakdown of muscle protein for amino acid use in the liver (Blok 1996; Gruys 2005). TNF $\alpha$  is the most prominent cytokine linked to muscle breakdown with inflammatory conditions (Pajak 2008). TNF $\alpha$  is associated with a decreased amount of anabolism. An important example is with insulin signalling pathway, where TNF $\alpha$  causes an inhibition of auto-phosphorylation of the tyrosine residues on the insulin receptor (Hotamisligil 1994). Additionally, TNF $\alpha$  has been shown to decrease skeletal muscle protein synthesis at the translational level, via the impairment of translation initiation (Lang 2002; Lang 2007). The decrease in muscle protein synthesis with inflammation has been demonstrated in previous studies, (Mansoor 1997; Jahoor 1999; Mackenzie 2003), which suggest a change in protein metabolism whereby amino acids are prioritized for acute phase protein synthesis at the expense of muscle protein synthesis. Cytokine induced changes in hepatically synthesized plasma protein concentrations associated with the acute phase response include; increased concentration of so-called “positive” acute-phase proteins such as fibrinogen, C-reactive protein and serum amyloid A, and a decreased concentration of “negative” acute-phase proteins, most particularly albumin (Gauldie 1994; Gruys 2005). However, there is evidence demonstrating that these changes in concentration may not be attributed directly to changes in their synthesis rates (Mackenzie 2003).

### **2.1.3 Hypoalbuminemia and IBD**

Albumin has several important physiological roles including the maintenance of oncotic pressure, binding and transportation of molecules, scavenging of free radicals,

inhibition of platelet function as well as anti-thrombotic effects (Margaron 1998), and involvement in the harmful effects of increased capillary permeability, including edema, and with transport and diffusion of oxygen, carbon dioxide, energy substrates and metabolites (Zikria 1989). Albumin status is often impaired in patients with inflammatory bowel disease (O'Sullivan 2006) and this can have detrimental results as hypoalbuminemia is a strong predictor of morbidity and mortality in hospitalized patients (Vincent 2003) as well as in patients recovering from surgery (Gibbs 1999). The concentration of circulating albumin, as well as other acute phase proteins, is mediated by protein catabolism, synthesis, exchange with the extravascular spaces, and losses (Fleck 1985). Increased catabolism, decreased synthesis, and extravasation have been suggested to account for the decrease in albumin concentration during episodes of stress, trauma and inflammation (Mendez 2005). However, vascular loss of acute phase proteins has been shown to increase in patients with sepsis and cancer and this loss may precede alterations in protein synthesis and catabolism (Fleck 1985). The rate of loss of albumin to the tissue spaces was measured experimentally as the transcapillary escape rate (TER) at various intervals up to 17 days following septic shock, as well as within 7 hours following surgery in cancer patients. TER was determined by measurement of the radioactivity of albumin in plasma following the insertion of radioiodinated albumin into an intravenous catheter in a forearm vein. The TER increased by more than 300% in patients with septic shock, and increased within 3 hours of surgery in cancer patients, followed by an increase to 100% by 7 hours. Considering that the normal rate of loss of albumin to the tissues is 5%/hour, the large increase resulting from the study indicate that increased vascular permeability plays a significant role in lowered albumin concentration frequently observed in disease states (Fleck 1985). Furthermore, there is evidence

supporting that the increase in TER after surgery occurs prior to the changes in plasma protein concentration associated with the acute-phase response. Positive-acute phase proteins do not demonstrate an increase (Colley 1983), and albumin concentrations do not show a decrease (Myers 1984), as quickly as TER increases following surgery (Fleck 1985). Increased loss is likely attributed to increased excretion, catabolism, or both. Cytokine mediated membrane permeability of the intestinal wall is believed to be responsible for albumin loss (Mahmud 1996). Coincidentally, loss of albumin into the urine via TNF $\alpha$  mediated glomerular leakage (Derici 2008) and loss of intact and catabolised albumin in the stool (Steinfeld 1960) in patients with IBD has been illustrated. Furthermore, in piglets with dextran-sulphate induced colitis, albumin synthesis is doubled in response to inflammation, though albumin concentrations remain unchanged, suggestive of increased loss of albumin as well as prioritization of albumin synthesis versus muscle protein synthesis (Mackenzie 2003). It has been suggested that prioritization of acute-phase protein synthesis occurs at the expense of skeletal muscle protein synthesis during episodes of stress and infection (Reeds 1994).

#### **2.1.4 Malnutrition and Inflammatory Bowel Disease**

Chronic diseases, such as IBD, are often complicated by impaired nutritional status (Reimund 2005), including decreases in body weight, muscle mass (O'Keefe 1994), as well as vitamin and mineral deficiencies (Goh J. 2003). Anorexia, chronic diarrhea, increased metabolic demands, compromised nutrient absorption (Reimund 2005), and drug-nutrient interactions are factors that contribute to malnutrition that can occur with IBD (Goh J. 2003).

The cytokine response to inflammatory stress plays an important role in malnutrition in inflammatory bowel disease (Reimund 2005). An increase in the production of systemic and tissue inflammatory mediators may propagate a vicious cycle of wasting in response to IBD and malnutrition (Citters 2003; Reimund 2005). In combination with this cytokine-induced malnutrition there is a concurrent malnutrition-induced increase in cytokine expression whereby malnutrition increases the expression of cytokines such as IL-1 $\beta$  (Cederholm 1997) and IL-6 (Lyouni 1998). The metabolic consequences of this and the resultant production of acute-phase proteins are vast (Citters 2003). These include the catabolism of muscle protein for amino acid use in the liver, sequestration of iron and zinc, increased gluconeogenesis, increased loss of body nitrogen, retention of water and sodium, increased loss of vitamins as well as potassium, phosphate, magnesium and zinc (Beisel 1995).

### **2.1.5 Dextran Sulphate Sodium Animal Models of Inflammatory Bowel Disease**

Several animal models of intestinal inflammation have been developed that are similar to human IBD. Some means by which animal models develop intestinal inflammation are by administration of a toxic chemical such as acetic acid or dextran sulphate (DS), genetic manipulation such as with strains of 'knockout' mice, and alterations of the immune system, including intravenous injection of preformed immune complexes (Hodgson 1978). Although these animal models of intestinal inflammation resemble inflammatory bowel disease, it is difficult to assess their values because the pathology and etiology of IBD remain unclear (Taylor 2003). However, for studies examining the roles of lipid mediators in inflammatory bowel disease, virtually all animal

models are valid because lipid mediators are involved in the final common pathway of all varieties of intestinal inflammation (Stenson 1994).

Dextran sulphate sodium (DSS) is a sulphated polysaccharide which, when typically administered in the drinking water of some laboratory animals, can be used to induce ulcerative colitis-like lesions in the large intestine (Okayasu 1990; Yamada 1992). Although the mechanism of action of DSS remains unclear (Cooper 1993), the intestinal inflammation that ensues when administered to hamsters, rats and mice is characterized by diarrhea, bloody stools, weight loss, shortening of the colon, mucosal ulceration and infiltration of neutrophils. Increased inflammatory mediators such as with leukotriene B<sub>4</sub> (LTB<sub>4</sub>), IL-2, IL-4, and IL-6 are present in established lesions (Elson 2003). In response to DSS, intestinal inflammation occurs in severe combined immunodeficient (SCID) mice, suggesting that this model of IBD is not appropriate for studies involving the acquired immune system (Dieleman 1994). However, due to the simplicity, reproducibility, and ability to quantify disease severity with this method, it remains an efficient animal model for studies involving intestinal inflammation (Elson 2003).

#### **2.1.6 Guinea Pig Model of DSS-Induced Inflammatory Bowel Disease**

Inducing IBD via DSS in the guinea pig has been shown to be a practical animal model due to the quick and reliable induction of the disease (Watt 1971; Grasso 1973; Iwanaga 1994; Hoshi 1996). The guinea pig intestine is rich in lamina propria macrophages in the healthy state (Sawicki 1977). There is evidence demonstrating that macrophages in the lamina propria take up sulphated polysaccharides, such as DSS (Watt 1971; Iwanaga 1994; Hoshi 1996). Thus guinea pigs are particularly susceptible to IBD due to this large population of macrophages in their gut. Despite the high susceptibility

of guinea pigs to DSS-induced IBD, few studies have used this model for studies involving intestinal inflammation. Two studies have been conducted evaluating the characteristics and the course of action of DS-induced colitis in the guinea pig. Iwanaga et al. (1994) and Hoshi et al. (1996) terminated guinea pigs at specific time points following administration of a 3% DSS solution administered in the drinking water, with their cecums and colons removed for histological analysis. Food intake was not recorded. Clinically, the guinea pigs produced bloody and loose stools between 48 and 72 hours and greater than 90% of the animals died within the 96 hour time point. In the early stages of the disease progression, almost all of the cryptal lumina in the cecum were destroyed, which ultimately resulted in the loss of the crypts. Furthermore, hemorrhaging and lesions were detected in the cecum and colon and the amount of lamina propria macrophages were increased following DSS administration. Further investigation revealed that the macrophages were found to be most prominent in the cecum and decreasing in abundance towards the rectum.

One limitation of the studies by Iwanaga et al. (Iwanaga 1994) and Hoshi et al. (Hoshi 1996) is that the volume of drinking water consumed, and therefore the volume of DSS solution consumed, was not reported though can be presumed to be highly variable based on drinking behavior of guinea pigs and the severity of disease. Consequently, the dose of DSS to be administered to guinea pigs, which results in IBD, has never been determined. It cannot be confidently determined how much DSS solution guinea pigs consume when the DSS is administered in the drinking water as guinea pigs tend to play with their water and consequently drain their water bottles into their cages (Terril 1998). Elucidating the dose of DSS that results in a moderate non-lethal IBD condition in the

guinea pig would be beneficial in developing a more reproducible, efficient, and practical animal model of this disease.

### **2.1.7 Evaluation of Techniques to Measure Protein Synthesis in the Guinea Pig**

Individual tissues in the body have different rates of protein turnover, and these rates vary under different physiologic conditions, such as with inflammation and nutritional status. Due to this, measurement of protein synthesis at the tissue level as opposed to the whole body level is fundamental for determining the tissues' contribution to protein turnover (Davis 2001). The two most common methods for measuring protein synthesis are the constant infusion method and the flooding dose technique, which measure the incorporation of tracer amino acids (isotopically labelled amino acid) into protein in order to quantify tissue and individual plasma protein synthesis (Fu 1998; Caso 2000; Mackenzie 2003). These methods have both been implemented in humans as well as animals (Hernandez 2000; Davis 2001; Mackenzie 2003).

The constant infusion method involves infusion of a tracer amino acid at a constant rate for typically 4-8 hours in order to obtain steady-state labelling of the free amino acid pool. This lengthy infusion period is advantageous for measuring slow turning over proteins such as muscle proteins. However, the use of this method in small animal studies is inconvenient as it requires surgery for catheterization (Wykes 1993). This method is invasive and requires multiple catheterizations for the duration of the study. Furthermore, the inflammation resulting from surgery may bias the outcomes.

Alternatively, the flooding dose method was first used in the 1950's by Loftfield et al. (Loftfield 1954), and was further refined by Garlick et al. (Garlick 1980) and involves the injection of a large dose of the tracee amino acid along with the tracer amino acid for a much shorter labeling period (10-30 minutes) than with the constant infusion method. One of the objectives of this technique is to rapidly increase the labeling of the intracellular amino acid pool to allow for the shorter labeling period. Furthermore, this method aims to solve the challenge of sampling from the true aminoacyl-tRNA precursor pool by minimizing the variation in isotopic enrichments between the intra/extracellular and aminoacyl-tRNA pools (Davis 2001).

Use of the flooding dose in small animals has distinct advantages over the use of the constant infusion method. The greatly reduced periods (10-30 minutes versus 4-8 hours) provide more efficient sampling to occur, and due to this short labelling time the flooding dose method is advantageous for measuring rapidly turning over proteins. Furthermore, the flooding dose requires a single injection of tracer (Davis 2001) as opposed to the constant infusion method, which requires surgery to insert catheters for prolonged infusions of the tracer (Wykes 1993). The flooding dose method is an efficient and convenient method presuming that the flooding conditions are met. These conditions are that the period of labelling be kept short (~30 minutes), and that the dose of tracee amino acid be sufficiently large (~5-10 times the endogenous flux of the tracer). The result is that there is little variation in the isotopic enrichment of the precursor pool during the infusion as well as there is a linear increase in the enrichment of the protein bound amino acid (Davis 2001).

Despite the advantages of using the flooding dose technique in small animals, this method has never previously been used in studies involving guinea pigs. The development of a model of protein synthesis in the guinea pig was based on previous studies using the flooding dose technique in small animals. Evaluation of studies in rodents and pigs indicated that many of the studies utilized a tracer/tracee solution concentration of 0.15 mol/L (Davis 1999; Welle 2006; Bregendahl 2008; Gasier 2009) a dose of 0.01 L/kg (Davis 1999; Bregendahl 2008; Gasier 2009) and, in a study utilizing L-[ring-<sup>2</sup>H<sub>5</sub>]-phenylalanine, a molar enrichment of 40% (Bregendahl 2008). These concentration, dose, and molar enrichment were tested successfully in a pilot study with the guinea pigs, and consequently, used for the entire study.

## **2.2 Potential Role of Fatty Acids in Inflammatory Bowel Disease**

Inflammatory bowel disease is a chronic disease for which the current medical therapy is limited by numerous stressful and harmful potential side-effects (Feldman 1994). For example, long term use of corticosteroids for both ulcerative colitis and Crohn's Disease may result in numerous adverse effects including, but not limited to, osteoporosis (Compston 1987), hypertension, glucose intolerance, and infection (Hanauer 1994; Plevy 1994). These and other undesirable side-effects emphasize the need for less harmful therapies. One such potential therapy that has been a popular area for research is dietary polyunsaturated fatty acids (Belluzzi 2000). Polyunsaturated fatty acids are believed to play a role in inflammation via the action of their soluble mediators termed eicosanoids. Prostaglandins, leukotrienes and thromboxanes produced from membrane phospholipids exert immunomodulatory effects, which support a role for these eicosanoids in the pathogenesis of IBD. More specifically, after an initiating event

triggers the inflammatory process the inflammatory response will then be amplified through the release of pro-inflammatory eicosanoids, which attract circulating leukocytes to the site of injury. Subsequent tissue injury is produced by infiltrating neutrophils and their release of proteases and reactive oxygen metabolites by myeloperoxidase. Eventually damage will occur to the epithelial layer as the neutrophils travel across the epithelium and form crypt abscesses. The eicosanoid leukotriene B<sub>4</sub>, released from neutrophils, is a particularly powerful stimulus of neutrophil secretion, aggregation, adherence and migration (Wallace 1994). In addition to this, LTB<sub>4</sub> is capable of increasing the production of stimulators of the acute phase response such as TNF $\alpha$ , IL-1 $\beta$  (Rola-Pleszczynski 1988), IL-6, as well as increasing vascular permeability (Calder 2002), increasing the amount of interferon gamma, interleukin-2 (IL-2), modifying the proliferation of B lymphocytes (Rola-Pleszczynski 1988), and stimulating the release of reactive oxygen species by neutrophils as well as proteases and myeloperoxidase (Wallace 1994). Tissue injury can be produced by protease and myeloperoxidase enzymes, with the latter playing a particularly significant role as it catalyzes the oxidation of chloride by hydrogen peroxide to generate hypochlorous acid (Wallace 1994). Hypochlorous acid is an extremely cytotoxic substance as it is extremely reactive with a variety of biological substrates (Yamada 1994).

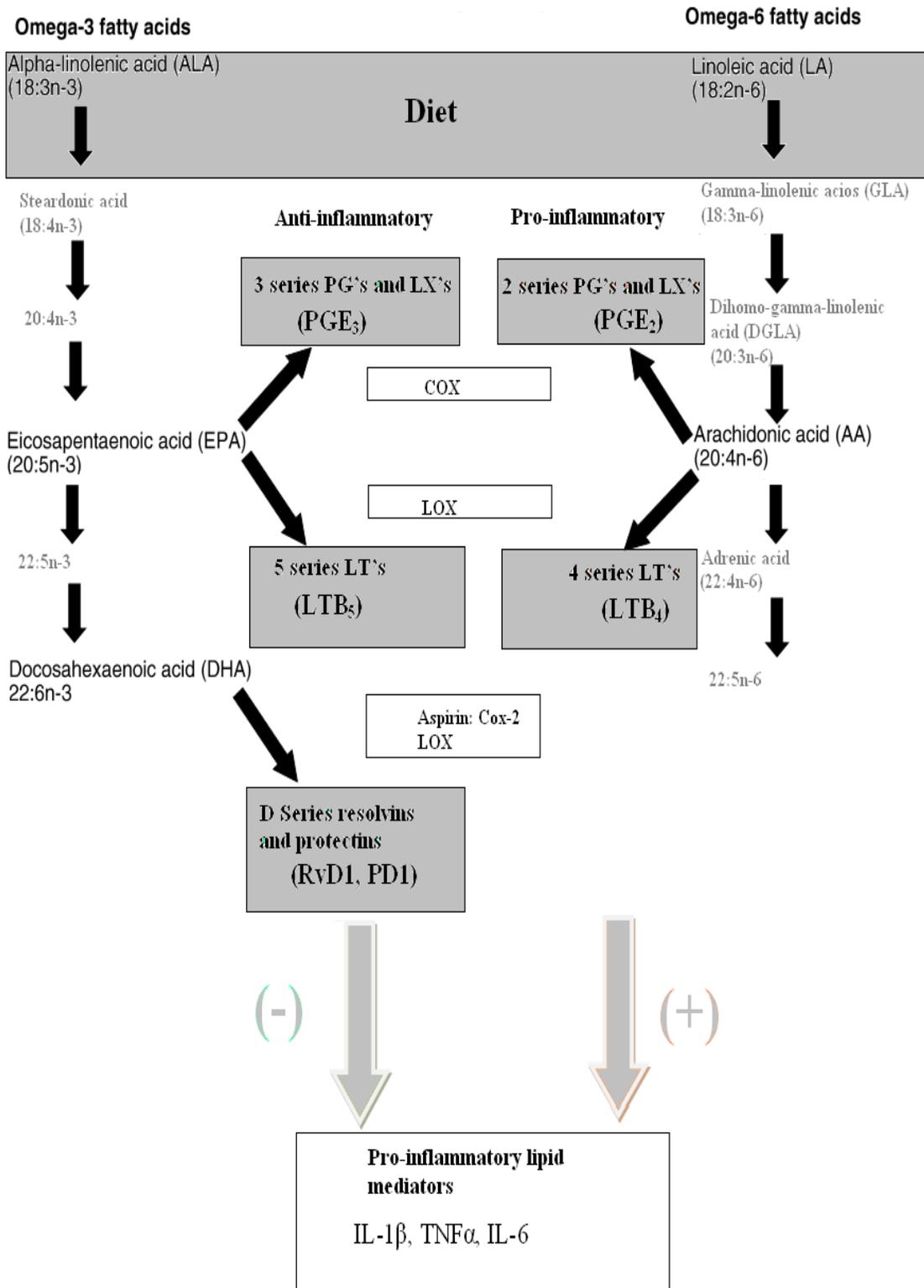
### **2.2.1 Role of Omega-3 and Omega-6 Fatty Acids in Inflammatory Bowel Disease**

In Japan the recent declining consumption of fish, which is rich in omega-3 PUFA, and the increasing intake of animal protein have been linked to the increasing incidence of inflammatory bowel disease (Shoda 1996). Furthermore, there is evidence

demonstrating that patients with IBD have altered plasma phospholipid profiles, such that the omega-3 phospholipids are decreased due to altered metabolism (Geerling 1999). The metabolic pathways of omega-3 and omega-6 polyunsaturated fatty acids and their relationships with inflammatory mediator production are well understood (**Figure 1.1**). Polyunsaturated fatty acids of the omega-3 and omega-6 series are substrates for eicosanoid production (Razack 2007). Linoleic acid (18:2 n-6, an omega-6 PUFA) is metabolized to arachidonic acid (AA) (20:4 n-6), which is further metabolized to the pro-inflammatory eicosanoids such as LTB<sub>4</sub>, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) as well as other 2 and 4 series eicosanoids. Alternatively, alpha-linolenic acid (18:3 n-3, an omega-3 PUFA) is metabolized to eicosapentaenoic acid (EPA) (20:5 n-3) and docosahexaenoic acid (DHA) (22:6 n-3) (Simopoulos 2002; Razack 2007). Whereas the omega-6 PUFA are metabolized to pro-inflammatory eicosanoids, EPA is further metabolized to the anti- or less pro-inflammatory eicosanoids such as leukotriene B<sub>5</sub> (LTB<sub>5</sub>) and prostaglandin E<sub>3</sub> (PGE<sub>3</sub>) as well as other 3 and 5 series eicosanoids (Simopoulos 2002). Similarly DHA is metabolized to anti-inflammatory compounds termed the docosatrienes and D series resolvins (Hong 2003; Hudert 2006). These resolvins and protectins play a role in the resolution phase of inflammation (Serhan 2008). The resolution phase of inflammation is considered a separate process from the anti-inflammatory process due to the combined action of reduction of neutrophil infiltration and the promotion of macrophage activity via pro-resolution molecules (Schwab 2007). This process begins with prostaglandins and leukotrienes activating and amplifying inflammatory signals, and then is followed by the promotion of the synthesis of resolvins, protectins, and lipoxins (Levy 2001; Serhan 2008). The mechanism of action of the D series resolvins and protectins, biosynthesized

from DHA, in resolving inflammation involves the inhibition of neutrophil migration (Kasuga 2008).

In addition to the production of anti-inflammatory mediators, the omega-3 PUFA, EPA and DHA, are believed to exert an anti-inflammatory effect by partially replacing AA in the membranes of immune cells. The resulting altered ratio influences enzymatic competition between the omega-6 and omega-3 fatty acids and thereby eicosanoid metabolism. More specifically, a decreased amount of AA in cell membranes has been shown to result in a decreased production of potent pro-inflammatory mediators, such as LTB<sub>4</sub> (Simopoulos 2002). In addition to this, EPA competes with AA at an enzymatic level the enzymes cyclooxygenase (COX) and lipoxygenase (LOX) use both these PUFA in eicosanoid metabolism. Both EPA and DHA have been shown to suppress the production of the cytokines IL-1 $\beta$ , TNF $\alpha$  (Simopoulos 2002), and IL-6 (Khalifoun 1997) which play central roles in the stimulation of the acute phase protein response (Castell 1989). Furthermore, omega-3 PUFA have also been shown to exert anti-inflammatory effects via increased membrane fluidity (Tappia 1997), to alter lipid raft fatty acid composition (Stulnig 2001), signal transduction (Speizer 1991), gene expression (Kliwer 1997), and gastrointestinal flora (Bomba 2003), to modulate immune response by alteration of antigen-presenting cell function (Hughes 1996), and finally to activate PPAR  $\gamma$  expression, which promotes healing of the epithelial barrier in the colon (Bassaganya-Riera 2006). However, despite these potential explanations for the effect of omega-3 PUFA on inflammation the definitive mechanism remains unclear.



**Figure 1.1:** The metabolic pathways of omega-3 and omega-6 PUFA and their relationship with inflammatory mediators. Adapted from (James 2000; Mills 2005; Serhan 2006).

### **2.2.2 Polyunsaturated Fatty Acid Studies in Inflammatory Bowel Disease**

Several studies have investigated the effects of polyunsaturated fatty acids on inflammation and in IBD in particular. The majority of these studies use fish oil as the source of EPA and DHA, since these are the two main omega-3 PUFA found in fish oil, which is also the most concentrated dietary source. Data obtained from animal studies have yielded promising results in finding therapeutic effects of fish oil on inflammatory bowel disease, whereas human studies have demonstrated conflicting results. Despite evidence that EPA and DHA exert anti-inflammatory effects by different mechanisms, most clinical trials investigate the combined effect of these two PUFA on IBD. Even though DHA has been shown to have anti-inflammatory effects, no study to date has been conducted examining the effect of DHA in the absence of EPA on inflammatory bowel disease.

### **2.2.3 Guinea Pig Model for Lipid Studies**

Guinea pigs are considered a good model for studies involving lipid research due to the fact that their blood lipid profiles and responses to dietary lipid interventions are similar to those of humans (Fernandez 2001). Furthermore, for studies involving eicosanoid metabolism, guinea pigs have demonstrated the decreased production of pro inflammatory lipid mediators in response to omega-3 PUFA. Further supporting this model for use in studies investigating eicosanoid responses to dietary lipid treatment (Mayatepek 1994) is the reduction of thromboxane A<sub>2</sub> (Ewart 2002), leukotriene B<sub>4</sub> and prostaglandin E<sub>2</sub> in guinea pigs fed n-3 PUFA.

### **2.2.4 Animal Studies Investigating Effects of Omega-3 PUFA on IBD**

Several animal studies have yielded evidence of a beneficial effect of omega-3 polyunsaturated fatty acids on disease progression as well as production of cytokines and eicosanoids in models of inflammatory bowel disease. Rats with trinitrobenzene sulphonic acid (TNBS)-induced colitis consuming a cod liver oil diet rich in omega-3 PUFA, EPA and DHA (5.95 mg EPA/g diet and 6.91 mg DHA/g diet), versus a sunflower oil diet rich in omega-6 PUFA had colons virtually absent of inflammation and ulcerations (Vilaseca 1990). This histological improvement may have been mediated by the lower levels of the pro-inflammatory eicosanoid thromboxane B<sub>2</sub> (TXB<sub>2</sub>) present in the fish oil group as compared to the sunflower oil group. Improved colonic damage may also be mediated by reduced levels of the pro-inflammatory eicosanoid prostaglandin E<sub>2</sub>. Rats fed a fish oil diet (4.16 mol EPA/100 mol fatty acid methyl esters and 3.01 mol DHA/100 mol fatty acid methyl esters) were found to have lower levels of this eicosanoid in comparison to rats fed an olive oil diet (Nieto 2002). In a rat TNBS colitis model, the APR-activating cytokine IL-6 was reduced in rats fed the omega-3 PUFA diet as compared to those fed omega-6 PUFA diet. This reduction in IL-6 may have been associated with the decreased colonic damage (Andoh 2003). In summary, animal studies have indicated a therapeutic effect of omega-3 PUFA on intestinal inflammation. This effect may be associated with omega-3 PUFA derived anti-inflammatory lipid mediators as well as the blockage of APR-inducing cytokine secretion. A question that remains unresolved is: are other potent pro-inflammatory cytokines and eicosanoids, such as TNF $\alpha$  and LTB<sub>4</sub>, also reduced by omega-3 PUFA diets? Furthermore, how omega-3 PUFA will affect inflammation-induced changes protein metabolism, including APR protein, such as albumin and fibrinogen. It remains to be seen if omega-3 PUFA are capable of blunting the APR, via the decrease in pro-inflammatory lipid mediators, such

as LTB<sub>4</sub>. A decrease in LTB<sub>4</sub> may translate to decreased cytokine stimulators of the APR, including TNF $\alpha$ .

### **2.2.5 Human Studies Investigating the Effects of DHA and EPA on IBD**

Although animal studies have yielded promising results, results from human studies investigating the effects of EPA and DHA on IBD have been inconsistent. Patients with ulcerative colitis receiving 2.7 g EPA/day and 1.8 g DHA/day for 2 months demonstrated moderate improvement in indicators of clinical disease activity (Salomon 1990). Additionally, patients in a randomized, double-blind, placebo-controlled, crossover trial, with active ulcerative colitis receiving fish oil supplementation (containing 3.24 g of EPA and 2.16 g of DHA) for four months achieved improved rectal dialysis levels of LTB<sub>4</sub> and less histological damage (Stenson 1992). In a similarly controlled trial, Aslan et al. (Aslan 1992) found that mean disease activity index declined by 56% for patients with active UC receiving 4.2 g omega-3 PUFA/day for 3 months (placebo only declined by 4%). Furthermore, they found a 72% decrease in steroid usage in patients consuming fish oil. In contrast to the study by Stenson et al., however, Aslan et al. did not find a reduction in mucosal LTB<sub>4</sub> levels. A double-blinded trial (Almallah 2000) provided further evidence of the beneficial effects of omega-3 PUFA in patients with distal proctocolitis. Those receiving a fish oil extract (EPA 3.2 g, DHA 2.4 g) daily for six months had improvements in disease activity, histological scores and immune reactivity compared to those given sunflower oil. Seidner et al (Seidner 2005) conducted a randomized controlled trial in patients with UC demonstrating the benefit of oral supplementation of combined fish oil, soluble, and antioxidants on improved clinical response and decreased use of corticosteroids. A particularly well-designed clinical trial

conducted by Belluzzi et al (Belluzzi 1996) aimed to determine if fish oil could reduce the frequency of long term relapse rates in patients with IBD. They performed a one-year, double-blinded, placebo-controlled study with patients in remission from Crohn's disease and at a high risk for relapse. The treatment contained 2.7 g daily of omega-3 fatty acids (40% EPA, 20% DHA) in capsules that were covered with an enteric coating such that the capsules were protected against gastric acidity for at least 30 minutes and therefore targeted the site of inflammation. After one year the remission rate of the omega-3 PUFA group was significantly greater (59%) as opposed to the 26% remission rate in the placebo group. These studies indicate a potential clinical benefit of omega-3 PUFA supplementation on IBD patients.

Unlike in animal studies, multiple human studies do not consistently indicate a therapeutic effect of EPA and DHA on inflammatory bowel disease. For example, Middleton et al (Middleton 2002) found that disease relapse rates are similar between UC patients receiving 270 mg EPA/day, 45 mg DHA/day and 1.6 grams per day of gamma-linolenic acid for 12 months. Alternatively, Lorenz et al (Lorenz 1989) found that UC patients receiving fish oil (3.2 grams fatty acids/day) for 7 months showed a reduction in inflammatory lipid mediators. However, a clinical effect was not seen in the CD patients receiving the same diet. Reduced inflammatory mediators from EPA and DHA is further supported in a study (Hawthorne 1992), in which UC patients were treated with either fish oil (20 ml HiEPA with 25% EPA and 6% DHA) or olive oil for one year. However, this only translated into a limited clinical benefit. The investigators concluded that the supplementation provided a modest corticosteroid sparing effect, however there is no benefit in maintenance therapy. Similarly, Loeschke et al (Loeschke

1996) noted no benefit from maintenance therapy of 5.1 g of combined EPA and DHA/day or a placebo for two years, to patients with UC in remission. Lorenz-Mayer et al (Lorenz-Meyer 1996) investigated the effect of EPA and DHA on Crohn's disease patients recovering from a relapse. The length of remission was not extended in the patients as a result of receiving 5 g of an omega-3 fatty acid compound (containing 50% EPA and 30% DHA) for one year. More recent studies have also found little evidence supporting the use of omega-3 PUFA clinically. One such study compared the use of fish oil (5.4 g/d) with sulfasalazine (2 g/d) for two months in a randomized cross-over trial in patients with UC (Dichi 2000). This resulted in a greater disease activity index at the end of the trial with the omega-3 PUFA supplementation. Furthermore, when previous studies examining the effects of omega-3 PUFA for maintenance of remission in CD (Turner 2009) and UC (Turner 2007) were analyzed, the authors concluded that the current data from these studies do not provide sufficient evidence for the routine use of omega-3 PUFA for maintenance treatment.

### **2.2.6 Summary of Clinical Studies**

It is evident that there are conflicting results concerning the effects of omega-3 PUFA on IBD, some studies having indicated a therapeutic benefit of EPA and DHA on IBD, while others demonstrated limited clinical improvements. Many suggestions have been proposed to explain this inconsistency. Cross-over studies often had short wash-out periods of 1-2 months between treatments and this short wash-out period did not allow for a complete displacement of the extra fatty acids from the cell membranes (Belluzzi 2000). Another reason why studies get differing results is that most are not sufficiently powered to account for the large variability between patients' cytokine levels (Yaqoob

2003). Furthermore, compliance becomes an issue in many of the trials as fish oil has an unpleasant taste and many of the patients were able to tell if they were receiving the fish oil treatment or placebo (Belluzzi 2000). Finally, one of the major reasons why these studies have conflicting results is due to the differing doses and compositions of the fish oils used. Little is known whether the anti-inflammatory effects demonstrated by fish oil can be contributed to some amount of EPA or DHA or a combination of both (Yaqoob 2003). However, what is known is that EPA and DHA, although members of the omega-3 PUFA family, do not have identical anti-inflammatory effects. This emphasizes the need for studies further investigating the differential effects of DHA or EPA on inflammation.

### **2.2.7 Separate DHA and EPA Studies**

Only a few studies have been conducted investigating the differential effects of DHA or EPA. Kew et al (Kew 2004) investigated the effects of supplementation with either an EPA rich or DHA rich oil on a range of immune outcomes in 42 healthy volunteers. In a placebo-controlled, double-blinded, parallel study, the volunteers were supplemented with either olive oil, EPA (4.7 g/day) or DHA (4.9 g/day) for 4 weeks. Each diet altered the fatty acid composition of the plasma phospholipids and neutrophil concentrations were altered by the EPA or DHA as expected. However, DHA, but not EPA, supplementation decreased T lymphocyte activation. Kelley et al (Kelley 1999) examined the effects of DHA supplementation (6g DHA/day) in 11 healthy men for 120 days on fatty acid composition, eicosanoid production, and select activities of human peripheral blood mononuclear cells (PBMNC). DHA supplementation resulted in an increased DHA content in PBMNC, as well pro-inflammatory eicosanoids PGE<sub>2</sub> and

LTB<sub>4</sub>, natural killer cell activity and in vitro secretion of IL-1 $\beta$  and TNF $\alpha$  were decreased. Therefore, these studies indicate that although EPA and DHA are members of the omega-3 PUFA family and are often used in combination in studies, they exert different inflammatory effects. They demonstrate that DHA may have potent anti-inflammatory effects on its own and that its effects may be mediated by reduction in PGE<sub>2</sub>, LTB<sub>4</sub>, IL-1 $\beta$ , and TNF $\alpha$ . The question remains as to whether DHA can similarly reduce pro-inflammatory eicosanoid and cytokine production in a model of intestinal inflammation and whether this translates into a therapeutic benefit.

### **3. Rationale**

Chronic intestinal inflammation is often complicated by impaired nutritional status (Reimund 2005), including decreases in body weight and muscle mass (O'Keefe 1994), as well as hypoalbuminemia (O'Sullivan 2006). Furthermore, patients with IBD endure clinical and physical symptoms of intestinal inflammation, including bloody stools, abdominal pain, weight loss, diarrhea, and fever (Baumgart 2007). Food avoidance, cytokine-induced anorexia (Kelley 1999), chronic diarrhea, increased metabolic demands, compromised nutrient absorption (Reimund 2005), and drug-nutrient interactions are factors that contribute to malnutrition and decreased intake that can occur with IBD (Goh J. 2003). One of the consequences of this chronic inflammation is a cytokine-mediated acute phase response (APR), which results in such complications as weight loss, hypoalbuminemia (Bistrain 1999), and increased catabolism of muscle protein for amino acid use in the liver (Blok 1996; Gruys 2005).

Omega-3 PUFAs EPA and DHA derived from fish oil are known as anti-inflammatory and have been considered as a nutritional therapy for IBD. The basis of the anti-inflammatory effects of EPA and DHA has largely been attributed to their effects on inflammatory lipid mediator metabolism. EPA and DHA are believed to decrease the amount of arachidonic acid-derived pro-inflammatory lipid mediators such as leukotriene B<sub>4</sub> as well as an increase in the anti-inflammatory lipid mediators derived from EPA and DHA, termed the resolvins and protectins (Simopoulos P. 2002) (Hong Song 2003). This may have a beneficial effect on inflammation as pro-inflammatory lipid mediators, such as LTB<sub>4</sub>, function to increase: the production of cytokine stimulators of the APR (Rola-Pleszczynski 1988) neutrophil activity, vascular permeability (Calder 2002) and the

release of reactive oxygen species by neutrophils as well as proteases and myeloperoxidase (Wallace 1994). Additionally, the resolvins and protectins metabolized from EPA and DHA are involved in the resolution phase of inflammation (Serhan 2008).

Data from animal studies have supported a potential therapeutic effect of the omega-3 PUFA EPA and DHA on IBD. EPA and DHA have been found to decrease intestinal damage (Vilaseca 1990), decrease pro-inflammatory eicosanoid production (Vilaseca 1990; Nieto 2002), and decrease the secretion of pro-inflammatory cytokines and stimulators of the acute phase protein response such as IL-6 (Andoh 2003).

Although some human studies have shown agreement with the outcomes of the animal studies, there is far more inconsistency that has resulted from these trials. One suggestion to explain this inconsistency may be that the omega-3 PUFA treatments vary greatly in their dose and composition amongst the various studies. EPA and DHA have been shown to exert different anti-inflammatory effects (Kew 2004), and the lipid metabolite of DHA, termed resolvin D1 has been shown to inhibit neutrophil migration (Kasuga 2008). Despite this, clinical trials utilize mixtures of both EPA and DHA. Furthermore, the only trials that have investigated DHA as a supplement on its own, were conducted in the healthy state, rather than in an inflamed one (Kelley 1999; Kew 2004). Thus, studies investigating the effects of DHA on clinical disease severity, GI inflammation, cytokine production, and the acute phase response remain to be conducted.

The consequences of omega-3 PUFA on the acute phase response and protein metabolism may translate into a blunted APR, thereby decreasing the extent to which muscle protein synthesis is compromised and albumin synthesis is prioritized. The utilization of the flooding dose technique and the development of a reproducible guinea

pig model of colitis, will allow us to examine the relationship between DHA and its impact on protein kinetics with intestinal inflammation. Furthermore, the question remains whether DHA can be effective in improving clinical and physical symptoms associated with IBD and whether this is associated with decreased pro-inflammatory lipid production. In order to investigate this question in the adult guinea pig, a reproducible model of DS-induced colitis needs to be adapted, as current DS models do not characterize the dose and duration of DS administration (Iwanaga 1994; Hoshi 1996).

## **4. HYPOTHESIS AND OBJECTIVES**

### **4.1 Hypothesis**

A diet rich in DHA increases the amount of DHA available to the system, thereby inhibiting the amount of pro-inflammatory lipid mediators produced. The anti-inflammatory properties of DHA translate into improved clinical and physical symptoms of IBD. The decrease in pro-inflammatory lipid mediators blunts the colitis-induced acute phase response, such that there is a decreased flow of amino acid use in the liver at the expense of muscle protein.

### **4.2 Objectives**

#### **4.2.1 Main Objectives**

The objectives of this study were to develop a reproducible guinea pig model of dextran sulphate-induced colitis, and to determine the effects of a diet rich in DHA on inflammatory mediator production, plasma and tissue protein synthesis, and clinical and physical symptoms of IBD.

#### **4.2.2 Specific Aims**

The specific aims of this study were to examine the combined and independent effects of DHA and intestinal inflammation on:

- (i) Development of colitis
- (ii) Clinical and physical symptoms of colitis
- (iii) Dietary intake, weight loss, and body composition

- (iv) Hepatic lipid profile
- (v) Inflammatory mediator production (cysteinyl leukotrienes, LTB<sub>4</sub>, TNF $\alpha$ )
- (vi) Fractional and absolute synthesis rate of total protein and albumin in plasma
- (vii) Fractional synthesis rate of tissue protein (liver, skeletal muscle, cecum, colon, small intestine).

**Docosahexaenoic acid improves clinical and physical symptoms of intestinal inflammation in a guinea pig model of dextran sulphate-induced colitis**

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## 5.1 Abstract

**Background:** Inflammatory bowel disease (IBD) is associated with several clinical and physical symptoms that affect nutritional status and quality of life. Omega-3 polyunsaturated fatty acids (PUFA), found in fish oil, have been shown to ameliorate some of the complications of IBD. Despite this, no studies have been conducted using dietary docosahexaenoic acid (DHA), a major PUFA in fish oil, in intestinal inflammation. **Objective:** To develop a model of dextran sulphate (DS) induced colitis in the guinea pig to determine if a diet rich in DHA improves clinical symptoms of colitis, and decreases inflammatory lipid mediator production, intestinal damage, and further to determine the effects on protein kinetics including tissue and acute phase protein synthesis. **Design:** In a 2x2 factorial design, guinea pigs (n=35) were randomized to receive either a control or a DHA rich diet for 44 d, and either water or DS treatments for the final 8 days. A flooding dose of L-[ring-<sup>2</sup>H<sub>5</sub>]phenylalanine was administered in order to determine the fractional synthesis rates (FSR) of selected tissues and albumin and total plasma protein (TPP). **Results:** DHA improved clinical and physical symptoms of colitis (p<0.02), as well as increased dietary intake (p<0.03) but did not decrease intestinal damage nor blunt the acute phase response. Inflammatory lipid mediator production tended to be less in the DHA group. Colitis resulted in an increase in TPP FSR (p<0.05) and a doubling of albumin FSR (p<0.02) at the expense of skeletal muscle FSR (p<0.007). **Conclusion:** DHA improves clinical and physical symptoms associated with IBD, including less compromised dietary intake. This may be linked with decreased inflammatory mediator production; however there does not appear to be an association with the acute phase response nor intestinal healing.

## 5.2 Introduction

IBD is associated with several clinical and physical symptoms, such as diarrhea, weight loss, fecal blood (Cooper 1993), as well as additional complications including cytokine-induced anorexia and food avoidance (Rigaud 1994), malnutrition (Reimund 2005), and muscle wasting (Flores 1989; Gruys 2005). The cytokine response to inflammation plays an important role in the complications associated with IBD. Pro-inflammatory cytokines released during intestinal inflammation result in an acute phase response characterized by increased plasma concentrations of positive acute phase proteins and decreases in concentrations of negative acute phase proteins (Gauldie 1994; Gruys 2005). The cytokines IL-6, IL-1 $\beta$  and TNF $\alpha$  mediate changes associated with the systemic phase of the acute phase response, which results in the breakdown of muscle protein for amino acid use in the liver (Blok 1996; Gruys 2005). TNF $\alpha$  is the most prominent cytokine linked to muscle breakdown with inflammatory conditions (Pajak 2008). Additionally, the cytokine response plays an important role in malnutrition in IBD (Reimund 2005). The metabolic consequences of cytokine-induced malnutrition and food avoidance and the resultant production of acute-phase proteins include the catabolism of muscle protein for amino acid use in the liver, sequestration of iron and zinc, increased gluconeogenesis, increased loss of body nitrogen, retention of water and sodium, increased loss of vitamins as well as potassium, phosphate, magnesium and zinc (Beisel 1995).

The undesirable side-effects of typical steroid therapy for IBD (Feldman 1994) emphasize the need for less harmful therapies. The omega-3 PUFA, EPA and DHA of fish oil, have been shown to consistently ameliorate some of the complications of IBD in

animal studies (Vilaseca 1990; Nieto 2002; Andoh 2003), likely due to their soluble lipid mediators termed eicosanoids (Wallace 1994). The lipid mediators biosynthesized from EPA and DHA, termed the resolvins and protectins, have been of particular interest in recent studies due to their potent anti-inflammatory properties (Hong 2003; Serhan 2006; Kasuga 2008; Serhan 2008). In addition to the production of anti-inflammatory mediators, the omega-3 PUFA, EPA and DHA, are believed to exert an anti-inflammatory effect by partially replacing arachidonic acid (AA) in the membranes of immune cells. The resulting altered ratio influences enzymatic competition between the omega-6 and omega-3 fatty acids and thereby eicosanoid metabolism. More specifically, a decreased amount of AA in cell membranes has been shown to result in a decreased production of potent pro-inflammatory mediators, such as leukotriene B<sub>4</sub> (LTB<sub>4</sub>) (Simopoulos 2002). LTB<sub>4</sub> is a powerful stimulus of neutrophil activity (Wallace 1994) and is capable of increasing the production of cytokine mediators of the acute phase response, such as TNF $\alpha$ , IL-1 $\beta$  (Rola-Pleszczynski 1988), and IL-6 (Calder 2002).

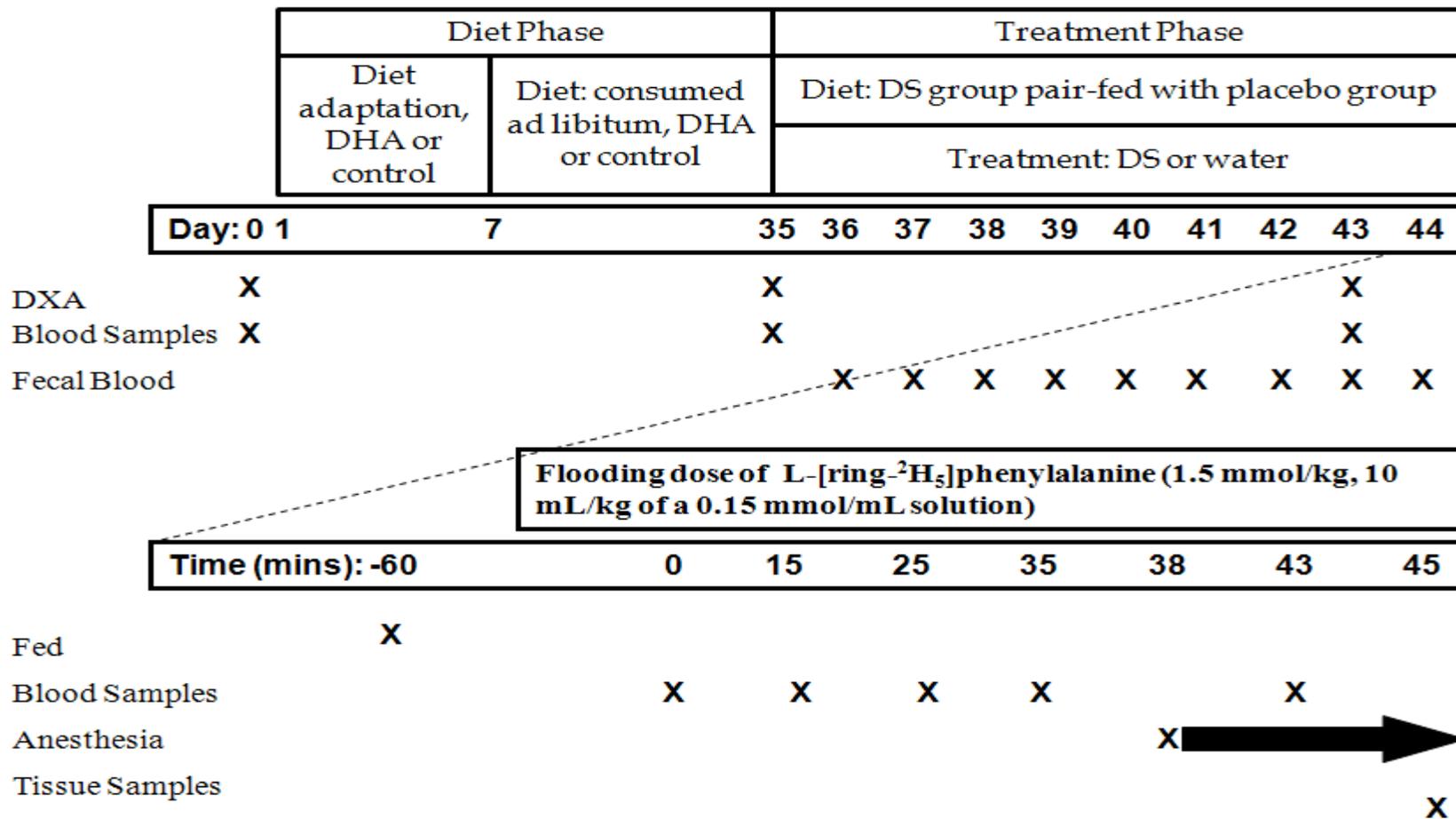
Although EPA and DHA are members of the omega-3 PUFA family, they do not exert identical anti-inflammatory effects. DHA, in the absence of EPA has been shown to decrease T lymphocyte activation (Kew 2004), PGE<sub>2</sub>, LTB<sub>4</sub>, IL-1 $\beta$ , and TNF $\alpha$  in healthy volunteers (Kelley 1999). Furthermore, resolvin D1, a lipid mediator biosynthesized from DHA, has been shown to inhibit neutrophil migration in inflammatory exudates (Kasuga 2008). Despite the evidence of the differential effects of EPA and DHA, little evidence is available for DHA in inflammatory states. Our aim was to develop a guinea pig model of acute and severe colitis in order to determine the effects

of DHA on clinical and physical outcomes of IBD, as well as to investigate its effect on inflammatory mediator production, protein kinetics, and intestinal damage.

## 5.3 Materials and Methods

### Experimental Protocol

Female, retired breeders guinea pigs (n=35; 50-69 wk; Pigmented) were obtained from Elm Hill Laboratories (Boston, USA). They were randomized to one of four experimental groups in a 2 x 2 factorial design: Diet (control vs. DHA) and inflammation (water vs. DS-induced colitis). The guinea pigs were housed individually at a room temperature of 21-22°C and a relative humidity of 50% on a 12h/12h light/dark cycle. They were adapted to their study diet over a one week period and then continued to consume their diet for the remainder of the 44 d study (**Figure 5.1**). On d 36, the guinea pigs received a DS solution (0.25g/(kg·d)) or deionized water administered orally twice daily for up to 8 days. Body composition was determined by dual X-ray absorptiometry (DXA) on d 0, 35 and 43. Protein synthesis was studied by administration of a flooding dose of L-[ring-<sup>2</sup>H<sub>5</sub>]-phenylalanine with serial blood sampling and post mortum tissue sampling. 45 minutes following the administration of the flood, guinea pigs were killed by overdose of AErrane isoflurane gas (Baxter Inc., Mississauga, ON, Canada) and cecum, colon, liver, quadriceps and small intestine tissues were sampled and snap frozen in liquid nitrogen. Ethical approval was obtained from the McGill University Animal Care Committee, and all practices were in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council On Animal Care 1993).



**Figure 5.1:** Study protocol of the study up to and including the flooding dose study day

## **Diet**

Guinea pigs were randomized to receive either the control diet (product 5TYJ, Purina Testdiet) or a diet enriched with 1.094 g/kg DHA (product 5C2Y, Purina Testdiet with the DHA oil received from Martek Biosciences Corporation, Columbia, MD). Both diets were in pellet form and balanced for energy, vitamins, minerals and macronutrients except for DHA (**Table 5.1**). The diets contained the antioxidant ethoxyquin for preservation. They were stored in a -20 °C freezer in order to prevent lipid peroxidation. Water was provided ad libitum in water bottles equipped with ball-bearing sipper tubes, which were filled once daily. The control and DHA diets were composed of 193 g/kg protein, 513 g/kg carbohydrate, and 61 g/kg fat and were designed to meet the nutritional requirements for growth (**Table 5.2**) due to lack of sufficient data to determine requirements for maintenance. However, the diets were considered representative for the adult guinea pig (National Research Council 1995).

The guinea pigs were randomized into 9 blocks, with 4 per block and each block containing one guinea pig from each experimental group, except for the final block, which did not have a control diet, water treated guinea pig. Within blocks, the colitis guinea pigs were initiated on their experimental diet 2 days prior to the non-colitis group in order to allow for pair feeding following DS administration. Guinea pigs were adapted to their study diets over the course of 7 days by increasing the proportion of study diet offered, as of d 1, versus chow (approximately 70 grams of total diet was offered daily). They continued to consume the study diets for the duration of the study. Diet consumption was monitored by daily weighing of the remaining food from the previous day, taking into account any food pellets scattered on the cage floor as well as removal of

**Table 5.1:** Diet Ingredients of Control and DHA Study Diets

<b>Ingredient</b>	<b>Control (g/kg diet)</b>	<b>DHA (g/kg diet)</b>
<b>Sucrose</b>	294	294
<b>Corn Starch</b>	218	218
<b>Soy Protein Isolate</b>	167	167
<b>Powdered Cellulose</b>	140	140
<b>Soybean Oil</b>	60	60
<b>Casein-Vitamin Free</b>	50	50
<b>Dicalcium Phosphate</b>	26.4	26.9
<b>Potassium Citrate, Tribasic Monohydrate</b>	11.3	11.0
<b>Calcium Carbonate</b>	11.3	11.0
<b>Potassium Carbonate Salt</b>	6.8	-
<b>Artificial Flavors</b>	5.9	5.9
<b>Choline Chloride</b>	-	5
<b>Ascorbic Acid</b>	2.6	2.6
<b>Magnesium Oxide</b>	2.3	2.3
<b>Vitamin and Mineral Premix<sup>1</sup></b>	1.5	4.0
<b>Vitamin K Premix<sup>2</sup></b>	1.5	1.3
<b>L-methionine</b>	0.6	0.6
<b>Ethoxyquin</b>	0.5	0.5
<b>DHASCO<sup>3</sup></b>	0.1	0.1
	-	1.094

<sup>1</sup>Vitamin and mineral premix contains (per kg diet): vitamin A 9900 µg/kg; vitamin E 30000 µg/kg for control diet and 37000 µg/kg for DHA diet; thiamin hydrochloride 0.0093 g/kg; riboflavin 0.0062 g/kg; niacin 0.066 g/kg; pantothenic acid 0.019 g/kg; folic acid 0.003 g/kg; pyridoxine 0.0041 g/kg; biotin 300 µg/kg; vitamin B<sub>12</sub> 10 µg/kg; choline chloride 1.85 g/kg; ascorbic acid 2.3 g/kg; 10.8 g/kg; phosphorous 6.5 g/kg; phosphorous (available) 5.2 g/kg; potassium 8.0 g/kg; magnesium 1.0 g/kg for control diet and 2.5 g/kg for DHA diet; sodium 3.5 g/kg; chlorine 4.0 g/kg; fluorine 0.016 g/kg; iron 0.075 g/kg; zinc 0.07 g/kg; manganese 0.076 g/kg; copper 0.013 g/kg; cobalt 0.0031 g/kg; iodine 590 µg/kg; chromium 0.0016 g/kg; molybdenum 580 µg/kg; selenium 300 µg/kg.

<sup>2</sup>Vitamin K (as menadione) 240 µg/kg.

DHASCO: Docosahexaenoic acid single cell oil, extracted from laboratory-grown algae.

**Table 5.2:** Nutrient Content of Study Diets and Nutritional Requirements for Guinea Pigs<sup>1</sup>

<b>Nutrient</b>	<b>RDC<sup>4</sup></b>	<b>Control</b>	<b>% RDC</b>	<b>DHA</b>	<b>% RDC</b>
<b>Fat (g/kg)</b>	NA	61	NA	62	NA
<b>Carbohydrate (g/kg)</b>	NA	513	NA	500	NA
<b>Protein (g/kg)</b>	180	193	107.2	193	107.2
<b>Energy (kJ/kg diet)<sup>2</sup></b>	12 560 (minimum)	14 025	111.7	13 900	110.7
<b>Fiber (g/kg)</b>	150	140	93.3	140	93.3
<b>FAT</b>					
Cholesterol (g/kg diet)	NA	0	NA	0	NA
Linoleic Acid (g/kg)	1.3-4.0	30.7	767.5-2192.9 <sup>3</sup>	30.7	767.5-2192.9 <sup>3</sup>
Linolenic Acid (g/kg)	NA	4.7	NA	4.7	NA
Arachidonic Acid (g/kg)	NA	0	NA	0	NA
DHA (g/kg)	NA	0	NA	1.094	NA
DHA (g/kg)	NA	0	NA	8	NA
Total Saturated Fatty Acids (g/kg)	NA	8.8	NA	8.8	NA
Total Monounsaturated Fatty Acids (g/kg)	NA	12.6	NA	12.6	NA
Total Polyunsaturated Fatty Acids (g/kg)	NA	35.3	NA	36.4	NA
<b>MINERALS</b>					
Calcium (g/kg)	8	10.8	135	10.8	135
Phosphorous (g/kg)	4	6.5	162.5	6.5	162.5
Phosphorous (available, g/kg)	NA	5.2	NA	5.2	NA
Potassium (g/kg)	5	8	160	8	160
Magnesium (g/kg)	1	1	100	2.5	250
Sodium (g/kg)	0.5	3.5	700	3.5	700
Chlorine (g/kg)	0.5	4	800	4	800
Fluorine (g/kg)	NA	15.9	NA	15.9	NA
Iron (mg/kg)	50	75	150	75	150
Zinc (mg/kg)	20	70	350	70	350
Manganese (mg/kg)	40	76	190	76	190
Copper (mg/kg)	6	13	216.7	13	216.7

Cobalt (mg/kg)	NA	3.1	NA	3.1	NA
Iodine (mg/kg)	0.15	0.59	393.3	0.59	393.3
Chromium (mg/kg)	NA	1.6	NA	1.6	NA
Molybdenum (mg/kg)	0.15	0.58	386.7	0.58	386.7
Selenium (mg/kg)	0.15	0.30	200	0.30	200
<b>VITAMINS</b>					
Vitamin A (g/kg diet)	0.0066	0.0099	150	0.0099	150
Vitamin D <sub>3</sub> (added, µg/kg diet)	25	30	120	30	120
Vitamin E (g/kg diet)	0.027	0.03	111	0.037	137
Vitamin K (as menadione, mg/kg diet)	5	2.4	48	2.4	48
Thiamin Hydrochloride (mg/kg diet)	2	9.3	465	9.3	465
Riboflavin (mg/kg diet)	3	6.2	206.7	6.2	206.7
Niacin (mg/kg diet)	10	66	660	66	660
Pantothenic Acid (mg/kg diet)	20	19	95	19	95
Folic Acid (mg/kg diet)	3.0-6.0	3	50-100	3	50-100
Pyridoxine (mg/kg diet)	2.0-3.0	4.1	136.7-205	4.1	136.7-205
Biotin (mg/kg diet)	0.2	0.3 (150)		0.3 (150)	
Vitamin B <sub>12</sub> (mg/kg diet)	NA	0.01	NA	0.01	NA
Choline Chloride (mg/kg diet)	1800	1850	102.8	1850	102.8
Ascorbic Acid (mg/kg diet)	200	2300	1150	2300	1150

<sup>1</sup> Based on nutrient composition of diet ingredients and nutritional requirements of the growing guinea pig, all NRC values are based on requirements for growth, except where indicated (NRC, 1995).

<sup>2</sup>Based on requirements for maintenance where customized or purified diets are recommended to contain minimum 3000 kcal ME/kg diet.

<sup>3</sup>The amount of linoleic acid in study diets was much greater than requirement; however the amount of soybean oil provided is in range of other accepted guinea pig diets (NRC, 1995).

<sup>4</sup>Recommended Diet Composition

any wood shavings or fecal pellets from the feed dispenser. The remaining food was discarded before offering fresh food. In order to create a fed state on the flooding study day, guinea pigs were manually administered diet (pre-weighed then moistened with water) via needle-less syringe approximately one hour prior to injection of the tracer. Guinea pig weight was monitored twice weekly prior to DS/water treatments and following d 36 they were weighed daily and percent weight loss recorded. Percent weight losses of 10% or greater following d 36 resulted in early termination. No guinea pigs were excluded from the study.

### **Dextran Sulphate Induced Colitis**

In rodent models, DS is typically administered in the drinking water as a 3-5% solution (Kawada 2007). However, since guinea pigs play with their water and can therefore drain a significant amount of their water into their cages (Terril 1998), an actual treatment dose of DS cannot be determined from this method. Therefore, on d 36, the guinea pigs were manually given an oral dose (0.25 g/(kg/d)) of DS (MP Biomedicals Inc. 40 000 MW) solution (200 g/L solution) by needle-less syringe (disposable 1 ml with tuberculin slip tip). This method was chosen over the traditional gavage administration as the guinea pigs were not familiarized with gavage and thus would have been more stressful for them. DS was administered twice daily for up to 8 days. The placebo guinea pigs were administered deionized water at the same dose and duration as the DS guinea pigs. The animals were closely observed while on DS; their feces were tested for occult blood (Hemocult<sup>®</sup> test packs, Beckman Coulter, Mississauga, Ont.), their health (feed and water intake, feces and urine production, eye discharges, % body weight change, hair loss) and behavior (activity, interest in environment, general demeanor) were

monitored. If significant changes in the above criteria were observed the DS doses were stopped and the guinea pig monitored every 4-7 hours. If the symptoms persisted 24-48 hours later, the guinea pig was killed early.

### **Dual X-Ray Absorptiometry (DXA) and Routine Blood Sampling**

On d 0, 35, and 43, body composition was determined with DXA (Discovery Series 4500A densitometer, Hologic Inc., Bedford, MA) under isoflurane anesthesia.

Anesthesia was induced with an induction chamber with 5% isoflurane and followed by 2% maintenance using a cone mask. The guinea pigs were scanned prone with limbs extended in whole body modes with analysis using the small animal software (QDR 4500A small animal software version 12.5). Guinea pigs were anesthetized for approximately 20 minutes. Blood was drawn from the lateral saphenous vein, also on d 0, 35, and 43, between 0630 and 0830 h into prechilled microtainers that were either empty or contained EDTA. All blood samples were centrifuged at 3000 x g for 30 minutes in order to obtain plasma or serum and then stored at -80°C.

### **Protein Fractional Synthesis Rates**

A flooding dose of L-[ring-<sup>2</sup>H<sub>5</sub>]-phenylalanine (40 mol %, Cambridge Isotope Laboratories, Cambridge, MA), in unlabeled phenylalanine (Ajinomoto U.S.A Inc., Raleigh, N.C.), was prepared in sterile saline for a final concentration of 0.15 mol/L and passed through a 0.22 µm syringe filter. The solution was injected intraperitoneally at a dose of 0.01 L/kg body weight with a 20 G Insyte needle (Becton Dickinson & Co., Franklin Lakes, NJ) over a 10-s period in order to determine protein fractional synthesis rates of total and albumin plasma proteins and of the colon, cecum, small intestine, liver,

and quadriceps proteins. A 1 ml blood sample was drawn at baseline, 15, 25, 35, and 45 minutes following isotope injection into pre-chilled microtainers containing EDTA and soybean trypsin inhibitor (Jahoor 1999). At 45 minutes the guinea pigs were killed and tissue samples of the distal colon, cecum, small intestine, liver, and quadriceps were snap frozen in liquid nitrogen and stored at -80°C. Furthermore, samples of each of the gut tissues were stored in 10% phosphate buffered formalin for histological analysis. Whole liver was weighed and then discarded.

### **Liver Fatty Acids Analysis**

Lipids were extracted from homogenized liver in a chloroform/methanol (2:1) solution containing 0.01g/L butylated hydroxytoluene (BHT) and centrifuged at 486 x g for 10 minutes. The supernatant was washed with chloroform and 7.3 g/L NaCl, centrifuged, and the upper layer discarded. The bottom layer was washed twice with a chloroform/methanol/water solution (3:48:47) and the extracted lipids were then methylated with toluene and methanolic HCl and heated at 80°C for one hour. Water was added, and the lipids were centrifuged for 5 minutes. The layers were separated, with the bottom layer combined with petroleum ether, centrifuged, and the new upper layer combined with the previously removed upper layer. Water was added to the combined upper layers, centrifuged, and the top layer reserved for analysis by gas chromatography (CP 3800, Varian Inc., Walnut Creek, CA).

### **Biochemistry**

Plasma Cysteinyl leukotriene concentration, in plasma from d 43, was assessed via Luminex® cysteinyl leukotriene competitive immunoassay (Catalog No. 10007577,

Cayman Chemical, Ann Arbor, MI), having a specificity of 100% for leukotriene (LT) C<sub>4</sub>, 93% for LTD<sub>4</sub>, 23.1% for LTE<sub>4</sub> and 0.12% for LTB<sub>4</sub>. Similarly, LTB<sub>4</sub> concentration was assessed with an LTB<sub>4</sub> competitive enzyme immunoassay (EIA, Catalogue No. KGE006, R&D Systems Inc., Minneapolis, MN). The assay had no significant cross-reactivity or interference. Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) concentrations were analyzed in cecum tissue homogenates using the Luminiex® multiplex bead immunoassay extracellular protein buffer reagent kit (Catalogue No. LGB0001, Invitrogen Corporation, Carlsbad, California), with the guinea pig TNF $\alpha$  antibody bead kit (Part LGC3011, Invitrogen Corporation, Carlsbad, California), having significant cross-reactivity only with mouse TNF $\alpha$ .

## **Sample Analysis**

### *Evaluation of Disease Severity*

A disease activity index (DAI) was calculated based on Stucchi et al's (Stucchi 2000) criteria of; % weight loss (from initial day on DS/water to final day), stool consistency, and presence of fecal blood. This index was adapted for each guinea pig by the addition of a behavioral component and the final score was determined by the average of scores (0-4) for each of the DAI criteria (**Table 5.3**). In assessing the criteria the examiner was not blinded.

Colon and cecum segments were embedded in parafilm and stained with hematoxylin and eosin. A blinded pathologist (Dr. Marilene Paquet, McGill University, QC) analyzed the slides and graded the disease severity based on; inflammation, crypt damage and the percent involvement of both the inflammation and the crypt damage, according to Carrier

**Table 5.3:** Scoring of Disease Activity Index<sup>1</sup>

<b>Score</b>	<b>Weight Loss</b>	<b>Stool Consistency</b>	<b>Presence of Fecal Blood</b>	<b>Behaviour</b>
<b>0</b>	None	Normal	Normal	Normal
<b>1</b>	1-5%			
<b>2</b>	5-10%	Loose Stools	Hemoccult +	Lethargic
<b>3</b>	10-20%			Lethargic/Shaking
<b>4</b>	>20%	Diarrhea	Gross Bleeding	Sudden Death

<sup>1</sup>The disease activity index is the summed scores of weight loss, stool consistency, presence of fecal blood, and behavior divided by 4. Adapted from Stucchi et al (Stucchi 2000).

et al (Carrier 2001) and Murthy et al (Murthy 1993) (**Table 5.4**). The average score for each of the features graded was used as the histological score.

#### *Serum Protein Concentrations*

Serum concentrations of total protein and albumin were determined using Vitros 350 Chemistry System (Ortho-Clinical Diagnostics, Markham, On.).

#### *Tissue and Plasma Protein Preparation*

Approximately 100-200 mg of frozen tissue was homogenized in cold 100 g/L trichloroacetic acid (TCA) solution. The homogenates were centrifuged at 604 x g for 15 minutes and the supernatant was collected for analysis of free amino acids. The protein pellet was washed in cold 100 g/L TCA, the supernatant discarded, and the protein pellet hydrolyzed with 4 mol/L HCl overnight at 110°C. Total protein was precipitated from 100 µl of plasma with 100 g/L TCA and hydrolyzed as reported previously (Mackenzie 2003).

Fibrinogen was precipitated from 100 µl of thawed plasma with an ethanol/saline (1:8) solution and refrigerated overnight. To isolate albumin, proteins were precipitated from the fibrinogen-free supernatant with 100 g/L TCA. Both fibrinogen and albumin were processed and resolved separately with sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis on a MINI-PROTEAN II System (Bio-Rad Laboratories), as described previously (Harding 2008).

#### *Isolation and Derivatization of Amino Acids*

**Table 5.4:** Inflammation, Crypt Damage and Percent Involvement Histology Grading<sup>1</sup>

<b>Feature Graded</b>	<b>Grade</b>	<b>Description</b>
<b>Inflammation</b>	0	None
	1	Multifocal inflammatory cells infiltration including polymorphonuclear
	2	Inflammation, gland dropout and crypt abscesses
	3	Mucosal erosion(s)
	4	Mucosal Ulcer(s)
<b>Percent Involvement (Inflammation)</b>	0	N/A
	1	1-25% (minimal)
	2	26-50% (mild)
	3	51-75% (moderate)
	4	76-100% (severe)
<b>Crypt Damage</b>	0	Intact crypts
	1	Loss of bottom third of crypts
	2	Loss of bottom two thirds of crypts
	3	Loss of entire crypts with surface epithelium intact
	4	Loss of entire crypt and surface epithelium (erosion)
<b>Percent Involvement (Crypt Damage)</b>	0	N/A
	1	1-25% (minimal)
	2	26-50% (mild)
	3	51-75% (moderate)
	4	76-100% (severe)

<sup>1</sup>Based on Carrier et al (Carrier 2001) and Murthy et al. (Murthy 1993). Average score of each feature graded is used as the histological score.

Amino acids from tissue and plasma proteins (free and bound) were purified using cation exchange chromatography (Dowex-50-X8, Bio Rad Laboratories, Hercules, CA).

Phenylalanine was esterified and derivatized to its n-propyl ester heptafluorobutyramide derivative using n-propanol and acetyl chloride, and then heptafluorobutyric anhydride in ethyl acetate as previously described (Mackenzie 2003).

### *GCMS*

Phenylalanine tracer:tracee ratios were determined by methane negative chemical ionization GCMS (Hewlett-Packard 5988A GC/MS, Palo Alto, CA). A DB-1 column (30 m x 0.25 mm x 1 µm) was used with helium as the carrier gas. The phenylalanine ions were detected at mass to charge ratios (m/z) from 383 to 388.

### *Calculations*

The tracer:tracee ratios (phenylalanine:L-[ring-<sup>2</sup>H<sub>5</sub>]-phenylalanine) were determined from the raw ion abundances from triplicate injections. Fractional synthesis rate (FSR) of mixed proteins in each tissue was calculated using the following:

$$\text{FSR}(\%/d) = \frac{(\Delta E_{\text{bound}}) \cdot (24 \text{ h/d}) \cdot (100\%) \cdot (60 \text{ min/h})}{E_{\text{free}} \cdot (t_2 - t_1 \text{ min})}$$

Where  $E_{\text{bound}}$  is the increase in the tracer:tracee ratio of tissue protein bound phenylalanine (at approximately 48 minutes) above the baseline (zero enrichment), and  $E_{\text{free}}$  is the tracer:tracee ratio of tissue free phenylalanine (at approximately 48 minutes).

FSR of plasma proteins (total pool, albumin, fibrinogen) were calculated using:

$$\text{FSR}(\%/day) = \frac{(\Delta E_{\text{bound}}) \cdot (24 \text{ h/d}) \cdot (100\%) \cdot (60 \text{ min/h})}{E_{\text{free}} \cdot (t_2 - t_1 \text{ min})}$$

Where  $E_{\text{bound}}$  is the increase in the tracer:tracee ratio of plasma protein bound phenylalanine between time 0 minutes and 45 minutes, and  $E_{\text{free}}$  is the tracer:tracee ratio of plasma free phenylalanine.

Absolute synthesis rate (ASR) of plasma proteins were calculated using:

$$\text{ASR (g/kg/day)} =$$

$$[(\text{FSR } \%/day)/100\%] \cdot (\text{concentration g/L}) \cdot (\text{plasma volume ml/kg}) \cdot 1\text{L}/1000 \text{ ml}$$

Plasma volume was assumed to be 38.8 ml/kg based on values in healthy, adult, male guinea pigs (Sisk 1976).

### *Statistics*

SAS (Version 9.1, SAS Institute Inc., Cary, NC) was used for all statistical analysis.

Differences were considered statistically significant at  $p < 0.05$  and data was expressed as mean  $\pm$  SEM. Data were tested for normality by the Shapiro-Wilk test and were log transformed if not normally distributed. Muscle and cecum fractional synthesis rates, albumin absolute synthesis rate, total plasma protein concentration, cysteinyl leukotriene concentration,  $\text{LTB}_4$  concentration, % alpha linolenic acid, and %DHA in the liver, histological scores for cecum inflammation and colon inflammation and crypt damage, and the absolute values for lean, fat and BMC mass, failed normality tests and were thus

log transformed, except for cecum crypt damage and repeated measures of body composition where the transformation did not improve the normality. The maximum likelihood method was used for tests of homogeneity of variances, where the best fitting model was used for the analysis using Bayesian Criteria. The data were analyzed for the main effects of diet (DHA or control diet) and treatment (DS or water treatment), as well as their interaction using two-way ANOVA in SAS mixed procedure. Tukey's test was used to assess post hoc differences. Spearman's test was used for correlations involving cecum inflammation; otherwise Pearson's test was used for normal data. Sample size (n) was estimated as minimum  $n = 8/\text{group}$  based on detecting a difference of 20% total plasma protein synthesis with a standard deviation of 14,  $\alpha = 0.05$  and Power = 0.80.

## 5.4 Results

DS-induced colitis in guinea pigs was used as a model of IBD to evaluate the effect of a DHA diet on several outcomes of disease progression, protein synthesis, and inflammation. These outcomes included clinical and physical outcomes, histological parameters of inflammation and crypt damage, alterations in protein synthesis associated with the acute phase response, and inflammatory lipid mediators.

### Disease Activity Index

DAI was assessed on the final study day, in order to evaluate clinical and physical signs of colitis, based on % weight loss, stool consistency, presence of fecal blood and an adapted parameter assessing behaviour such as lethargy or sudden death. Each parameter was gauged on a scale from 0 (least severe) to 4 (most severe), and the average of the total score represented the DAI. There was a main effect of dietary DHA ( $p < 0.018$ ) to decrease DAI regardless of DS treatment and of DS treatment ( $p < 0.0001$ ) regardless of the diet. The interaction of DS and the control diet resulted in greater physical signs ( $p < 0.044$ ) (**Figure 5.2**). Sudden death occurred with two guinea pigs receiving the control diet during the DS treatments and were unable to be included for outcomes assessed on the final study day such as; histology, percent composition of fatty acids in the liver, and protein synthesis.

### Characteristics of Colitis

Hematoxylin and eosin stained sections of the cecum and colon were analyzed by a blinded pathologist (Dr. Marilene Paquet, McGill University) in order to assess severity and extent of inflammation and crypt damage (**Figure 5.3**). Cecum and colon segments

of guinea pigs receiving water had intact crypts and minimal evidence of inflammatory cell infiltration. DS resulted in loss of crypt structure ranging from the destruction of the bottom third of the crypt (score of 1) to the entire crypt in combination with epithelial erosion (score of 4), as well as increased inflammatory cell infiltration and mucosal erosions and ulcers. Regardless of diet, a main effect of dextran sulphate administration resulted in elevated inflammatory scores in both the cecum ( $p<0.003$ ) and the colon ( $p<0.002$ ). Similarly, a main effect of DS also resulted in increased crypt damage scores for both the cecum ( $p<0.003$ ) and the colon ( $p<0.0003$ ) for both dietary groups (**Table 5.5**). There was no interaction of the main effects. Histological scores of cecum inflammation ( $r=0.45$ ,  $p<0.017$ ) and crypt damage ( $r=0.66$ ,  $p<0.0001$ ) correlated positively with clinical parameters of DAI.

### **Feed Intake, Weight Change and Body Composition**

Feed intake was monitored daily over the course of the entire study, while body weight was monitored twice-weekly prior to DS and water treatments, and daily following these treatments. Body composition was assessed by DXA at baseline as well as before and after treatments. Alterations in nutritional status and metabolism were assessed by calculating changes in feed intake, weight and body composition for the entire study (**Table 5.6**).

Initial weight ( $1.1\pm 0.18$  kg), lean mass ( $0.76\pm 0.01$  kg), fat mass ( $0.28\pm 0.02$  kg), and bone mineral content (BMC) ( $0.03\pm 0.01$  kg) were not different among experimental groups. Guinea pigs offered free access to their diet consumed approximately 50% of their

estimated energy requirement (National Research Council 1995). The reduced feed intake resulted in similar initial weight loss amongst the experimental groups.

Following treatment administration and pair feeding within diet groups on day 36, guinea pigs consuming the DHA diet had a greater intake than control diet guinea pigs ( $p < 0.025$ ). As a result of this main effect of diet intake, control diet guinea pigs met approximately 26% of their estimated energy requirements, whereas DHA guinea pigs met approximately 40% of their estimated energy requirements.

Regardless of experimental group, guinea pigs lost weight over the course of the study. Despite pair feeding, there was a main effect of DS in colitis guinea pigs, who lost more weight ( $p < 0.002$ ) and lean mass ( $p < 0.011$ ) than guinea pigs receiving water when post-treatment intake was used as a covariate as intake differed between diet groups following treatment administration. Furthermore, percent change of initial lean mass correlated negatively with DAI ( $r = -0.67$ ,  $p < 0.0001$ ). Finally, guinea pigs receiving DS, regardless of diet group, lost more weight in the final three days of the disease progression ( $p < 0.0001$ ) (**Figure 5.4**). Additionally, weight lost in the final 3 days of the study was correlated negatively with DAI ( $r = -0.88$ ,  $p < 0.001$ ), and histological scores of crypt damage ( $r = -0.56$ ,  $p < 0.015$ ). Lean mass for all experimental groups decreased significantly over time (**Table 5.7**). However, changes in fat and BMC mass were not significantly different amongst groups. There was no interaction of the main effects for any of the intake, weight and body composition results.

### **Percent Composition of Fatty Acids in the Liver**

Percent composition of total fatty acids in the liver was determined using GC in order to assess the transfer of fatty acids from the diet into the guinea pig system. Post-treatment intake was used as a covariate for all fatty acids assessed in the liver, as DHA guinea pigs consumed more than control guinea pigs. The percent composition of the essential fatty acids linoleic acid and alpha-linolenic were unaffected by neither diet nor treatment. Oral administration of DS resulted in decreased % arachidonic acid ( $p < 0.034$ ) (**Table 5.8**) and consumption of DHA resulted in increased % DHA composition ( $p < 0.002$ ). There were no interaction effects for the percent of total fatty acids in the liver. Other fatty acids measured in the liver included myristic acid (~1%), myristoleic acid (~0.1%), palmitic acid (~16%), palmitoleic acid (~1%), stearic acid (~16%), and oleic acid (~15%).

### **Tissue Protein Fractional Synthesis Rates**

A flooding dose of L-[ring-<sup>2</sup>H<sub>5</sub>]-phenylalanine was used to measure protein synthesis rates in order to determine the changes in protein synthesis associated with the acute phase response. Statistical analysis of all fractional synthesis rates utilized post-treatment intake as a covariate, due to the fact that DHA guinea pigs consumed more feed than non DHA guinea pigs. Liver (**Figure 5.5**) and colon fractional synthesis rates were unaffected by either the diet consumed or the treatment administered (**Table 5.9**).

Although cecum FSR (**Figure 5.5**) was not statistically different among study groups, it correlated negatively with DAI ( $r = -0.45$ ,  $p < 0.009$ ), histological scores of cecum crypt damage ( $r = -0.55$ ,  $p < 0.003$ ), and correlated positively with % of initial lean mass change ( $r = 0.38$ ,  $p < 0.035$ ) and weight change in the final 3 days of the study ( $r = 0.54$ ,  $p < 0.001$ ). Muscle protein synthesis (**Figure 5.5**) was reduced with colitis as indicated by a main effect of DS ( $p < 0.007$ ). Protein synthesis of the small intestinal mucosa showed increased

values in the DHA diet group ( $p < 0.026$ ). There were no interaction effects demonstrated for tissue protein synthesis.

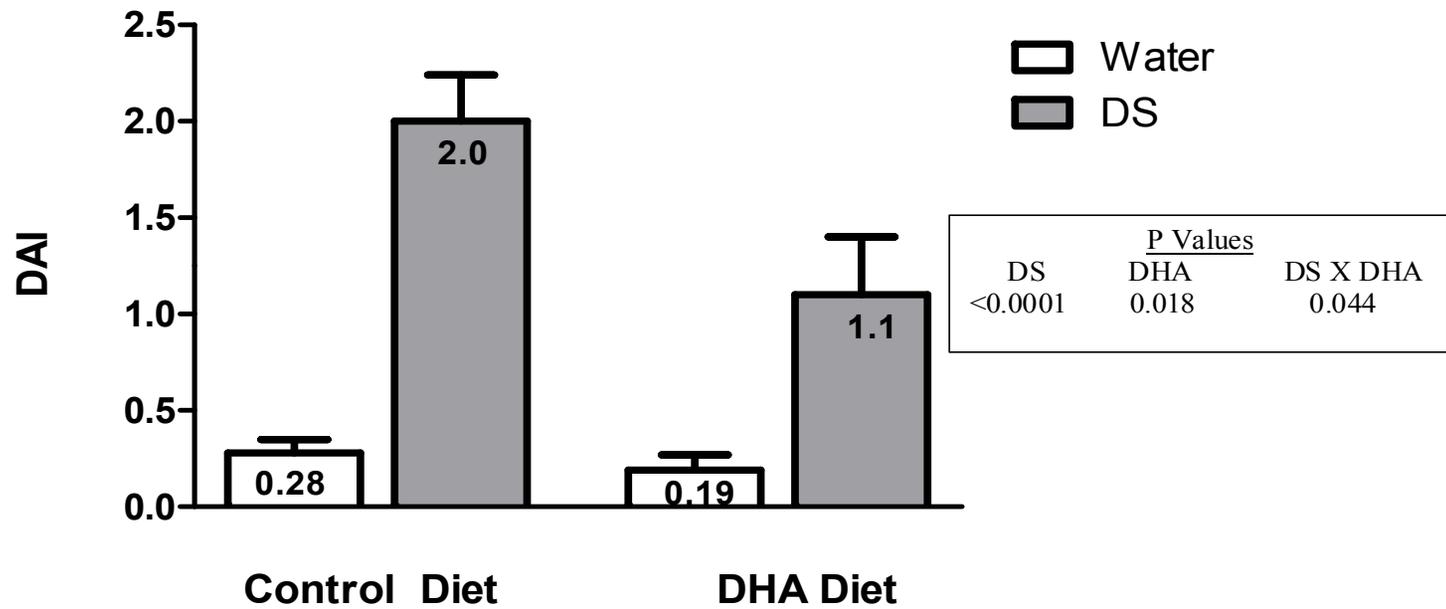
### **Plasma Protein Synthesis**

Post-treatment intake was used as a covariate for all plasma protein synthesis data due to differences in feed intake in the DHA diet group. The concentration of albumin and total protein in serum and their fractional and absolute synthesis rates in plasma are shown in **Figures 5.6 & 5.7**. The concentration of total protein in serum was unchanged across treatment and diet groups despite increased fractional ( $p < 0.054$ ) and absolute ( $p < 0.034$ ) synthesis rates in plasma with a main effect of colitis. Similarly, albumin concentration in serum was unaffected by neither diet nor treatment group, whereas both fractional ( $p < 0.017$ ) and absolute ( $p < 0.0004$ ) plasma synthesis rates were increased with a main effect of colitis, though there was no effect of diet. Plasma protein and albumin FSR correlated with several indices of nutrition and disease. Total plasma protein FSR was directly proportional to DAI ( $r = 0.74$ ,  $p < 0.0001$ ), changes in lean mass ( $r = -0.38$ ,  $p < 0.037$ ), and histological damage as indicated by scores of cecum inflammation ( $r = 0.39$ ,  $p < 0.041$ ) and colon crypt damage ( $r = 0.47$ ,  $p < 0.051$ ). Similarly, albumin FSR was directly proportional to DAI ( $r = 0.69$ ,  $p < 0.0001$ ), changes in lean mass ( $r = -0.46$ ,  $p < 0.011$ ), and histological damage as assessed by cecum inflammation ( $r = 0.69$ ,  $p < 0.0001$ ), cecum crypt damage ( $r = 0.63$ ,  $p < 0.0005$ ), colon inflammation ( $r = 0.69$ ,  $p < 0.0001$ ), colon crypt damage ( $r = 0.69$ ,  $p < 0.002$ ). Additionally, albumin FSR and total plasma protein FSR were positively correlated with each other ( $r = 0.38$ ,  $p < 0.034$ ). No interaction effects were seen with plasma protein synthesis outcomes.

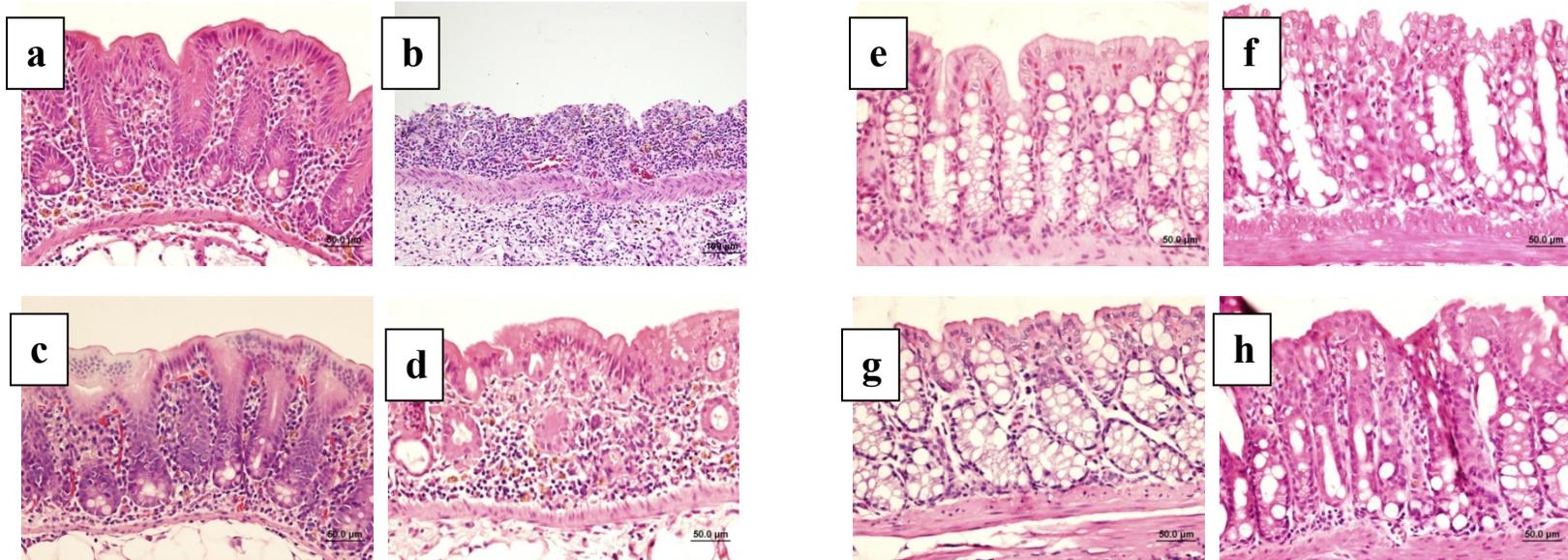
## **Inflammatory Mediators in Plasma**

Concentrations of inflammatory lipid mediators were determined via Luminex and EIA as further evidence in determining the extent of the inflammatory response amongst experimental groups. Plasma concentrations of cysteinyl leukotrienes were increased with colitis regardless of the diet group ( $p < 0.005$ ) (**Figure 5.8**), and were associated with increased DAI ( $r = 0.53$ ,  $p < 0.007$ ) and decreased muscle FSR ( $r = -0.46$ ,  $p < 0.030$ ). Both cysteinyl leukotrienes and  $LTB_4$  concentrations were highly variable, particularly in the control diet, DS group. No differences in  $LTB_4$  concentrations were found across experimental groups.  $LTB_4$  was positively correlated with total plasma protein FSR (0.35,  $p < 0.054$ ). There were no significant interaction effects.

## Disease Activity Index (DAI) of Guinea Pigs on Final Study Day



**Figure 5.2:** Disease activity indexes on the last study day of either water or dextran-sulphate treatment in guinea pigs consuming either a control or DHA-enriched diet. Group means are  $\pm$  SEM, n=8 for control diet, water group, n=9 for all other groups.



**Figure 5.3:** Cecum and colon segments from guinea pigs after up to 8 days of treatment with DS or water. (a) Cecum – control diet, water (b) cecum – control diet, DS (c) cecum – DHA diet, water (d) cecum – DHA diet, DS (e) colon – control diet, water (f) colon – control diet, DS (g) control – DHA diet, water (h) colon – DHA diet, DS. Segments (b), (d), (f), and (h) illustrate crypt damage, inflammatory cell infiltrate and epithelial erosion characteristic of colitis.

**Table 5.5:** Histology Scores of Cecum and Colon Inflammation and Crypt Damage<sup>3</sup>

	CONTROL DIET, WATER	CONTROL DIET, DS	DHA DIET, WATER	DHA DIET, DS	<u>P-values</u> DS
Cecum, Inflammation <sup>1</sup>	2.0±0.4	6.4±1.4	2.1±0.6	5.6±1.4	0.003
Cecum, Crypt Damage <sup>1</sup>	1.3±0.3	9.2±2.7	1.6±0.26	7.3±2.4	0.003
Colon, Inflammation <sup>2</sup>	0.5±0.2	1.3±0.3	0.8±0.2	3.7±1.1	0.002
Colon, Crypt Damage <sup>2</sup>	0.1±0.1	1.3±0.5	0.3±0.2	3.7±1.8	0.0003

Group means are ± SEM, n=9 except where indicated.

<sup>1</sup>n=7 for control diet, water, n=5 for control diet, DS, n=8 for DHA diet for both water and DS

<sup>2</sup>n=8 for control diet, water, n=7 for control diet, DS

<sup>3</sup>Histological scores were calculated by: Inflammation or crypt damage grade x percent of involvement of the damage. Both were analyzed on a scale of 0 (least inflammation/crypt damage or percent involvement) to 4 (greatest inflammation/crypt damage or percent involvement).

**Table 5.6:** Diet Intakes, Percent Energy Requirements<sup>4</sup>, and Body Composition Data for the Entire Study

	CONTROL DIET, WATER	CONTROL DIET, DS	DHA DIET, WATER	DHA DIET, DS	P-values	
					DS	DHA
Pre-Treatment Diet Intake (g/kg BW•d) <sup>1</sup>	21.6±1.4	21.0±1.2	22.0±1.5	23.6±1.3		
Pre-Treatment % of Energy Requirements (%) <sup>1,4</sup>	53.5±3.3	51.5±2.9	52.7±3.4	57.8±3.0		
Post-Treatment Diet Intake (g/kg BW•d) <sup>1</sup>	10.5±0.9	10.5±1.9	14.1±2.3	17.2±3.0		0.025
Post-Treatment % of Energy Requirements (%) <sup>1,4</sup>	25.8±2.1	25.9±3.8	36.7±5.0	42.0±6.9		0.013
% Change of Initial Lean Mass <sup>2</sup>	-6.5±2.2	-13.2±3.6	-6.1±1.7	-12.8±3.9	0.011	
% Change of Initial Fat Mass <sup>3</sup>	-18.7±5.4	-23.9±4.4	-26.4±7.8	-15.5±10.8		
% Change of Initial Bone Mineral Content <sup>2</sup>	2.11±0.6	2.5±1.8	2.4±1.0	1.1±0.8		
% Change of Initial Weight <sup>1</sup>	-9.8±2.8	-16.9±2.0	-9.0±1.7	-14.7±2.1	0.002	

Group means are ± SEM, n=9 except where indicated.

<sup>1</sup>n=8 for control diet, water

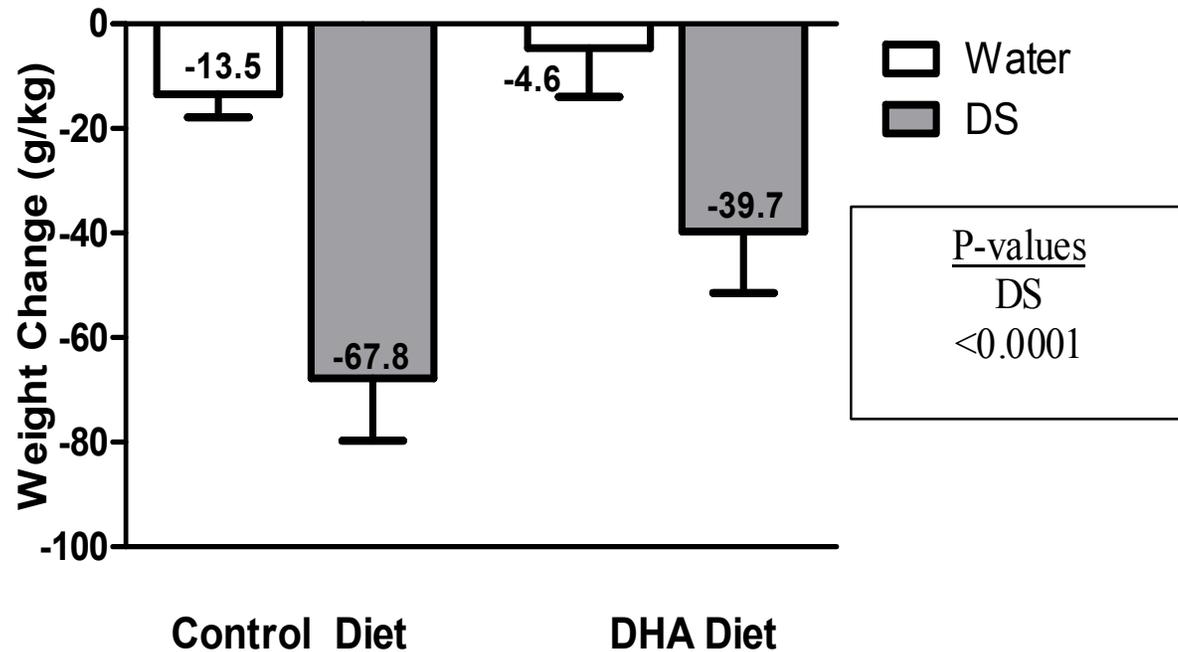
<sup>2</sup>n=8 for control diet, water and DHA diet, DS, n=7 for control diet, DS

<sup>3</sup>n=8 for control diet, water, n=7 for control diet DS, and DHA diet, DS

<sup>4</sup>The % Kcal requirement was calculated with:  $\frac{\text{Kcal intake} \cdot 100\%}{\text{Kcal requirement}}$ , where kcal intake is based on the amount of feed intake multiplied

by 3.33 Kcal/g energy density of the diets, and kcal requirement was based on  $136 \text{ kcal/BW}^{0.75}$  (NRC 1995).

## Weight Change Over the Final Three Days of the Study in Guinea Pigs Fed a Control or DHA Diet and Administered Water or DS



**Figure 5.4:** Weight change over the final three days of the study in guinea pigs fed either a control or DHA diet and administered either a water or DS treatment. Group means are  $\pm$  SEM,  $n=9$  except for control diet, water group where  $n=8$ .

**Table 5.7:** Body Composition Data for Days 0, 35, and 43

	CONTROL DIET, WATER	CONTROL DIET, DS	DHA DIET, WATER	DHA DIET, DS
Lean Mass (kg):				
Day 0 <sup>1</sup>	0.78±0.03	0.75±0.01	0.72±0.03	0.79±0.02
Day 35 <sup>2</sup>	0.75±0.03	0.71±0.02	0.67±0.04	0.71±0.02
Day 43 <sup>3</sup>	0.73±0.03 <sup>a</sup>	0.65±0.02 <sup>a</sup>	0.68±0.03 <sup>a</sup>	0.70±0.03 <sup>a</sup>
Fat Mass (kg):				
Day 0 <sup>1</sup>	0.31±0.04	0.30±0.04	0.25±0.04	0.28±0.03
Day 35 <sup>4</sup>	0.26±0.03	0.25±0.03	0.20±0.03	0.26±0.03
Day 43 <sup>3</sup>	0.25±0.03	0.23±0.02	0.19±0.04	0.21±0.03
Bone Mineral Content (kg):				
Day 0 <sup>1</sup>	0.033±0.001	0.033±0.001	0.032±0.0004	0.033±0.001
Day 35 <sup>4</sup>	0.034±0.001	0.034±0.001	0.033±0.0004	0.034±0.001
Day 43 <sup>5</sup>	0.034±0.001	0.033±0.001	0.033±0.001	0.033±0.001

Group means are  $\pm$  SEM, n=9 except where indicated. Group means within columns with different superscript letters indicate a main effect of time via repeated measures analysis ( $p < 0.05$ ).

<sup>1</sup>n=8 for control diet, water

<sup>2</sup>n=7 for control diet, water, n=8 for control diet, DS and DHA diet, water

<sup>3</sup>n=8 for control diet, water and DHA diet, DS, n=7 for control diet, DS

<sup>4</sup>n=7 for control diet, water, n=8 for control diet, DS

<sup>3</sup>n=8 for control diet water and DS, DHA,DS.

**Table 5.8:** Percent Composition of Fatty Acids in the Liver

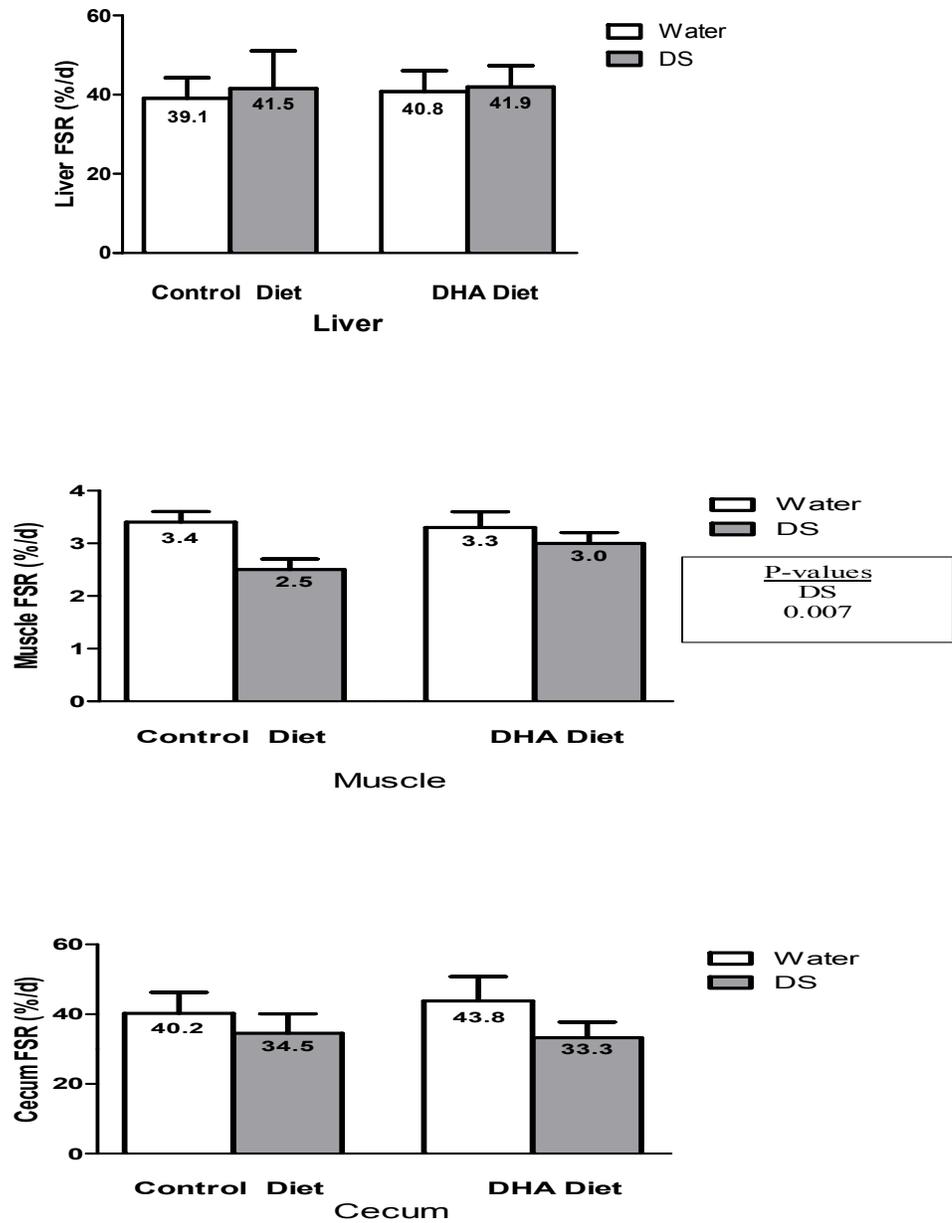
	CONTROL DIET, WATER	CONTROL DIET, DS	DHA DIET, WATER	DHA DIET, DS	P-values	
					DS	DHA
$\omega$ -6 Fatty Acids:						
% Linoleic Acid (18:2) <sup>1</sup>	41.69±0.89	41.56±0.90	38.22±1.43	38.54±1.20		
% Arachidonic Acid (20:4) <sup>2</sup>	2.56±0.25	2.28±0.50	3.58±0.40	2.42±0.44	0.034	
$\omega$ -3 Fatty Acids:						
% Alpha-Linolenic Acid (18:3) <sup>2</sup>	3.53±0.33	3.02±0.14	3.20±0.48	2.93±0.22		
% Docosahexaenoic Acid (22:6) <sup>2</sup>	0.20±0.03	0.34±0.19	1.25±0.19	1.11±0.29		0.002

Group means are  $\pm$  SEM, n=9 except where indicated.

<sup>1</sup>n=7 for control diet, water and control diet, DS, n=8 for DHA diet, water

<sup>2</sup>n=8 for control diet, water, n=7 for control diet, DS

**Tissue Fractional Synthesis Rates from Guinea Pigs Fed a Control or DHA Diet and Administered Water or DS**



**Figure 5.5:** Fractional synthesis rates (FSR) of mixed proteins in liver, muscle, and cecum of guinea pigs fed either a control or DHA diet and administered either water or DS treatments. For liver FSR: n=7 for control diet, water and DHA diet, water, n=5 for control diet, DS, n=8 for DHA diet, DS. For muscle FSR: n=7 for control diet, water and control diet, DS. For cecum FSR: n=8 for DHA diet, DS, n=7 for control diet, DS.

**Table 5.9:** Colon and Small Intestinal Mucosa Protein Fractional Synthesis Rates as Determined by a Flooding Dose of L-[ring-<sup>2</sup>H<sub>5</sub>]-Phenylalanine

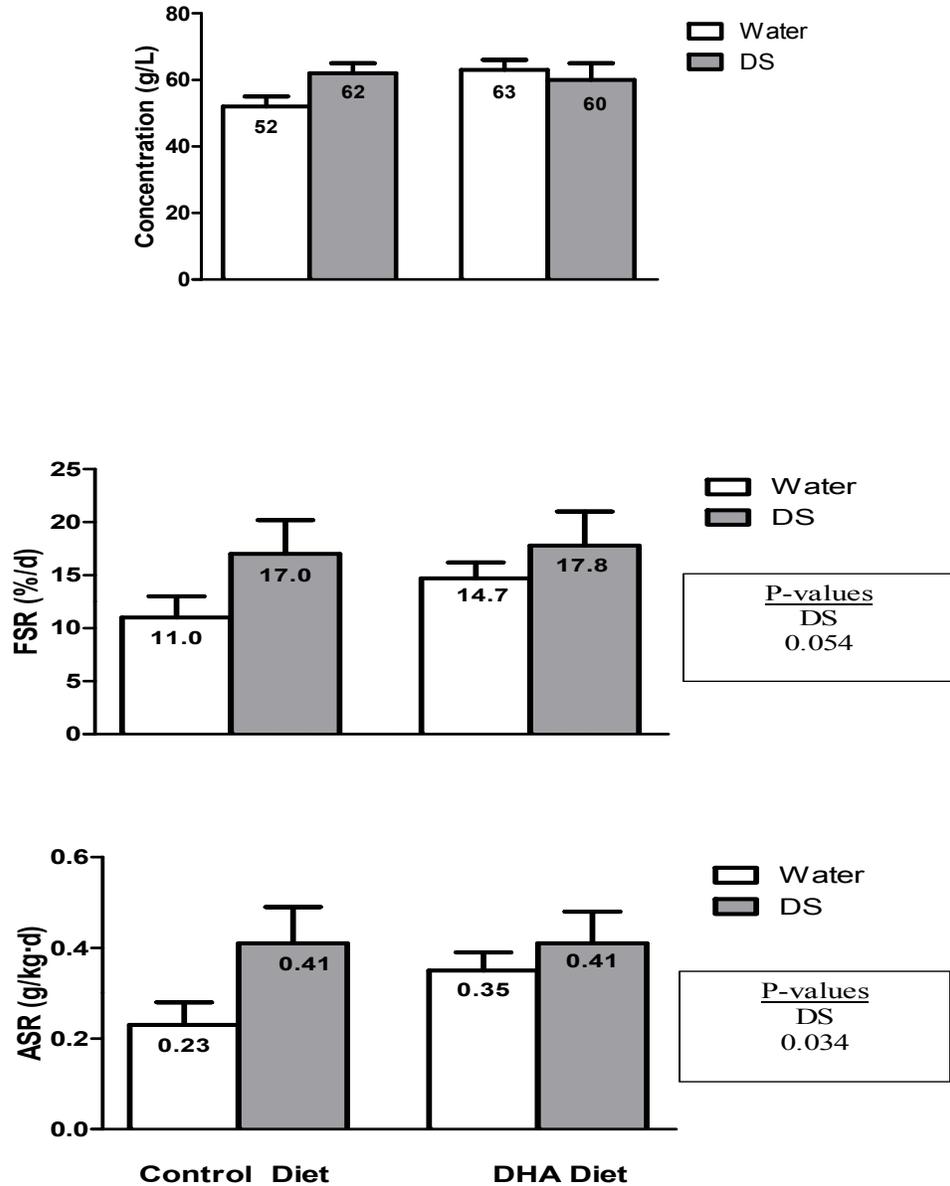
	CONTROL DIET, WATER	CONTROL DIET, DS	DHA DIET, WATER	DHA DIET, DS	<u>P-values</u> DHA
Tissue:					
Colon FSR (%/d) <sup>1</sup>	21.2±2.7	21.3±1.6	25.2±2.7	20.5±2.6	
Small Intestinal Mucosa FSR (%/d) <sup>2</sup>	31.0±1.9	31.4±2.6	36.4±1.3	32.5±2.2	0.026

Group means ± SEM, n=9 except where indicated.

<sup>1</sup>n=7 for control diet, water and control diet, DS

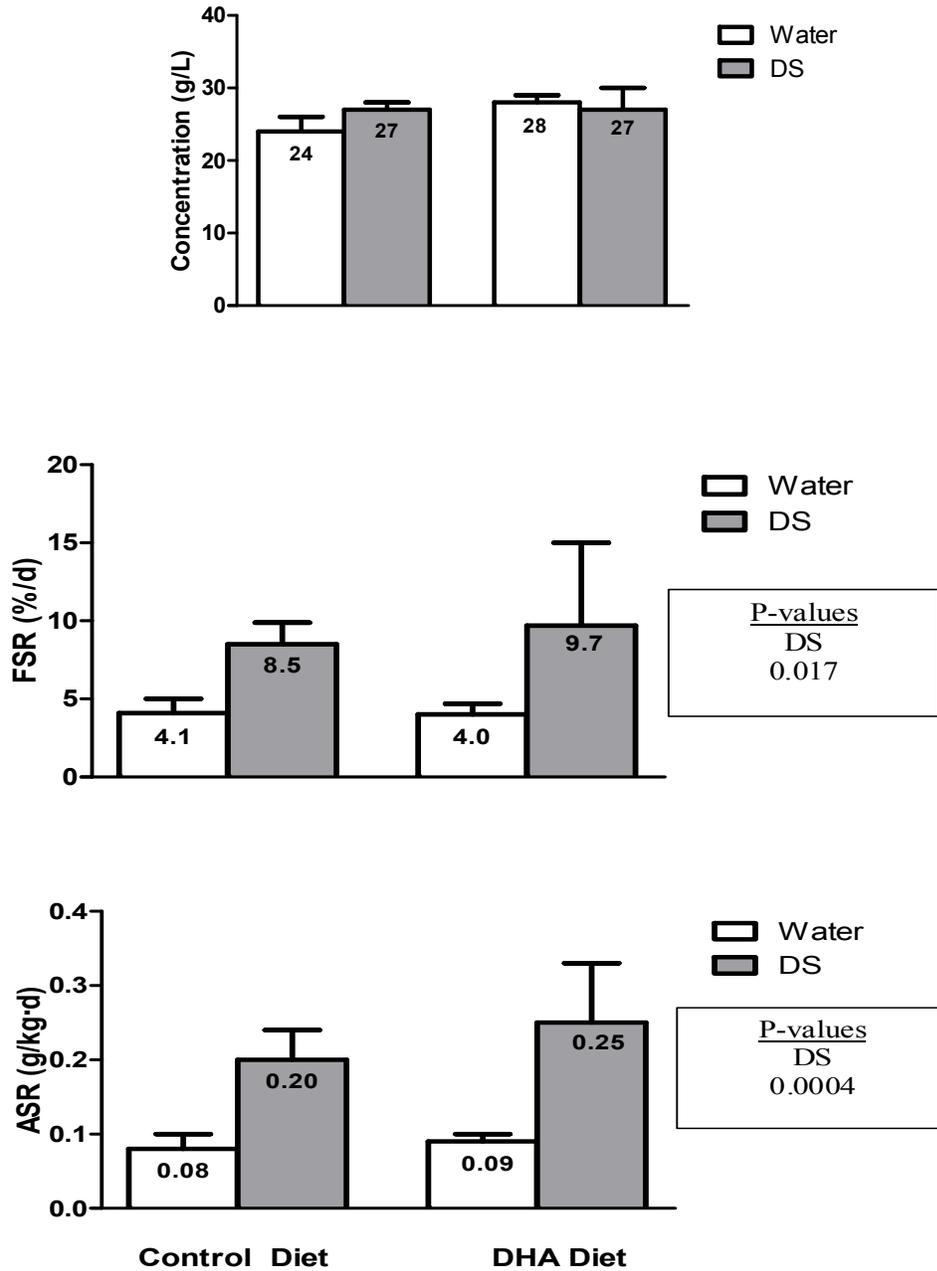
<sup>2</sup>n=8 for DHA diet, DS, n=7 for control diet, DS

**Concentration, Fractional and Absolute Synthesis Rates of Total Protein in Plasma in Guinea Pigs Fed a Control or DHA diet and Administered Water or DS**



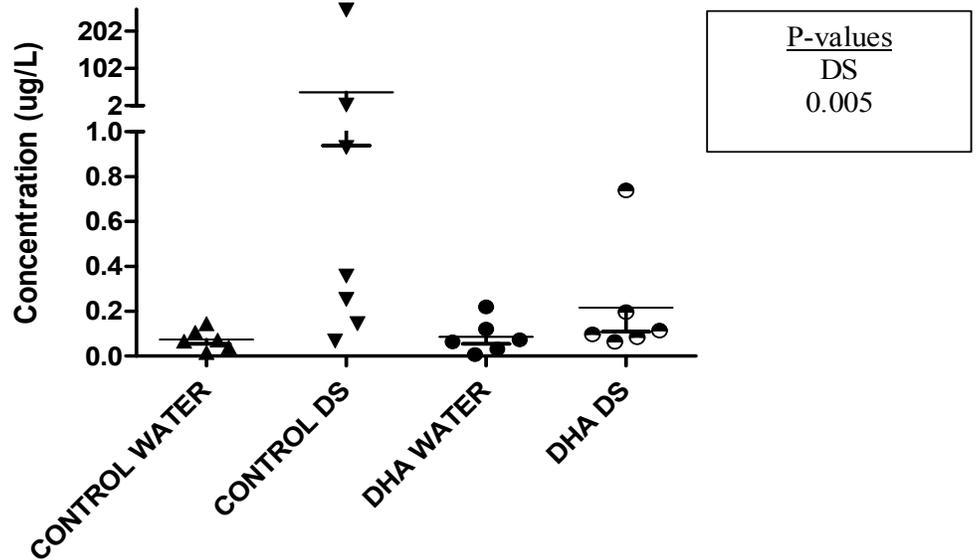
**Figure 5.6:** Concentration, fractional synthesis rates (FSR), and absolute synthesis rates (ASR) of total plasma protein of guinea pigs fed either a control diet or a DHA diet and administered either water or DS treatment. Group means are  $\pm$  SEM. n=8 for control diet, water group, n=7 for control diet, DS and DHA diet, DS, n=9 for all other groups.

**Concentration, Fractional and Absolute Synthesis Rates of Albumin in Plasma in Guinea Pigs Fed a Control or DHA diet and Administered Water or DS**

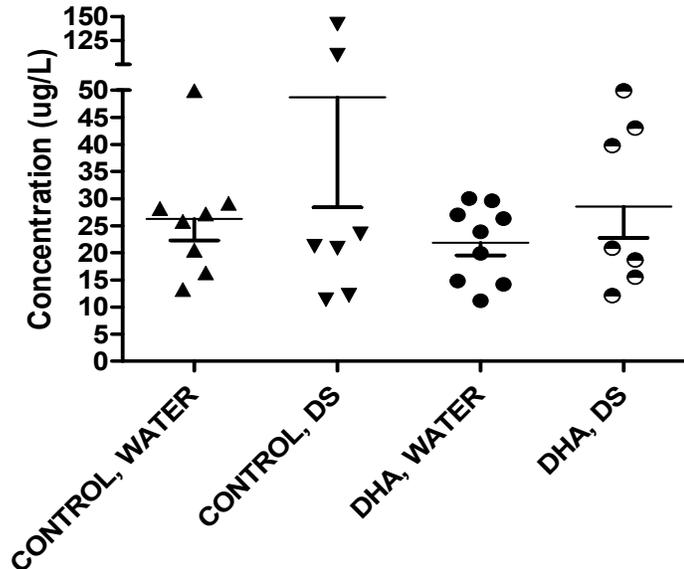


**Figure 5.7:** Concentration, fractional synthesis rates (FSR), and absolute synthesis rates (ASR) of albumin of guinea pigs fed either a control diet or a DHA diet and administered either water or DS treatment. Group means are  $\pm$  SEM. For concentration: n=8 for control diet, water group, n=7 for control diet, DS and DHA diet, DS. For FSR: n=7 for control diet, water, n=5 for control diet, DS and DHA diet, DS. For ASR: n=8 for control diet, water, n=7 for control diet, DS and DHA diet, DS.

### Plasma Cysteinyl Leukotrienes from Day 43



### Plasma Leukotriene B<sub>4</sub> from Day 43



**Figure 5.8:** Plasma Cysteinyl Leukotrienes and Leukotriene B<sub>4</sub> Concentration from Day 43. Group means are  $\pm$  SEM. Group means with different superscripts indicate a main effect of DS treatment ( $p < 0.05$ ).  $n = 6$  for all groups for cysteinyl leukotrienes.  $n = 8$  for control diet, water,  $n = 7$  for control diet, DS and DHA diet, DS for the leukotriene B<sub>4</sub> outcome.

## 5.5 Discussion

We adapted a guinea pig model of DS-induced intestinal inflammation to be reproducible and less severe. Furthermore, we modified the flooding dose technique for this guinea pig model in order to investigate protein kinetics and nutritional strategies for ameliorating gastrointestinal inflammation. The aim of the present study was to investigate if, a diet rich in DHA, improves disease severity and histological examination of cecum and colonic damage. Additionally, we aimed to examine if this improvement can be associated with altered lipid mediator production, and subsequent systemic effects on the APR and protein metabolism. Feeding of a DHA rich diet increased the percent of DHA in guinea pig livers (1.2% vs 0.3%). Furthermore, guinea pigs consuming the DHA diet, tended to have decreased inflammatory mediator production in DS guinea pigs in comparison to the control diet. Although this did not translate into a blunting of the acute phase protein response or moderated intestinal damage, DHA feeding did result in increased survival, and decreased clinical and physical symptoms of intestinal inflammation.

Our objective was to modify the guinea pig model of DS-induced colitis by administrating DS orally via needle-less syringe at a dose of 0.25 g/(kg·d) for up to 8 days, as opposed to administration in the drinking water *ad libitum*. Previous studies have induced colitis in guinea pigs via the oral administration of dextran sulphate as a 3% solution in the drinking water (Iwanaga 1994; Hoshi 1996). This model is considered a practical animal model due to the rapid and consistent induction of colitis (Hoshi 1996). Several studies have previously used a TNBS model of colitis in the guinea pig, which

results in a less rapid, more prolonged inflammatory response than with DS (Linden 2005; Lomax 2007).

Our adapted model resulted in a more accurate determination of a DS dose, which resulted in colitis with similar histological and clinical characteristics of some previous guinea pig (Iwanaga 1994), rodent (Cooper 1993), and piglet (Harding 2008) animal models. These characteristics included; crypt damage, inflammatory cell infiltrate, mucosal erosions and ulcerations, epithelial erosions, and bloody diarrhea. Consistent with previous guinea pig studies (Iwanaga 1994; Hoshi 1996), the histological damage was more apparent in the cecum rather than the colon. Dextran sulphate is a sulphated polysaccharide, which induces colitis in animal models of IBD (Okayasu 1990). The mechanism of action of DS is not clearly defined, however evidence suggests the mechanism is explained by several pathways, including; direct cytotoxic effects on epithelial cells, interference with the interaction between T lymphocytes and epithelial cells (Ni 1996), obliteration of crypts (Iwanaga 1994), and the activation of lamina propria macrophages (Iwanaga 1994; Hoshi 1996). Uptake of sulphated polysaccharides from macrophages is believed to cause mucosal injury via the release of lysosomal enzymes (Abraham 1974). The guinea pig is believed to be particularly susceptible to DS-induced colitis due to its especially large population of cecal lamina propria macrophages (Hoshi 1996). The clinical, physical, and survival characteristics of our guinea pig model of colitis were further investigated by the calculation of a disease activity index. DS resulted in a higher DAI than non-DS guinea pigs, as evidenced by greater weight loss, presence of fecal blood, diarrhea, and lethargic behavior. Sudden death occurred with two guinea pigs receiving the control diet and DS treatments,

presumably as a result of severe colitis. This loss of two animals may have biased the histological scores, resulting in a lower score for the control diet, DS group. Therefore, our model of DS-induced colitis resulted in an acute and severe model of IBD in the guinea pig. Furthermore, our model represented an adult model of DS-induced colitis, as opposed to the pediatric model, which has been applied previously in piglets (Harding 2008).

Guinea pigs ate less diet than expected during the dietary adaptation phase of the study as indicated by the consumption of approximately 50% of their estimated energy requirements (EER), as well as a decrease in weight in all animals. This decline in feed intake reflects the resistance of acceptance of diet changes with the guinea pig (National Research Council 1995), and furthermore, may be attributed to the lack of natural roughage present in the study diets. During the colitis phase of the study, guinea pigs receiving water were pair fed with guinea pigs receiving DS. The % of EER declined further in this phase, although DHA guinea pigs were more nourished (meeting approximately 40% of their EER), compared to control diet guinea pigs (meeting approximately 26% of their EER). Although this considerably low % EER was unexpected, it resulted in a malnourished model used in this study, which is consistent with the pathology of clinical IBD (Lucendo 2009).

Malnutrition, including protein energy malnutrition (PEM), often accompanies IBD and this is caused by a variety of factors including; decreased oral intake, increased metabolic demands, and gastrointestinal losses. PEM likely coincided with this malnourished model of colitis in the guinea pig, as guinea pigs met approximately half of their protein requirements following DS and water treatments. The implications of PEM

with IBD include intestinal malabsorption, decreased efficiency of the mucosal barrier (which causes a decrease in the functionality of the mucosa-associated lymphoid tissue), increased risk of infection (Lucendo 2009), and reduced protein turnover (Wykes 1996). Furthermore, in piglets with macronutrient restriction in combination with colitis, muscle protein synthesis was shown to decrease to support an increase in fibrinogen and albumin synthesis. This emphasizes the role of adequate nutritional status in preventing compromised protein status typical of IBD (Mackenzie 2003). Further exacerbating the effects of malnutrition, inflammatory mediators involved in IBD, including TNF $\alpha$  and IL-6, can result in anorexia and catabolism (Pajak 2008; Lucendo 2009). Although TNF $\alpha$  was unable to be detected in the cecum tissue, indicators of catabolism were evident. Although all guinea pigs lost weight and lean mass, DS treatment resulted in a greater loss in weight and lean mass. This finding is consistent with Crohn's disease data in humans, which demonstrated that patients with CD experience lower lean body mass than healthy patients (Jahnsen 2003). Using DXA to determine body composition is limited by hydration status where a subject containing excess or decreases in water will over- or under- estimate the fat content (Laskey 1996). This limitation may affect the accuracy of the body composition results from this study, as neither hydration status nor edema was considered. Many of the colitis guinea pigs may have been dehydrated, as many of them reduced their water intake as they got sick. Furthermore, edema may have been present in the guinea pig, as it can occur with clinical IBD due to cytokine-mediated increased vascular permeability and endothelial cell damage (Grisham 2003).

The DHA diet increased the % liver DHA composition in guinea pigs. Furthermore, guinea pigs consuming the DHA diet were more nourished following treatment administration. This finding is likely linked with the decreased DAI in the

DHA group, indicating that DHA guinea pigs had less severe clinical and physical symptoms associated with colitis, thus inclining them to eat more.

In response to inflammation, activated immune cells such as neutrophils, produce and release inflammatory mediators (Blok 1996; Gruys 2005). Pro-inflammatory leukotrienes are a group of acute inflammatory mediators produced from arachidonic acid released from membranes of leukocytes (Samuelsson 1987). The enzyme 5-lipoxygenase (5-LO) converts AA into leukotriene A<sub>4</sub> (LTA<sub>4</sub>). The combination of LTA<sub>4</sub> to glutathione results in the production of leukotriene C<sub>4</sub> (LTC<sub>4</sub>). LTC<sub>4</sub> is subsequently metabolized to leukotriene D<sub>4</sub> (LTD<sub>4</sub>) and leukotriene E<sub>4</sub> (LTE<sub>4</sub>) (Campbell 1990). In a separate pathway, LTA<sub>4</sub> is converted to LTB<sub>4</sub> (Simopoulos 2002). LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> are collectively referred to as cysteinyl leukotrienes, and these cysLT can accumulate in the effusion fluids associated with inflammation (Westcott 1990). In a similar vein, LTB<sub>4</sub> is a potent stimulator of neutrophil and cytokine activity with inflammation (Rola-Pleszczynski 1988; Calder 2002). Omega-3 PUFA, such as DHA are believed to play an anti-inflammatory role via the decreased production of these pro-inflammatory mediators (Simopoulos 2002). Furthermore, the omega-3 PUFA are metabolized to anti- or less pro-inflammatory eicosanoids such as leukotriene B<sub>5</sub> (LTB<sub>5</sub>) and prostaglandin E<sub>3</sub> (PGE<sub>3</sub>) as well as other 3 and 5 series eicosanoids (Simopoulos 2002).

In the present study DHA has been hypothesized to blunt the cytokine-induced acute-phase response via decreased pro-inflammatory lipid mediator production.

Decreased inflammatory lipid production may translate into decreased cytokine activation of the APR, as pro-inflammatory lipid mediators have been shown to have the capability of increasing the production of APR-stimulating cytokines (Rola-Pleszczynski 1988) (Calder 2002).

Although plasma concentrations of cysteinyl leukotrienes and LTB<sub>4</sub> were not statistically different between diet groups, this likely was a result of the large variability as well as the decreased sample size in the control diet, DS group due to the two sudden deaths. Upon examination of **Figure 5.8**, it is evident that in the control diet, DS group there exists data points, which represent high concentrations of both cysLT and LTB<sub>4</sub>. In comparison, the DHA diet, DS group had data points representing lower concentrations of inflammatory mediators. Thus, although there are no statistical differences between the diet groups, the differences are biologically significant, as the control guinea pigs tended toward greater inflammatory production, and this may have been statistically evident with lesser variability. It is of note that one guinea pig in the control diet, DS group had both high cysLT and LTB<sub>4</sub> concentrations, more specifically, 258.74 ug/L cysLT and 109.66 ug/L LTB<sub>4</sub>.

The hepatic fatty acid profile was measured in the liver, as the liver is one of the major sites for biosynthesis of eicosanoids (Kmieć 2001), as well as the liver is the major site for processing of fatty acids from the diet. The DHA diet increased the % composition of DHA in the liver of guinea pigs. Furthermore, the lower % AA in the liver of DS guinea pigs compared to control guinea pigs suggests that AA was used more with intestinal inflammation, likely for increased pro-inflammatory mediator production. Further investigations of inflammatory mediators revealed a tendency for DHA guinea

pigs with colitis to have reduced levels of cysLT and LTB<sub>4</sub> than those with the control diet. This was expected as omega-3 PUFA are believed to play a role in reducing pro-inflammatory lipid mediators, by partially replacing arachidonic acid in the membranes of immune cells (Simopoulos 2002). Furthermore, despite the decreases in inflammatory mediator production with the DHA diet, this did not translate to differences in intestinal damage. Pro-inflammatory lipid mediators, like LTB<sub>4</sub>, are capable of increasing the production of stimulators of the acute phase response, such as TNF $\alpha$  (Rola-Pleszczynski 1988; Simopoulos 2002). In the current study, it was expected that a decrease in inflammatory mediator production would result in a blunted acute phase response. However, albumin and total plasma protein FSR was increased to similar values in both diet groups and the reduction in muscle FSR was also similar.

The cytokine-induced acute phase protein response to inflammation classically includes the increase in concentration of positive acute-phase proteins such as fibrinogen, and the decrease in negative acute-phase proteins, such as albumin (Bistrian 1999). In the present study the concentration of albumin and total proteins was the same in both the control and colitis guinea pigs, regardless of study diet. Further investigation using a flooding dose of phenylalanine revealed increased total plasma protein FSR. Fibrinogen FSR and ASR was unable to be reliably calculated due to insufficient quantity of fibrinogen to be detected via GCMS. The blood sampled from the guinea pigs clotted rapidly, most likely contributing to a loss of fibrinogen as fibrin in clot formation. Furthermore, it is likely that there was increased fibrinogen loss in guinea pigs experiencing the GI damaging effects of DS, as fibrinogen is utilized in wound healing and blood clotting (Wolberg 2007). The classic acute phase protein response has been

characterized in pigs whereby, 48 hours following the inflammatory stimulus, there is an increase in positive acute phase proteins, including C reactive protein, and decreases in negative acute phase proteins, such as albumin and alpha-lipoprotein (Lampreave 1994). Furthermore, malnourished rats demonstrate rapid decreases in albumin concentration following an inflammatory stimulus (Jennings 1992). The mechanism for this hypoalbuminemia with inflammation has been further explored by Ruot et al. (Ruot 2003) whereby rats with sepsis were found to have an accelerated absolute plasma efflux of albumin during the early phase of sepsis. Thus, in the early phase of inflammation, the catabolism of albumin that is retained in the extravascular space likely plays a role in hypoalbuminemia.

The finding of decreased albumin concentration with inflammation contradicts the current study findings whereby albumin concentration was unaltered following inflammation. However, this contradiction is only apparent and is explained further via the protein kinetics data. Colloid osmotic pressure (Yamauchi 1992) and cytokines (Brenner 1990; Castell 1990) are important regulators of albumin synthesis. Increased osmotic pressure inhibits albumin gene transcription via the suppression of albumin promoter activities (Tsutsumi 1993). Cytokines such as TNF $\alpha$ , and IL-6, also regulate albumin synthesis at the mRNA level by inhibiting albumin gene transcription (Castell 1989; Brenner 1990). Leukotrienes may have an indirect effect on acute phase protein synthesis as LTB<sub>4</sub> is capable of increasing the production of the above-mentioned stimulators of the acute phase response such as TNF $\alpha$ , IL-1 $\beta$  (Rola-Pleszczynski 1988), and IL-6 (Calder 2002).

Albumin FSR has been shown to increase with several inflammatory conditions, such as in patients with cancer (Fearon 1998), head trauma (Mansoor 1997), and hemodialysis (Giordano 2001). Furthermore, in piglets with DS-induced colitis, albumin FSR is doubled, while albumin concentration remains the same for both control and colitis piglets (Mackenzie 2003). This is consistent with the current study, where albumin FSR was doubled in guinea pigs with colitis, while albumin concentration remains unchanged. This increase in albumin FSR, despite albumin being a negative-acute phase protein, demonstrates that measuring acute phase protein concentrations alone is an unreliable determinant of plasma protein metabolism with IBD. Additionally, the lack of a difference in albumin and total protein concentration with increased FSR in the current and Mackenzie et al (Mackenzie 2003) study, is suggestive of increased albumin loss with IBD. Although this loss was not apparent as hypalbuminemia in this study, the concentration of albumin may have decreased had the study continued for a longer period of time. Intuitively, increased albumin loss can be attributed to increased albumin excretion, catabolism, or both. Although this cannot be determined from the current study, some previous studies have investigated albumin metabolism. Albumin's abundance in plasma, as well as its low molecular weight (approx. 65 000 Da) (Nakamoto 2004), illustrates its susceptibility to loss from the body with inflammation. Cytokine mediated membrane permeability of the intestinal wall is believed to be responsible for albumin loss (Mahmud 1996). Coincidentally, loss of albumin into the urine (Deric 2008) and stool (Steinfeld 1960) in patients with IBD has been illustrated. The loss of albumin into the urine in patients with active IBD was determined as urinary albumin excretion rate (UAER) and was increased in patients with active IBD versus healthy controls (17.9 vs 8.4 mg/g creatinine) (Deric 2008). This glomerular leakage of

albumin is believed to be mediated by TNF $\alpha$  effect on permeability via increasing superoxide production in glomerular epithelial cells, as well as cytokine-induced inhibition of glycosaminoglycans metabolism (Yu 1983; Derici 2008).

The catabolism of albumin is not well defined, however, the study by Steinfeld et al. (Steinfeld 1960) additionally provides evidence of the catabolism of albumin in the gut with IBD. Furthermore, the high sulfur content of albumin (Barker 1984) may render its catabolism beneficial to provide cysteine for glutathione synthesis during oxidative stress in IBD (Sido 1998).

It should be noted that plasma protein ASR was also calculated in this study based on the serum protein concentration and blood volume. However, the use of this outcome is limited as it likely underestimates the actual ASR, especially considering the above-mentioned potential of albumin loss in guinea pigs with colitis.

The present study utilized a flooding dose of phenylalanine in the guinea pig in order to determine the protein kinetics in response to inflammation and a diet rich in DHA. This method proved efficient allowing for rapid blood and tissue samples to be taken, as well as providing an accurate estimate of the free amino acid precursor pool in order to calculate protein fractional synthesis rates. Most notable was the decrease in muscle FSR with colitis, regardless of the dietary group. This outcome is consistent with previous studies (Mansoor 1997; Jahoor 1999; Mackenzie 2003), which suggest a change in protein metabolism whereby amino acids are prioritized for acute phase protein synthesis at the expense of muscle protein synthesis. The prioritization of acute phase

protein synthesis is supported by the important roles of acute phase proteins in the resolution of inflammation via the clearance of pathogenic particle, debris, facilitation of the healing process, and modulation of the immune response (Gauldie 1994).

Compromised muscle synthesis likely corresponds with increased muscle proteolysis.

The muscle proteolysis that can accompany inflammation is well documented (Wray 2002; Costelli 2003), as well as evidence demonstrating the increased flow of amino acids for plasma protein synthesis (Mansoor 1997; Béchet 2005; Raj 2006; Fleet 2008).

The effect of trauma on protein kinetics was investigated by Mansoor et al. (Mansoor 1997) via a constant infusion of L-[1-<sup>13</sup>C]leucine. Following injury muscle protein FSR decreased by 50% whereas fibrinogen and albumin FSR was doubled and increased by 60%, respectively. Thus, this provides further evidence of the prioritization of acute

phase protein synthesis at the expense of muscle protein synthesis following injury. The amino acid compositions of the major acute-phase proteins comprise a significant portion of phenylalanine, tryptophan, and tyrosine (Reeds 1994). Therefore, it has been

suggested that increased muscle proteolysis is associated with the increased demand for these amino acids in order to provide sufficient amounts for plasma protein synthesis

(Reeds 1994). Muscle proteolysis was not measured in this study, however, there were significant losses in lean mass over time, as well as increased losses of lean mass in guinea pigs with colitis.

Although the FSR in the cecum tended to be lower in guinea pigs with colitis, this difference was not significant. However, this may be biologically significant as the cecum was the site of origin of the inflammation due to macrophages taking up the DS. Furthermore, lower cecum FSR was associated with several parameters of inflammatory

disease such as DAI, histological scores, loss in lean mass and weight. This suggests that cecum FSR may be compromised with inflammation.

The lack of significant differences between the two diet groups for several of the outcomes may be attributed to biological reasons, however it is of importance to address the statistical component as well. Firstly, two guinea pigs with colitis and consuming the control diet died following DS treatments. This may have biased several of the outcomes where these guinea pigs could not be included otherwise such as; histology, tissue and plasma protein synthesis, and inflammatory mediator production. The two guinea pigs likely died as a result of severe intestinal inflammation. Thus their contribution to the above-mentioned outcomes may have reflected this severity, and therefore that data may have been sufficient in revealing a statistical effect of diet. Furthermore, the smaller sample size in the control, DS group may have biased the statistical analysis. This is particularly of note with the cecum FSR and inflammatory mediators, whereby the numerical differences between the diet groups appear large but did not approach significance. The large variability with these outcomes likely contributes to their lack of significance, and the smaller sample size may have further prevented statistical significance. Furthermore, the guinea pig model used in this study was representative of acute and severe intestinal inflammation. Consequently, a strong effect of the DS treatment was seen for the majority of the outcomes. In order to elucidate differences in the diet with further outcomes, it may be advantageous to use an intestinal model with a more prolonged and less severe inflammatory response, such as with TNBS-induced colitis (Linden 2005; Lomax 2007).

The present study is the first of its kind to investigate the effects of DHA, in the absence of other omega-3 PUFA, on protein kinetics, disease progression, and

inflammation in a guinea pig model of IBD. Previous human studies have provided evidence on the ability of DHA to exert anti-inflammatory characteristics (Kelley 1999; Kew 2004); however these studies were implemented in the healthy state as opposed to a diseased population. The principal benefits of a diet rich in DHA observed in this study were the improvements in physical and clinical symptoms of intestinal inflammation, such as diarrhea, bloody stools, behavior (shaking, lethargy), and survival. This likely contributed to the improvement in appetite demonstrated by the DHA guinea pigs increase in feed intake following DS and water treatment administration. It is important to note however, that the potentially therapeutic DHA diet was given prior to the onset of colitis, which has limitations in clinical relevance to humans with colitis. This improvement in symptoms of IBD was demonstrated despite the severity and the acuteness of this colitis model. The guinea pigs consumed their study diets for 35 days prior to the induction of colitis. Despite this lengthy diet adaptation period, DHA did not prevent the onset of IBD. This is consistent with a study by Bassaganya-Riera et al. (Bassaganya-Riera 2006), whereby omega-3 PUFA (as fish oil) failed to avert the onset of IBD in pigs. Interestingly however, is that omega-3 PUFA supplement did manage to promote remission possibly through a peroxisome proliferator-activated (PPAR)  $\gamma$  dependent mechanism, as omega-3 PUFA upregulated the gene expression of colonic PPAR  $\gamma$ . Additionally, Omega-3 PUFA, such as with DHA, are believed to be beneficial in the resolution of inflammation indirectly via reducing the production of pro-inflammatory lipid mediators (Simopoulos 2002), and directly via the production of anti-inflammatory lipid mediators (Serhan 2008). Despite that the role of omega-3 PUFA in the resolution of inflammation is well documented (Schwab 2007; Kasuga 2008; Serhan 2008), human studies have found little evidence demonstrating a clinical benefit of

supplementation in patients with IBD (Turner 2007; Feagan 2008; Turner 2009). One possible explanation for this is that human intake of omega-3 PUFA varies, whereas in animal studies the experimental diet is fixed. DHA is metabolized into its anti-inflammatory mediators; the D series resolvins and protectins. These mediators are initially biosynthesized in inflammatory exudates during the resolution of inflammation and act directly on target cells to shield from excessive neutrophil infiltration and organ damage (Kasuga 2008). In the present study however, DHA did not protect the cecum and the colon from damage, despite a trend for decreased inflammatory mediator production.

In conclusion, a model of DS-induced acute and severe intestinal inflammation was developed in the guinea pig for the investigation of protein kinetics in nutritional interventions. Feeding of DHA increased the DHA content of the liver, and furthermore, resulted in a tendency for a decreased production of pro-inflammatory mediators. Despite this decrease in inflammatory mediator production, the acute phase protein response was not affected differently by the DHA diet, nor were there improvements in intestinal healing. Overall, a diet rich in DHA has clinical benefits in IBD due to the improvement of physical symptoms associated with colitis, including diarrhea, fecal blood, lethargy, survival, and dietary intake. The evidence presented here supports further investigations on the mechanism of action of DHA selectively improving physical symptoms of IBD.

## **6. FINAL CONCLUSIONS**

### **6.1 Summary of Results**

A guinea pig model of acute and severe intestinal inflammation was developed and characterized by extensive cecum and moderate colon damage as illustrated by inflammatory cell infiltrate, crypt damage, and bloody diarrhea. DHA decreased clinical and physical symptoms of colitis and promoted dietary intake in DS guinea pigs. Although inflammatory lipid mediator production tended to be lessened with DHA, the clinical benefit did not translate into a blunted acute phase response or improvement in intestinal damage.

### **6.2 Future Research**

Clinical IBD is characterized by chronic recurring episodes of gastrointestinal inflammation (Knigge 2002). The guinea pig model of IBD developed in this study was representative of acute and severe intestinal inflammation. Since omega-3 PUFA are believed to play a role in the resolution phase of inflammation (Serhan 2008), it would be of merit to extend this model of IBD to one that involves periods of remission as well as episodes of intestinal inflammation. Allowing a resolution phase of inflammation in the current model may unveil further clinical benefits of DHA with intestinal inflammation. Furthermore, it would permit further investigation on whether DHA's role in the resolution of inflammation involves the blunting of the acute phase response such that muscle protein synthesis would be less compromised.

Recently, the discovery and role of the omega-3 anti-inflammatory lipid mediators, the resolvins and the protectins, have been investigated (Hong 2003; Hudert 2006; Serhan 2008). It has been shown that the DHA lipid metabolite resolvin D1 is

initially synthesized in inflammatory exudates and consequently protects organ damage via the inhibition of neutrophil migration (Kasuga 2008). Although intestinal damage was not shown to be improved with DHA in the current study, it would be interesting to measure resolvin D1. Further insights on whether resolvin D1 was present in inflammatory exudates in the guinea pig colitis model, may aid in revealing the role of action of DHA in improving clinical and physical symptoms of colitis or demonstrating why intestinal damage was not improved in the current study. Additionally, although pro-inflammatory leukotrienes were measured in this study, it may be of merit to measure the anti-inflammatory leukotrienes as well, such as LTB<sub>5</sub>.

TNF $\alpha$  mediates changes associated with the systemic phase of the acute phase response, which results in the breakdown of muscle protein for amino acid use in the liver (Blok 1996; Gruys 2005). TNF $\alpha$  in the cecum of guinea pigs was measured in this study, however no measureable levels were detected in any experimental group. This was the first time that an attempt was made to measure TNF $\alpha$  in the guinea pig cecum, therefore it is possible that TNF $\alpha$  was not available in detectable amounts in this tissue. However, it would be of merit to further attempt to measure TNF $\alpha$  in other tissues or plasma, as it is a principle cytokine involved in the acute phase response.

### **6.3 Significance**

The guinea pig model of colitis characterized in this study can be used to investigate nutritional interventions in acute and severe intestinal inflammation. Furthermore, this represents a model of adult IBD as opposed to piglet pediatric models which have been used previously (Harding 2008). Of clinical significance was the finding that DHA improves clinical and physical symptoms of IBD, including improvement in dietary intake. Further investigation is required in the determination of the mechanism of action

of DHA in improving clinical signs of IBD, as in the current study this did not appear to involve improvements in gut healing nor blunting of the acute phase response.

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