M.Sc. Thesis

BONE HEALTH OUTCOMES IN PROTEIN DEFICIENT PIGLETS DURING THE ACTIVE AND RECOVERY PHASES OF DEXTRAN SULFATE SODIUM-INDUCED COLITIS

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ABSTRACT

Objectives: The global objective of this study was to characterize bone health outcomes in a protein deficient piglet model of acute irritable bowel disease (IBD) and to investigate anabolic or anti-inflammatory nutrition interventions during early recovery. Specific objectives were to: 1) characterize bone mineral accretion and biomarkers of growth and bone metabolism in protein deficient piglets with active colitis, and to determine their association with cytokines as markers of inflammation, 2) determine the impact of anabolic nutritional intervention or an anti-inflammatory N-acetyl-cysteine (NAC) supplementation in the early recovery phase of colitis on bone mineral accretion, bone geometry, biomarkers of growth and bone metabolism as well as cytokines.

Methods: Twenty one female piglets, 7 to 10 days old, weighing 3-4 kg were studied over 15 days. Piglets received a protein deficient enteral formula (EN) (15% of National Research Council requirement) and dextran sodium sulfate (DSS) (0.5 g/kg.d – b.i.d) intragastrically to induce colitis from day 3 until day 9. Piglets were then randomized into one of 4 groups: active control (ACT; n=5) were euthanized on day 10, adequate protein (AP; n=6) received EN supplying 100% of protein requirement, recovery (REC; n=5) continued protein deficient EN, and N-acetylcysteine (NAC; n=5) received protein deficient EN with NAC supplement. All recovery groups were euthanized on day 15. In-vivo bone quality assessments such as areal bone mineral density (aBMD) and bone mineral content (BMC) obtained from dual x-ray absorptiometry (DXA), as well as serum bone biomarkers and pro-inflammatory cytokines were measured on day 1, 9, and 14. DXA, micro computed tomography (μCT), bone length and width, as well as bone strength were measured ex-vivo in lumbar spine and femur.

Results: During the active phase of piglets receiving DSS, femur bone area (BA) and bone mineral content (BMC) continued to increase over time during the colitis phase while bone mineral density (BMD) decreased over time. BA of the spine was greater in AP piglets compared to REC piglets, with no differences noted in piglets receiving NAC supplementation. No significant differences were noted between groups for femur total bone volume or BMC, while AP piglets had a lower vBMD than piglets in the ACT

group. Bone markers revealed a decrease in the recovery phase compared to the active phase, however, no differences between groups were observed. Cytokines demonstrated no change over the study. While the groups showed no differences in the amount of energy to break the bone, AP piglets had a lower work to failure, lower stress max, lower Young's Modulus, as well as, less toughness than all other groups.

Conclusion: Despite having active colitis and receiving a protein deficient diet, piglets continued to grow, however, growth rate was greatest in AP piglets in the recovery phase. AP piglets in the recovery phase demonstrated the greatest growth in length, however, had the lowest vBMD. AP piglets revealed the lowest bone toughness and stiffness compared to ACT and REC groups, though, not in comparison to piglets receiving NAC. These nutritional interventions during recovery in IBD can provide indication for further studies on the strategies to optimize bone health outcomes and decrease disease severity in pediatric IBD.

RESUMÉ

Objectifs: L'objectif global de cette étude était de caractériser l'état de santé des os dans une protéine modèle porcelet déficiente de la maladie du côlon irritable aiguë (IBD) et d'enquêter sur les interventions nutritionnelles ou anti-inflammatoires pendant la récupération précoce. Les objectifs spécifiques étaient les suivants: 1) caractériser l'os accrétion minérale et des biomarqueurs de la croissance et le métabolisme des os dans un modèle de porcelet déficiente en protéines avec la colite active et leur association avec l'inflammation, comme indiqué par les cytokines pro-inflammatoires, 2) déterminer l'impact de l'intervention nutritionnelle anabolisants ou anti-inflammatoire N-acétylcystéine (NAC) supplémentation dans la phase de relèvement précoce de la colite en évaluant l'accrétion minérale osseuse, la géométrie de l'os, les biomarqueurs de la croissance et le métabolisme osseux, ainsi que les cytokines pro-inflammatoires.

Méthodes: 21 porcelets femelles, âgés de 7 à 10 jours, pesant 3-4 kg ont été étudiés pendant 15 jours. Les porcelets ont reçu une formule de protéine déficiente entérale (EN) (15% des besoins du Conseil National de Recherches) et du sulfate de sodium dextran (DSS) (0,5 g / kg.d – b.i.d) intra gastrique pour induire la colite jusqu'au jour 9. Les porcelets ont ensuite été randomisés dans l'un des 4 groupes: contrôle actif (ACT; n = 5) ont été euthanasiés au jour 10, les protéines adéquates (AP; n = 6) reçus EN fournir 100% des besoins en protéines, la récupération (REC; n = 5) protéine continue déficiente EN, et N-acétylcystéine (NAC; n = 5) ont reçu la protéine déficiente EN avec supplément NAC. Tous les groupes de récupération ont été euthanasiés au jour 15. In vivo évaluations de la qualité de l'os telles que la densité de surface minérale osseuse (aBMD) et la teneur minérale osseuse (BMC) obtenu à partir d'absorptiométrie double énergie à rayons X (DXA), ainsi que des biomarqueurs osseux sérum et les cytokines pro-inflammatoires étaient mesurés le jour 1, 9 et 14. DXA, micro tomodensitométrie (μCT), la longueur et la largeur de l'os, ainsi que la résistance osseuse ont été mesurés ex-vivo dans la colonne lombaire et du fémur.

Résultats: Au cours de la phase active des porcelets recevant DSS, zone fémur osseuse (BA) et la teneur minérale osseuse (BMC) a continué d'augmenter au fil du temps alors

que la densité minérale osseuse (DMO) a diminué au fil du temps. BA dans la colonne vertébrale était plus élevé chez les porcelets AP par rapport aux porcelets REC, sans différences constatées chez les porcelets recevant du NAC supplémentation. Aucune différence significative n'a été observée entre les groupes fémur pour le volume osseux totale ou BMC, alors que les porcelets AP avaient un plus bas vBMD que les porcelets dans le groupe ACT. Les marqueurs osseux ont révélé une diminution de la phase de récupération par rapport à la phase active, cependant, aucune différence entre les groupes n'a été observée. Les cytokines n'ont démontré aucun changement au cours de l'étude. Alors que les groupes n'ont montré aucune différence dans la quantité d'énergie pour briser l'os, les porcelets AP avaient un travail inférieur à l'échec, le stress max inferieure, module de bas Young inférieure, ainsi que, moins de dureté que tous les autres groupes.

Conclusion: Malgré la colite active et une alimentation déficiente en protéines, la croissance est encore positive, cependant, la croissance a été plus grande chez les porcelets AP dans la phase de récupération. Les porcelets AP dans la phase de récupération ont démontré la plus grande croissance en longueur, cependant, était la plus faible vBMD. Les porcelets AP ont révélé la plus faible résistance osseuse et de rigidité par rapport aux groupes ACT et REC, cependant, pas par rapport aux porcelets recevant du NAC. Ces interventions nutritionnelles pendant la récupération de IBD peuvent fournir une indication pour d'autres études sur les stratégies visant à optimiser les résultats de santé des os et diminuer la gravité de la maladie IBD pédiatrique.

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LIST OF ABBREVIATIONS

25OHD: 25-hydroxycholecalciferol

ACTH: adrenocorticotrophic hormone

AP: adequate protein

ATG16L1: autophagy-related protein 16-1

BA: bone area

BAP: bone-specific alkaline phosphatase

BGLAP: bone-gamma-carboxyglutamic acid-containing protein

BMC: bone mineral content

BMD: bone mineral density

BMI: body mass index

BS/TV: bone surface density of trabeculae

BV/TV: bone volume fraction

CARD15: caspase recruitment domain-containing protein 15

CD: Crohn's disease

CRF: corticotrophin releasing factor

DLG5: disks large homolog 5

DNA: deoxyribonucleic acid

DSS: dextran sulfate sodium

DXA: dual-energy x-ray absorptiometry

EIA: enzyme immunoassay

EN: enteral nutrition

GCs: glucocorticoids

GH: growth hormone

GI: gastrointestinal

Gla: gamma-carboxyglutamic acid

GSH: glutathione

GSSG: glutathione disulfide

HIV: human immunodeficiency virus

HPA: hypothalamic-pituitary-adrenal axis function

IBD: irritable bowel disease

IFNγ: interferon gamma

IGF -1: insulin-like growth factor-1

IL: interleukin

IRMA: immunoradiometricassay

JAK2: janus kinase 2

μCT: micro-computed tomography

MSC: mesenchymal stem cells

MYO9B: myosin-IXb

NAC: N-acetylcysteine

NADPH: nicotinamide adenine dinucleotide phosphate

NF-kB: nuclear factor-kappa B

NOD2: nucleotide-binding oligomerization domain-containing protein 2

NRC: National Research Council

NSAID: nonsteroidal anti-inflammatory drugs

NTx: N-terminal telopeptide

OC: osteocalcin

OCTN: organic cation transporter

OPG: osteoprotegerin

PBM: peak bone mass

Pi: inorganic phosphate

pQCT: peripheral quantitative computed tomography

RANKL: receptor activator of nuclear factor kappa-B ligand

RIA: radioimmunoassay

ROS: reactive oxygen species

SDS: standard deviation scores

STAT3: signal transducer and activator of transcription 3

Tb.N: total # of trabeculae

Tb.Th: thickness of trabeculae

Tb.Sp: separation of trabeculae

Th: T helper

TNFα: tumor necrosis factor alpha

TGHβ: transforming growth factor beta

TYK2: tyrosine kinase 2

UC: ulcerative colitis

vBMD: volumetric bone mineral density

LITERATURE REVIEW:

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is an autoimmune disease with chronic or recurring immune response and inflammation of the gastrointestinal tract that includes Crohn's disease (CD) and ulcerative colitis (UC) [1]. Although these conditions share basic features of remission and exacerbation of gastrointestinal symptoms, they differ in their manifestations. The key differences include the location of the disease as well as the extent of inflammation. In CD, chronic inflammation may affect any part of the gastrointestinal tract, from mouth to anus, and can penetrate through the entire thickness of the bowel wall. In UC, however, mucosal inflammatory changes are restricted exclusively to the colon and typically involve the inner lining (mucosa) [2]. Symptoms may range from mild to severe and can occur quite differently among people. Signs and symptoms that are common to both CD and UC can include diarrhea, fever and fatigue, abdominal pain and cramping or nausea, blood in the stool, a reduced appetite, as well as unintended weight loss. An individual with IBD is likely to alternate from periods of active illness (flare-up) followed by periods of remission. Adults and children with IBD may present with similar clinical features, however, children can acquire distinct complications including slowed growth, impaired bone development, and decreased stature [3].

In the absence of a cure, therapy is directed at achieving and maintaining remission, prevention of disease complications, control of postoperative disease recurrence, maintenance of normal growth and development, and maximization of quality of life. Most individuals affected will require ongoing medication and when this fails, surgery is often required. IBD is a lifelong disease and usually occurs in early adulthood in otherwise healthy, active individuals. However, IBD is increasingly being diagnosed in young children, identifying about 25% of cases in childhood and adolescence [3].

Epidemiology:

IBD is mainly found in developed countries, more commonly in urban areas and often in Northern climates with Canada having amongst the highest incidence of people with CD and UC in the world. There are approximately 233,000 Canadians living with IBD, 129,000 with CD and 104,000 with UC. There are over 10,200 new cases identified every year, 5,700 with CD and 4,500 with UC [4]. The incidence of the disease in Canada affects about 1 in every 150 Canadian adults. The incidence and prevalence of IBD in the pediatric population has shown significant increases. Canadian studies have reported an increase in new diagnoses from 9.5 per 100,000 in 1994 to 11.4 per 100,000 in 2005. The prevalence of the disease has risen 5% annually in children younger than 4 years of age and 7.6% annually in children ages 5 to 9 years [3]. In the US, 12.5 years is the mean age of identification of pediatric IBD, with 20% diagnosed before the age of 10 and fewer than 5% identified before the age of 5 [3]. In general, adult IBD affects males and females equally, however, in children, new cases of CD in males outnumber females and an equal number of males and females are diagnosed with UC [1, 3]. Pediatric IBD is more extensive at the time of presentation and demonstrates continued progression within the first 5 to 7 years after identification in comparison to adult onset IBD [5]. IBD affects more people of Caucasian and Jewish origin than in other racial and ethnic subgroups, yet these differences seem to be narrowing [4]. Both the incidence and prevalence of IBD has increased for Hispanics and Asians – demonstrating both groups are more likely to develop UC than CD [6]. Though IBD rates among African Americans now comes close to that of Caucasians, there are differences between racial and ethnic groups in IBD family history, disease location, and extra-intestinal manifestations [6]. In addition, IBD affects Canadians financially, with an estimated economic cost of $2.8 billion in Canada spent on IBD in 2012 equating to over $11,900 per person every year with IBD [4].

Pathophysiology:

The etiology of IBD is not known yet thought to be multifactorial and encompasses both innate and ecological mechanisms. Research has demonstrated that the pathogenesis of IBD consists of a combination of genetics, immunology, environmental and bacterial factors [1].

Genetics/immunology:

Family studies have demonstrated that approximately up to 25% of children who develop IBD have a positive family history of IBD and this link appears to be stronger for CD than UC [3, 7]. Among family members, the risk of developing IBD is greatest among first-degree relatives, especially siblings. In addition, twin studies have shown that genetics play an essential role in the etiology of IBD; however, it also suggests that genetic factors may be more important in CD than in UC [7]. Monozygotic twin concordance is approximately 50% for CD and only 20% for UC [3]. There are a variety of specific links involved in the pathogenesis of IBD, with more than 160 genes and loci for IBD revealed by genome-wide association studies [6]. Many share a connection to the immune system, the inflammatory response, or bacterial recognition pathways, which are the main aberrations in the pathogenesis of IBD. Most prominent among these loci include IBD1 encoding the protein NOD2/CARD15 in CD (bacterial recognition), IL23R/IL-17 in CD and UC (cytokine receptor), and ATG16L1 in CD (autophagy) [1, 6]. Altered IBD1 genes cause mutation of the protein NOD2/CARD15 and affects the ability of the host to localize and destroy bacteria that come into contact with the gut tissue [6, 7]. An abnormal inflammatory response occurs or the presence of antigen stimulates an adaptive immune response when the bacteria are not disposed of. NOD2/CARD15 was the first CD gene to be linked with a defect in this mechanism and has demonstrated that IBD pathogenesis potentially contains a weakness in bacterial identification and host innate immunity [7]. Interleukin-23 receptor (IL-23R) is the cytokine responsible for the differentiation of the pro-inflammatory effector T lymphocytes (TH17 cells) most correlated with CD. IL-23R has shown to aid in the balance of anti-inflammatory regulatory T cells and these pro-inflammatory effector T cells [7, 8]. Genes regulating IL17, IL-23 receptors, IL12B, STAT3, JAK2, and TYK2 are associated with both CD and UC via disruption of the adaptive immunity pathways [9]. ATG16L1 is a key gene in the process of autophagy and mutation in this gene causes autophagy failure and promotes inflammation [7, 9]. The significance of understanding these and related pathways are important to consider for future therapies for IBD.

Environmental/bacterial factors:

There is accumulating evidence that events early in life may have long-term effects on health and disease. Some factors in the environment have been linked with the diagnosis of IBD in the pediatric population. Studies have shown that high levels of hygiene may aid in reducing childhood exposure to bacteria and viruses and possibly change the type of bacteria found in the gut [9, 10]. Also, children who have had one or more prescription for an antibiotic within the first year of life are at risk of developing IBD [11]. The use of these antibiotics may lead to an alteration in certain gut micro flora that are crucial for the protective effects against IBD [11]. Other medications such as isotretinoin, used for acne treatment, have been associated with IBD by possible alteration of the innate or adaptive immune system [12]. Nonsteroidal anti-inflammatory drugs (NSAID) and aspirin can affect the interaction between the gut microbiome and immune cells in the gastrointestinal wall, as well as, alter platelet aggregation, releasing inflammatory mediators, which are key events in the pathogenesis of IBD [13]. In addition, as previously mentioned, with IBD being more common in developed countries, air pollution has also been investigated for a link to IBD and has been shown to only effect younger patients [4].

Lifestyle risk factors such as smoking/smoking byproducts, as well as second hand smoke, are known to have different effects on UC and CD. A higher risk is noted for developing CD, however, is not a risk factor for UC and may actually be protective of the development of UC [6, 7]. Diet has been recognized as a risk factor for the development of IBD suggesting food antigens are thought to trigger an immunologic response, however, specific pathogenic antigens have not been identified. Hypotheses have been made to suggest that a "Western" style diet (processed, fried, and sugary foods), or a high intake of total fat, polyunsaturated fatty acids, omega-6 fatty acids, and red meat is associated with an increased risk of developing IBD [14, 15]. Specifically, a study by *Geerling et al*. demonstrated high intakes of monounsaturated fat (OR: 33.9 [95% CI 2.6-443.1]), polyunsaturated fat (OR: 5.1 [95% CI 1.0-26.7]), and vitamin B_6 (OR: 6.9 [95% CI 1.6-30.7]) were associated with an increased risk to develop UC. Long-term intake of dietary fibre, in particular from fruit, has been connected with a decrease in risk of CD

but not UC [15]. A hypersensitivity to cow's milk protein in infancy has been suggested as a cause of IBD, and specifically with UC [16]. In addition, breast-feeding may be a defense mechanism against the development of IBD as it stimulates the development and maturation of the gastrointestinal mucosa of infants and may protect them from GI infections in infancy. As discussed, the risk of IBD is increased following any GI infection, which causes an imbalance in the gut microbiome, and specifically infections with either Campylobacter or Salmonella [1, 17]. Regular physical activity has been associated with a decrease in the risk of CD and individuals who had an appendectomy demonstrate to have a lower occurrence of UC [18]. Lastly, sleep deprivation has been linked with an increased risk of UC and disease flares in patients with IBD and further studies are needed to explore the mechanisms by which sleep may influence intestinal inflammation [19].

Inflammatory Response in IBD:

Due to the lack of studies preformed on the pediatric population when it comes to comprehending the inflammatory events occurring in the gut of an IBD patient, reliance on a mix of information on mucosal immunity gathered primarily from human adult studies and animal models is necessary. The mucosal immune system protects the intestinal system from a variety of challenges from the external environment, and in particular of the GI tract – foods, commensal microflora, microbial pathogens, and xenobiotics. Studies demonstrate that disturbance in the epithelial barrier may either initiate or spread chronic intestinal inflammation [20]. Abnormal intestinal permeability has been discovered in IBD patients, which may predispose the individual to excessive antigen uptake, continuous immune stimulation, and ultimately mucosal inflammation [20]. Such IBD associated genes, OCTN, DLG5, JAK2, MYO9B, are known to affect epithelial permeability, which leads to an inappropriate exposure of the mucosal immune system to luminal antigen. In response, activated immune cells secrete a mixture of soluble mediators of inflammation, including cytokines (tumor necrosis factor [TNF]-α, interferon-γ, transforming growth factor-β, and interleukin-2, -5, -6, -12, and -18), arachidonic acid metabolites, reactive oxygen intermediates, streptolysins, and growth factors [3, 21].

Bone Growth and Development in Pediatrics:

Bone development and remodeling takes place on a continual basis, from the prenatal period and early childhood to adulthood. Ossification, the process whereby tissue becomes bone, can take one of two forms depending on the type of bone that is forming. Flat bones, such as the ribs, ilium, sternum, and skull, undergo *intramembranous* ossification. This process is initiated by mesenchymal stem cells (MSCs), which are multipotent stromal cells that can differentiate into a variety of cell types such as osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells) [22]. Osteoblasts are essential to create ossification centers. In the matrix formation phase that follows, the osteoblasts begin to lay down osteoid, which is the organic portion of bone matrix, consisting of collagen fibres. Osteoblasts begin the process of forming bone tissue by secreting the osteoid as several specific proteins. Some of the osteoblasts are entrapped within the osteoid and become osteocytes. The osteoid then calcifies and forms slender needle-like structures or spongy bone called spicules, which aggregate to form trabeculae. Meanwhile, the blood vessels on the outside of the spongy bone condense and form the periosteum, the dense layer of connective tissue enclosing bones except at the surface of the joints. As trabeculae thicken, an interconnected network forms to create woven bone, disorganized collagen fibers and is mechanically weak. Eventually, this leads to the formation of lamellar bone around the newly formed spongy bone. Lamellar bone (compact bone), in contrast to woven bone, has regular parallel configuration of collagen into sheets (lamellae) and is mechanically strong [22, 23]. While flat bones are formed through intramembranous ossification, most bones of the skeleton, such as the long bones of the legs and arms, are formed by *endochondral* ossification. This process involves cartilage models that are eventually replaced with bony tissue. Beginning in the developing fetus, this process is divided into several stages. Around the 8^{th} week of development, chrondroblasts begin secreting cartilaginous matrix that will form the hyaline cartilage model for bone development. As with other hyaline cartilage tissues, chondrocytes are trapped in lacunae and the perichondrium surrounds the model. The next stage begins when chondrocytes within the center of the model hypertrophy and begin to resorb some of the surrounding cartilage matrix. As these chondrocytes enlarge, the matrix begins to calcify and chondrocytes

begin to die, as nutrients cannot diffuse though the newly formed calcified matrix. Next, MSC's within the perichondrium divide to form osteoblasts and a compact bone collar is formed around the calcified cartilage shaft. A periosteal bud consisting of capillaries and osteoblasts invades the core of the cartilaginous shaft, forming a primary ossification center. The remains of the calcified cartilage serve as a template in which osteoblasts can build bone. Bone mineralization extends from the primary ossification center towards the epiphyses. Most primary ossification centers are formed by the 12^{th} week of development. The same basic process of primary ossification is repeated in the epiphyses as secondary ossification centers are formed, replacing the calcified cartilage with mostly spongy bone. As the secondary ossification centers are formed, osteoclasts resorb bone within the diaphysis creating a medullary cavity (or marrow cavity). When secondary ossification is complete, cartilage is totally replaced by bone, except in two places: a region of cartilage remains over the surface of the epiphyses as the articular cartilage and another area of cartilage remains between the epiphyses and diaphysis. The later is referred to as the epiphyseal plate or growth plate and is the point at which bone growth and length occurs. In puberty, the epiphyseal plate completely ossifies and bone growth comes to a stop with exception of appositional expansion [23, 24].

Implications of IBD in Pediatrics

As previously stated, the incidence of IBD is rising in both adults and children of all ages. Although presenting symptoms and therapeutic options are comparable, there are important differences in the two groups in which applying separate approaches to treatment and management of the disease in children is necessary. Symptoms such as growth failure and malnutrition, bone resorption, weight loss, anemia, arthritis, and delayed puberty lead to a higher severity of morbidity in the pediatric population [25, 26].

Growth Failure:

The risk of growth failure in children and adolescence with IBD is of major concern. Growth failure has been reported in 10 to 88% of children with IBD [27]. One of the largest studies to investigate longitudinal growth patterns in pediatric IBD have

demonstrated that girls with IBD were 2.7 cm shorter than their healthy peers at a mean age of 16.5, and boys showing similar results at 2.6 cm shorter (at mean age of 18.4 years) [28]. A decrease in height velocity may occur prior common clinical symptoms; therefore, it is important to plot children's height and weight on appropriate growth charts to aid in early detection of the disease. In a study of 124 pediatric IBD patients, it was demonstrated that height velocity was < -2.0 standard deviation scores (SDS) during at least one period between 3 years of age and the end of puberty for 34% of children with UC and 65% with CD [27]. Subnormal growth velocity was most frequent during the pre-pubertal period, when 24% children with UC and 40% with CD had growth velocity < -2.0 SDS [27]. IBD is likely to have a great effect on nutritional status and growth due to the quick increase of lean body mass that normally occurs during the growth of a child. Nutritional requirements are increased during pediatric growth, specifically during pubertal growth, which accounts for 16% of adult height and is associated with a doubling of body weight. Difficulties are faced to meet nutritional requirements in a pediatric IBD patient, which can lead to issues of malnutrition including delays in skeletal maturation, onset of menarche, and epiphyseal fusion in the long bones [25]. Permanent growth impairment can result in IBD if delayed puberty occurs, in contrast to healthy children. This delay can also affect the normal mineral accumulation peak that follows the pubertal growth spurt by affecting hormones that are significant for normal bone mineralization and in turn, play a role in osteoporosis [25, 26]. It has also been reported that 85% of children with CD and 65% with UC demonstrate a weight loss at the time of identification of IBD. This weight loss and nutritional insufficiency commonly seen in pediatric IBD is mostly due to inadequate food intake (affected by abdominal pain and disease related anorexia) and malabsorption (iron, zinc, folate, and vitamin B_{12} due to small bowel mucosal disease) rather than excessive losses [25, 26]. Growth failure can be very insidious; therefore, it is important that these issues are addressed early in the disease to prevent permanent effects on height and maturation.

Bone Growth and Development:

Of particular concern, children with IBD demonstrate decreased bone mineral density (BMD) from a variety of risk factors such as inadequate nutrition, the inflammatory

process from the disease itself, and the use of corticosteroids [27, 29]. Low BMD in childhood is of great concern as it is associated with increased fracture risk and development of osteoporosis and osteopenia [29, 30]. *Gokhale et al.* demonstrated lower BMD Z scores in children with IBD at the lumbar spine and femoral neck compared to healthy sibling controls, even after correction for bone age [29]. For example, BMD Z scores of lumbar spine for boys with CD was -0.55 $\pm$ 1.30 (p=0.017) and for girls was 0.88 $\pm$ 1.28 (p=0.004). For boys, 34.3% of BMD Z scores was < -1 SDS while 17.1% was < -2 SDS. Slightly higher, but similar for girls, 36.4% of BMD Z scores was < -1 SDS and 18.2% was < -2 SDS [29]. The low BMD in the patients in this study was predominately seen at the lumbar spine, with less loss of bone at the femoral neck. Other studies have also shown mean SDS of the lumbar spine BMD to be significantly lower than reference values (-0.75 (SD 1.20)) with no significant difference seen between boys and girls [31]. These sites are commonly studied, as they are sites of rapid turnover of bone in children and consist predominantly of trabecular bone. Studies have shown that children with IBD, even at the time of diagnosis, demonstrate mild cortical bone loss and formation of trabecular bone is greatly affected throughout the disease [30, 31]. In addition, *Boot et al.* determined that the mean delay of puberty was 0.70 years (1.14) in IBD, with a greater delay related to lower lumbar spine BMD SDS (r=-0.45, p<0.01). Throughout childhood, bones grow through a process called modeling and this results in changes of bone shape, width, and size until skeletal maturity is completed. Bones of children with IBD are affected differently than adult IBD in that children will usually present with decreases in osteoblast and chondrocyte activity (arrested growth), especially bone formation, while adults show signs of bone resorption and reduced osteoblastic activity required to fill resorption pits [32, 33].

Malnutrition

Malnutrition is usually evident in IBD and can present in different forms. Whether it is through protein-energy malnutrition, an altered body composition, micronutrient deficiencies, or poor bone mineral accretion or BMD, when persistent it increases both morbidity and mortality [34]. Protein-energy malnutrition is a common symptom at the time of diagnosis for IBD and is usually accompanied by a history of weight loss, and

fluctuates during the course of the disease. It is common for IBD children to have abnormal anthropometry, demonstrate a low body mass index (BMI) and be underweight and thin, especially CD patients, as compared to their healthy peers [34, 35]. A decreased intake is usual for children with IBD and this can be due to anorexia, nausea, vomiting, impaired taste sensations, and dietary restrictions set by the physician or patient (in fear of abdominal pain or diarrhea). Due to inflammation of the small bowel mucosa or resection of small bowel segments for patients with CD, malabsorption can occur (particularly vitamin D, vitamin K, calcium, and magnesium). The relationship between vitamin D status and bone health has been controversial among adults and children with IBD. Vitamin D is essential for normal absorption of calcium from the intestine and for normal bone mineralization. Maintaining optimal vitamin D stores in IBD patients is difficult due to these GI losses and general malabsorption that is commonly seen in this disease. Studies show that IBD has been associated with hypovitaminosis D; however, serum 25-hydroxy-vitamin D (25OHD) concentration was not correlated with lumbar spine BMD Z scores [35, 36]. The limited pediatric research on vitamin D and its impact on bone health of IBD report no association between BMD and vitamin D status in children with CD.

Increased nutrient loss is observed as well. In particular, protein loss can be attributed by the inflamed mucosa, as well as, blood loss (increased loss of iron), diarrhea (increased loss of electrolytes – potassium, magnesium, and zinc), fistula drainage, and liver disease [36]. Studies show that protein deprivation may decrease serum levels of insulin-like growth factor 1 (IGF-1) and insulin binding proteins, causing a resistance to the biological effects of growth hormone (GH), which in turn, effects linear growth [35, 36]. GH is secreted by the pituitary gland and acts on the liver to generate IGF-1, which is necessary for the proliferation and differentiation of chondrocytes in the epiphyseal plate. GH also has a direct action on the growth plate by increasing its sensitivity to GH and generating local IGF-1. Overall, IGF-1 from serum or following GH stimulation in chondrocytes is crucial for longitudinal bone growth and formation. It also stimulates the production of osteoblasts, increases type 1 collagen synthesis, alkaline phosphatase activity (enzyme that helps break down proteins reflecting biosynthetic activity of bone

forming cells), and osteocalcin production (bone gamma-carboxyglutamic acid-containing protein) [37]. IGF-1 aids in stimulating the transport of inorganic phosphate (Pi) across the plasma membrane of osteoblastic cells and plays a key factor in the adjustment of calcium-phosphate metabolism, which is essential for normal skeletal mineralization during growth [37].

Plasma levels of IGF-1 are usually associated with the rate of growth [37]. Decreased levels of IGF-1 have been demonstrated in states of undernutrition such as marasmus, anorexia nervosa, celiac disease, and HIV patients, however, refeeding these patients results in increased IGF-1 levels. More specifically, an elevated protein intake on a hypocaloric diet is able to prevent the decrease in IGF-1. This demonstrates the hepatic production and plasma levels of IGF-1 are under the influence of dietary proteins. Low plasma IGF-1 levels that are seen in protein restriction may be due to a resistance to the action of GH at the hepatic level and by an increase of IFG-1 metabolic clearance rate. Human studies have demonstrated that there is a positive association between protein intake and bone mineral accretion and has been detected in both sexes at the level of the lumbar spine, the proximal femur and the mid-femoral shaft in healthy children and adolescents [37]. Attaining peak bone mass (PBM), the amount of mineral present at the end of skeletal maturation, is greatly affected by sufficient protein intake during the time of growth and is an important factor of osteoporotic fracture later in life. Although no gender difference has been detected in bone mass at birth, males have a more prolonged bone growth period than females, with a greater increase in bone size and in cortical bone thickness, in puberty [36, 37]. Several animal studies using rats have also demonstrated the detrimental effects of inadequate protein on bone health. *Ammann et al.* demonstrated that after 16 weeks, growing rats fed a protein restricted diet of 2.5% casein as compared to an adequate protein diet; BMD and bone strength was significantly decreased at skeletal sites (lumbar spine, femur, and tibia) containing trabecular or cortical bone. This study also confirmed lower plasma IGF-1 and estrogen levels in the protein-restricted group and resulted in induced bone loss or cessation of growth and increased bone fragility [38]. In particular, a decrease in periosteal formation and mineral apposition – indicating a reduced osteoblast recruitment and activity was found. This specific effect of

a low protein diet was determined without any interference of caloric intake, further determining that dietary proteins, by influencing both the production and action of growth factors, particularly the growth hormone (GH)-IGF system, could affect bone growth and metabolism [38]. In another study on rats, it was demonstrated that rats on a low protein diet (4%) as compared to controls on a 20% protein diet, showed a significantly reduced femur weight and length, as well as, a diminished diaphyseal cross-sectional area and ultimate strength [39]. The multiple animal studies consistently demonstrate that protein malnutrition affects growth, development, and both bone collagen and mineral content of long bones in rats. Lastly, protein undernutrition can also be associated with alterations of cytokine secretion – interferon gamma (INF-γ), tumor necrosis factor alpha (TNF- α), or transforming growth factor beta (TGH- β) [37].

Inflammation

The mechanism by which IBD affects bone growth has been associated with high levels of inflammatory cytokines such as TNF- α and interleukin-1beta (IL-1β) in the gut mucosa and of interleukin-6 (IL-6) in the peripheral blood [33]. TNF-α promotes activation of osteoclasts (bone resorption) by increasing the expression of receptor activator of nuclear factor kappa B ligand (RANK-L) in osteoblasts (bone formation/regulation). RANK-L is a protein expressed by osteoblasts and plays a key role in osteoclast formation, function, and survival. It binds to its receptor RANK on osteoclast precursors, which initiates formation of mature osteoclasts. With increased RANK-L, apoptosis of osteoclasts is inhibited and stimulates osteoblast apoptosis, which in turn, increases bone resorption and reduces bone formation. Increased expression of pro-inflammatory cytokines, IL-6 and IL-1β, which are produced by osteoblasts under the effect of TNF-α, also activates osteoclasts by activating RANK-L and elevated levels have been detected in IBD. IL-1β is also a potent inducer of nuclear factor (NF)-kB (a protein complex that controls transcription of DNA, cytokine production and cell survival) and high concentrations are found in both CD and UC [27]. TNF-α also inhibits osteoprotegerin (OPG), which is a decoy receptor that blocks the binding of RANK-L to RANK by reducing osteoclast activity. Excessive RANK-L prevails over OPG in terms of binding to RANK, which also increases osteoclast formation, increased bone

resorption and greater bone loss [21, 23, 33]. In summary, the balance between RANK-L and OPG determines if there will be net bone formation or resorption; under the influence of pro-inflammatory cytokines, resorption is likely to predominate.

It is confirmed that both malnutrition and inflammation and their interaction contribute to growth failure as seen in pediatric IBD. For example, pro-inflammatory cytokines will also act directly upon appetite centers through the mechanism involving changes in serotonin receptor stimulation [37]. This explains how inflammation reduces energy intake as well as directly affecting linear growth. Studies demonstrate that children with IBD have increased levels of pro-inflammatory cytokines and low levels of IGF-1 [37]. Providing enteral nutrition to treat inflammation has not only shown a positive response to reduce certain cytokines such as IL-6 and TNF- α, it also increases IGF-1 [40, 41]. In a study by *Bannerjee et al*., children with CD received a 6-week course of exclusive enteral nutrition (EN) using a polymeric formula. IL-6 was the first parameter to show significant improvement, in which a decrease occurred as early as day 3 after initiating an enteral diet. This was followed by an increase in IGF-1 by day 7. In contrast, none of the nutritional parameters significantly changed until after day 14 or later [41]. Although inflammation markers may change rapidly once inflammation subsides, it is hypothesized that nutritional restoration is the primary cause for reduction in inflammation and increases in IGF-1 must rely on adequate nutrition before or coincident with inflammatory changes.

Corticosteroids

In order for the body to maintain homeostasis, it is constantly adapting at a molecular, cellular, physiological, and behavioral levels to the many environmental alterations it faces. Cortisol, a naturally occurring glucocorticoid is a steroid hormone produced from cholesterol in the adrenal glands and regulates inflammation and other processes in the body [42]. It is normally released in response to events and circumstances such as waking up in the morning, exercising, and acute stress. Studies suggest that alterations in GI inflammation due to stress on the body may be facilitated through changes in the

hypothalamic-pituitary-adrenal (HPA) axis function. When the body reacts to stress, the peptide hormone and neurotransmitter involved in the stress response, corticotrophin releasing factor (CRF) is secreted from the hypothalamus. This causes the release of adrenocorticotrophic hormone (ACTH), a polypeptide trophic hormone, from the anterior pituitary gland, which then stimulates the excretion of cortisol [42, 43]. Cortisol is also increased by inflammatory cytokines as seen in IBD, particularly IL-6, as it aims to provide a negative feedback which reduces inflammation. Patients with IBD, having chronically increased levels of inflammatory cytokines in the blood, may attenuate the HPA axis response to both inflammation and acute stress. In addition, cortisol triggers bone mineral resorption to free amino acids for use as an energy source through gluconeogenesis. Cortisol indirectly acts on bone by blocking calcium absorption, which reduces bone cell growth [44]. With a disruption to serum calcium homeostasis, bone resorption increases and this ultimately reduces BMD. Cortisol primarily acts on the outer layer of bone (periosteum) by inhibiting osteoblast formation and cell proliferation; this less appositional growth and reduced cortical thickness. Synthetic glucocorticoids (GCs) are commonly used in the treatment of IBD to aid in reducing inflammation and symptoms when other medications, such as aminosalicylates, do not work. However, studies have demonstrated that chronic use in children increases the growth impairment associated with IBD, thus decreasing BMD [20, 26]. The growth suppressive effects are known to be multifactorial. Glucocorticoids inhibit osteoblast production of bone-matrix components and increases osteoblast apoptosis. The early phase of increased bone resorption can be due to the result of an increased expression of RANKL and decreased expression of OPG.

Corticosteroids also create a central suppression of GH, the extent of which is dependent on the dose, preparation, and timing of the medication. Furthermore, these medications create a decreased hepatic transcription of GH receptor; such that production of IGF-1 is decreased resulting in reduced IGF-1 binding in the cartilage and may increase bone resorption [26]. Even after cessation of glucocorticoid therapy, catch-up growth does not always fully counteract the growth deficits, especially when treatment occurs during puberty [26, 27].

IBD Management

Nutrition Management: Adequate Protein

As previously mentioned, there is no cure for IBD; therefore, the ultimate goal is directed at achieving and maintaining remission, prevention of disease complications, control of postoperative disease recurrence, maintenance of normal growth and development, and maximizing quality of life. With inadequate nutrition in pediatric IBD being of concern, multiple studies show that EN therapy using liquid formulas (elemental, semi-elemental, or polymeric) is beneficial for the induction of remission and management of the disease and now the standard of care [45, 46]. Formulas providing adequate protein with complete amino acid profile improves whole body protein turnover and restores tissue damage. Exclusive EN with adequate protein has been shown to decrease mucosal cytokine production (specifically IL-2, INF-γ, and TNF-α) to normal levels within 3 days, induce endoscopic healing, and aid with restoring body weight [41, 47, 48]. Studies have shown that exclusive EN was associated with a decrease in serum C-reactive protein and TNF-α (proteins involved in inflammation), while in the intestinal mucosa there was indication of mucosal healing together with the down regulation of the pro-inflammatory cytokines IL-β, IL-8, and interferon γ [48, 49]. These results emphasize the importance of focusing on mucosa when evaluating the response treatment in IBD. In addition, energy and protein are important in regulating IGF-1 since both are important for restoration of serum IGF-1 concentrations after fasting and IGF-1 is restored more readily by a diet rich in essential amino acids [50]. A diet deficient in both protein and energy results in a further decrease of IGF-1 in serum [50]. Exclusive EN has been proven to be an effective alternative to corticosteroid therapy and is now the treatment of choice for children with CD [45]. The downside of EN is that symptoms tend to recur quickly if the enteral feed is stopped and in order to facilitate growth, remission must be maintained [26]. In addition, exclusive EN is not well tolerated by all IBD patients [26].

Amino Acid Supplementation: NAC

Amino acids are used in every cell of the body to build the proteins needed for function and survival. They contain a basic amino group (NH2) and an acidic carboxyl group

(COOH), as well as a side chain specific to each amino acid. Studies have demonstrated effective therapies for IBD by supplementing with specific immunomodulatory amino acids [51]. The inflamed intestine in IBD is exposed to a significant amount of oxidative stress by the production of reactive oxygen species (ROS) and is highly toxic to cells, promoting injury to the gut [52]. Deterioration of the tripeptide glutathione (GSH), which is the most important intracellular antioxidant, has been reported in patients with IBD. GSH is also crucial for nutrient metabolism, and regulation of cellular events such as DNA and protein synthesis, cytokine production and immune response. It is synthesized from amino acids glutamate, cysteine, and glycine. When GSH reacts with ROS, GSH is oxidized to glutathione disulfide (GSSG). Once oxidized, GSH can be reduced back by glutathione reductase, using NADPH as an electron donor [52, 53]. GSH synthesis is controlled primarily by γ-glutamylcysteine synthetase activity, cysteine availability, and GSH feedback inhibition and adequate dietary protein is vital for GSH homeostasis. Studies have shown that supplementation of amino acids such as cysteine, methionine, N-acetyl-cysteine (NAC), and L-2-oxothiazolidine-4-carboxylate are efficient precursors of cysteine for tissue GSH synthesis [53]. By using NAC as a precursor of cysteine for synthesis of GSH, studies have shown that NAC decreases the production of free radical damage (ROS), as well as, the production of TNF-α, IL-1β, and IL-6 in the colonic tissue resulting in an improved remission for UC [54]. *Uraz et al.* was able to demonstrate significant decreases in pro-inflammatory cytokines using a colitis-induced model with acetic acid in rats. In this study, 32 rats were divided into 4 groups; induced colitis was performed in 2 of the groups while the other 2 groups were injected with saline solution. One of the induced colitis groups and one of the control groups were administered NAC supplementation. After only 4 days, pro-inflammatory cytokines were significantly elevated in the colitis group than all other groups ($p<0.001$). The NAC group with colitis suppressed cytokine production to control group levels and GSH was lowest in the colitis group compared to control and was restored by NAC supplementation.

NAC is a good precursor choice due to its small molecular weight, is soluble in water, and can rapidly enter the cytoplasm by means of organic anion transporters in the cell membrane. In addition to its antioxidant and detox functions, studies have demonstrated

that NAC functions as an osteogenesis-enhancing molecule, activating osteoblastic differentiation. NAC was shown to accelerate and enhance gene expression of representative bone matrix proteins such as collagen type I, osteopontin, and osteocalcin in osteoblastic culture compared to that in untreated culture at days 3, 7, and 14 [55]. Another study demonstrated that *in-vivo* exposure of calvarial bone to NAC resulted in a marked local increase in calvarial bone thickness and secondary bone marrows were observed inside the newly formed bone, indicating that NAC exerts a stimulatory influence on bone formation, as well as an inhibitory effect on osteoclastic bone resorption [56]. Within 12 hours of NAC treatment, cellular levels of total GSH increased and stimulation of osteoblastic differentiation [56].

IBD Research

Animal Model Research: DSS Induced Colitis Model:

Neonatal piglets have become an important model for pediatric IBD research given the developmental and physiological similarities between piglets and human infants, specifically similar GI and cardiovascular physiology functions and nutrient metabolisms. In addition, chemically induced piglet models of IBD are commonly used due to the moderately inexpensive rapid induction of colitis by chemicals such as dextran sulfate sodium (DSS) [57]. DSS is a chemical colitogen with anticoagulant properties, to stimulate disease [58]. It produces a toxic insult on the intestinal epithelium, interfering with epithelial cell barrier function. Impairment of mucosal barrier and exposure of the submucosa to luminal antigens creates an activation of innate immunity [59]. The efficiency of DSS to induce colitis depends on molecular weight (5 kDa is used for inducing mild colitis, whereas 40 kDa is for severe colitis), dosage (usually 1-5%), the duration (whether acute or chronic), strain and sex of the animal, as well as the microbial environment. By administering a DSS dosage 3-10% for a 7-10 day period can induce acute colitis and a cyclical regimen of 7 days DSS administration followed by 7 days rest can mimic a chronic relapsing colitis [60]. Piglets with DSS-induced colitis demonstrate the clinical features of colitis including a greater production of pro-inflammatory cytokines, such as IL-6, TNF-α, INF-γ, and IL-1. Piglet studies show that severe protein

deficiency dramatically impacts body weight gain, linear growth, and body composition of DSS-colitis piglets, as well as, compromise colon integrity [61]. In addition, research completed in murine models of DSS induced colitis has demonstrated a reduced bone mass, which resulted from suppressed bone formation, and increased bone resorption [59]. However, piglets are preferred models as they are sufficient in size, compared to rodents, to perform invasive procedures, such as surgical manipulation.

Investigation Methods

In the pediatric IBD population, malnutrition with growth failure is a frequent occurrence that can manifest as delayed onset of puberty and delayed skeletal maturation. These disease complications can lead well into adulthood, as seen by as many as 20% of children with IBD not achieving their adult height potential [35]. Common measurements of growth in children include weight, height/length, skeletal maturation and body composition to evaluate gain in lean body mass. In a piglet model, weight, snout-to-rump length, and chest circumference are basic anthropometric indicators that can be measured over time to assess growth rate. Specifically, investigating bone health is important in pediatric IBD to ensure adequate skeletal development and growth is achieved [26].

Dual-energy x-ray absorptiometry (DXA):

The most commonly used method, and gold standard, for measuring bone mass in people from birth to adulthood is DXA. The DXA machine works by emitting two X-ray beams, each with different energy levels, and has the capacity to differentiate soft tissue from mineralized tissue. The higher energy is able to penetrate bone whereas the lower energy is only able to penetrate soft tissue. DXA quantifies fat, lean and bone mineral masses and is the most commonly used technique that can measure regions in both the axial and appendicular skeleton. DXA is commonly used in the pediatric population for assessing bone health and has many advantages including: high accessibility, low ionizing radiation dose, precision, as well as the availability of pediatric reference data for measures of whole body, lumbar spine and hip. When investigating bone health in children,

measuring total body bone mineral content (BMC) is preferred over bone mineral density (BMD), which is a two-dimensional measure (not volumetric) calculated as BMC divided by bone area (BA) [62]. Positioning can make BMD values less accurate and analysis may lead to an underestimation of BMD in pediatric subjects who have a decrease in height velocity as seen in many IBD patients [63]. Due to the continuous change in bone geometry and the incapability to differentiate change due to bone growth or bone mineralization, BMD is not an ideal measure of bone mineral acquisition in childhood. Studies have demonstrated reliable measurement of BMC, lean body mass and fat mass in piglets using DXA [64, 65]. Another limitation of DXA is being less acurate in populations with abnormal hydration status such as patients with edema [64].

Microcomputed tomography (μCT):

In addition to using 2 dimensional imaging measures such as DXA, 3 dimensional x-ray imaging is also beneficial in further analyzing bone health. Studies involving the use of microcomputed tomography (μCT) have steadily increased as development has continued to demonstrate improved resolutions for *in vivo* imaging for small animals and *ex vivo* scanning of tissues for utilizations in experiments related to fields that study disease, organ structure, as well as drug treatments [66]. The μCT works by an x-ray beam first passing through a specimen onto a detector where it collects magnified projection images. While the specimen becomes rotated in the machine, capturing hundreds of angular views, a computer synthesizes a stack of virtual cross section slices through the object. This enables scrolling through the cross sections, interpolating sections along different planes, to inspect the internal structure [66]. μCT can be used to determine volumetric measurements of trabecular (or cancellous) bone at the ends of long bones and cortical (compact) bone which makes up the diaphysis on long bones as well as a protective shell around the ends of long bones and vertebral bodies in the spine. Measurements directly related to cortical bone include the cortical area, average cortical thickness, total cross-sectional area, and cortical bone volume fraction (BV/TV). In addition, measuring width, diameter, and thickness also gives information used to calculate material and structural properties. It is also possible to measure the volumetric bone mineral density (vBMD) of a cross section and converting into units of mass per

unit volume (g/cm^3). For trabecular regions of the bone, BV/TV is significantly lower due to its intricate structure. μCT images can measure the surface density of trabeculae (BS/TV), the total number of trabeculae (Tb.N) as well as their thickness (Tb.Th) and the separation (Tb.Sp) between each filament in the web. Another technique, peripheral quantitative computed tomography (pQCT), also exits to measure vBMD of extremities to assess growth, bone health, and detect signs of osteoporosis, however, does so at lower resolutions than a μCT [66, 67]. Numerous animal studies have shown the success and usefulness in measuring changes in BMD, BMC, and 3-dimensioanl map using μCT imaging of the femur and lumbar spine [68].

Bone Biomarkers:

Bone biomarkers (either from serum, plasma, or urine) include both enzymes and peptides derived from cellular and non-cellular compartments of bone and reflect both the formation by osteoblasts and resorption by osteoclasts. Due to skeletal growth and rapid bone modeling during childhood and adolescence, the pediatric population has significantly greater circulating concentrations of bone biomarkers than adults. The measurement of bone markers in both blood and urine is a means of assessing bone modeling and remodeling processes, whereas bone densitometry is an assessment of peak bone mass [62]. Concentrations of bone markers can be affected by both physiological and pathological conditions, however, are very important in the diagnosis, prognosis, and management of metabolic bone disease. Bone biomarkers markers may show more rapid changes soon after initiating treatment, which may be useful in the management of bone diseases. In addition, due to the difficulty to collect reliable consecutive urine samples from children, it is preferred to measure bone biochemical markers in serum samples. Both bone formation (active osteoblasts) and bone resorption markers (products of collagen degradation) can be collected. Some bone biomarkers that will be analyzed in this study include bone alkaline phosphatase (BAP), osteocalin (OC), and cross-linked telopeptides, such as N-terminal telopeptide (NTx).

Bone Alkaline Phosphatase (BAP)

Bone-specific alkaline phosphatase (BAP) is the bone specific isoform of alkaline phosphatase and is considered to be a highly precise early marker of the bone forming activity of osteoblasts. BAP is synthesized by the osteoblasts and is recognized to be involved in the calcification of bone matrix [69]. BAP is an isoenzyme that is made by bone and the level of the enzyme increases when bones are growing or if bone cells are active. BAP has been shown to be 1.5-2.5 times higher in growing children than in normal adults due to the presence bone growth. Decreases of BAP have been observed in situations such as cessation of bone growth, scurvy, severe anemia, kwashiorkor, vitamin B_{12} deficiency, and multi-nutritional deficiency of zinc or magnesium [69]. BAP as a bone marker has been successively used in the piglet model using enzyme-linked immunosorbent assays (ELISA). In addition, in GH deficient children, serum BAP levels significantly increase after GH therapy suggesting its use in monitoring response to therapy [62].

Osteocalcin (OC)

Osteocalcin (OC) is synthesized by osteoblasts and concentrated in bone matrix. It is also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP) and is a noncollagenous protein synthesized primarily in bone and dentin. This calcium binding bone protein is distinguished by its content of three gamma-carboxyglutamic (Gla) residues and thus its carboxylation is vitamin K dependent [70]. OC levels correlate with bone formation rates and serum concentrations of OC reflect the portion of newly synthesized protein that does not bind to the mineral phase of the bone but is released directly into the circulation. The peptide is rapidly degraded in the serum and intact and fragmented segments coexist in the serum. The resulting heterogeneity of the OC fragments in the serum can lead to limitations with the use of this marker. In contrast, OC as a bone marker has been shown to be a better maker of bone mineralization and turnover in pigs than serum alkaline phosphatase [71]. Both serum OC and Gla are currently used for the clinical assessment of calcium-phosphate metabolic disturbances of

bone in diseases such as growth disturbances, hyperparathyroidism, hypo- and hyperthyroidism, and osteoporosis. OC levels are elevated in situations of new bone formation (and high bone turnover) as seen in rapidly growing animals and in young children [72]. The serum concentration of OC is measurable by radioimmunoassay (RIA), immunoradiometricassay (IRMA), and enzyme immunoassay (EIA). The Pig Gla-Osteocalcin EIA kit measures bone osteogenesis via the specific and highly sensitive detection of porcine cOC (an active osteocalcin that exhibits a propensity towards osseointegration).

N-terminal telopeptide of type I collagen (NTx)

N-terminal telopeptide (NTx) is a telopeptide that can be used as a biomarker to measure the rate of bone turnover. These short proteins (peptides) are a component of the cross-links in type I collagen fibrils. Collagen fibrils are formed by cross-links comprised of proteins and NTx are found in the region of type I collagen where the crosslinks attach. They are produced during bone resorption and can be measured in the blood or urine, making the test one of the most reliable indicators of bone resorption. High NTx levels have been found to correlate with low BMD and to predict fracture risk [73]. Studies demonstrate that bone resorption, as measured by NTx, is significantly increased in IBD patients compared to normal controls [73]. NTx can be measured in serum with an enzyme immunoassay (EIA) that has been developed for humans and is sensitive in piglets to detect differences in bone resorption [74].

RATIONALE:

IBD, including CD and UC, is a condition with chronic or recurring immune response and inflammation of the gastrointestinal tract [1]. Although adults and children with IBD may present similar clinical symptoms, children can acquire distinct complications including slowed growth, impaired bone development, and a decreased stature [3]. While there is no cure for the disease, pediatric therapy is directed at achieving and maintaining remission by minimizing inflammation and ensuring adequate nutrition for the maintenance of normal growth and development. The majority of studies in children with IBD focus only on the active phase of IBD, which resembles more of an acute disease stage. By further exploring and illustrating both the active and recovery phase of the disease, it is possible to examine the effects of recurrent malnutrition that is typically seen in pediatric IBD, and more specifically, protein malnutrition. It will also enable further exploration of the effects of inflammation and nutrition and their interactions on bone health outcomes in children with IBD. With an increasing incidence of pediatric IBD worldwide, it is essential to investigate nutrition interventions for the maintenance of normal growth and development, as well as an optimal quality of life.

As inflammatory disease etiologies are multifactorial, the use of appropriate animal models is essential. Neonatal piglets have become an important model for pediatric nutrition research given the developmental and physiological similarities between piglets and human infants, specifically similar GI and cardiovascular physiology functions and nutrient metabolism. Administering DSS for six and half days followed by five and half days of recovery without DSS, it is possible to mimic acute recovery in colitis and explore the impact of nutrition interventions on bone accretion and inflammation during remission. Usually 20% concentrated DSS is given at 0.4 g/kg of body weight per day to ensure successful induction of colitis [75]. The length of recovery is based on diet related changes in digestive enzyme adaptation, colonic mucosa and small intestine epithelial turnover rates of approximately 3 to 8 days in healthy pigs [76]. In addition, with the rapid growth rate of piglets, 5.5 days is suggested to be sufficient to detect metabolic and physiologic changes during recovery in the short term [77].

Both malnutrition and inflammation contribute to growth failure in IBD. Protein malnutrition, in particular, can affect bone growth by affecting the GH/IGF-1 axis leading to lower mineralization, which in turn can increase the risk of osteoporosis and fractures in childhood and later in life [35, 36]. To mimic the human scenario of low protein intakes and malnutrition, protein malnutrition can be achieved by providing piglets with a severely protein deficient diet that meets 15% of the daily total protein requirement (National Research Council Nutrient Requirement of Swine 11th Edition) [78]. This degree of protein deficiency has demonstrated a significant decrease in protein synthesis in previous studies [79]. Protein malnutrition rather than protein-energy malnutrition is studied in order to examine the specific influence of protein on bone accretion and inflammation in the recovery phase of IBD, as well to decrease other potential complex interactions between nutrients and inflammation.

Malnutrition as well as growth failure cannot be treated with nutritional counseling alone. As seen in IBD patients, intake can be severely compromised and malabsorption can contribute to nutrient loss. Nutritional supplements, in addition to their regular diet, improves nutritional status and can help reduce some of the consequences of undernutrition, as well as maintaining remission and preventing relapses. Although using NAC has been proposed as a nutritional supplement in several diseases, limited research exists on its effect on bone, especially in human studies. Deterioration of GSH status has been reported in IBD. By using NAC as a precursor of cysteine for the synthesis of GSH, NAC decreases the production of free radical damage, as well decreases the production of pro-inflammatory cytokines associated with IBD, such as TNF-α, IL-1β, and IL-6 in the colon to improve remission for UC. The role of NAC on bone is further being explored to function as an osteogenesis-enhancing molecule, activating osteoblastic differentiation, thus improving bone growth [53, 54, 56]. It will be interesting to determine if NAC, even in a protein deficient state, can impact bone mineral accretion and increase bone formation biomarkers, as well as decrease inflammation. The dose of NAC will provide a total of 2.5 times the amount of cysteine in a normally adequate nutrition piglet diet based on studies previously completed in malnourished children [80]. This is considered

to be a high dose, however, it remains below the toxicity level to study its greatest effect on decreasing inflammation and promoting osteoblastic differentiation during recovery.

By studying a model of active and early recovery in colitis, it is possible to further investigate the effects colitis has on bone mineral accretion that is essential for bone health outcomes. Nutritional interventions such as adequate protein intake and NAC supplementation will be explored to optimize the body's potential to resume normal growth and bone mineralization as well to decrease inflammation to further prevent growth failure in children with IBD.

OBJECTIVES & HYPOTHESES:

Global objective:

The global objective of this study is to characterize bone health outcomes in a protein deficient piglet model of acute IBD and to investigate nutrition or anti-inflammatory interventions during early recovery.

Specific objective 1:

To characterize bone mineral accretion and biomarkers of bone growth and bone metabolism in a protein deficient piglet model with active colitis and their association with inflammation as indicted by pro-inflammatory cytokines.

Hypothesis 1:

It is hypothesized that rates of bone mineral accretion will be associated with changes in bone formation and resorption biomarkers and will correlate with proinflammatory cytokines levels (TNF-α, IL-1β, and IL-6) during the active phase of colitis.

Specific Objective 2:

To determine the impact of anabolic nutrition intervention or anti-inflammatory NAC supplementation in the early recovery phase of colitis by assessing bone mineral

accretion, bone geometry, biomarkers of growth and bone metabolism and pro-inflammatory cytokines.

Hypothesis 2:

It is hypothesized that piglets receiving an adequate protein diet will demonstrate an increase in bone mineral accretion, along with increases in bone strength, and bone formation biomarkers compared to piglets continuing to receive a low protein diet.

It is also hypothesized that piglets receiving NAC supplementation will demonstrate a decrease in pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and an increase in bone mineral accretion, bone strength, and bone formation biomarkers compared to piglets receiving a low protein diet.

METHODOLOGY:

Study Design:

Twenty-one female piglets (7 to 10 days old, 7 to 9 pounds or 3.2 to 4.1kg; Les Porcheries Chanca Inc.; St-Louis de Gonzague, QC, Canada) were used for the research study. Each piglet underwent surgery, using isoflurane (Baxter Healthcare Co., Richmond Hill, ON, Canada) as the anesthetic, for the implantation of catheter insertion into the stomach (for feeding and administration of DSS), the femoral vein (for isotopic tracer infusion) and the external jugular vein (for blood sampling). Enteral feedings were started on day 1, after surgery and as soon as recovery from anesthesia was confirmed. All piglets received a protein deficient EN formula (15% of National Research Council requirement) that was delivered intragastrically from day 1 to day 9. For further details on diet composition for this study for a protein deficient diet and adequate protein diet see **Tables 2-8**. On day 3 of the study period, each piglet received DSS (20% solution, 40,000 MW; ICN Biomedicals Inc., OH, USA), 0.4 g/kg/d split between 2 doses, intragastrically to induce severe colitis and was stopped on day 9. Disease activity was determined by stool consistency and fecal occult blood test. At the end of day 9, DSS was

discontinued and 21 piglets were randomized to 1 of 4 groups (n = 5-6 each) with one group immediately removed to harvest tissues and 3 groups continuing in the recovery stage. Recovery groups received either

a) Continuing the low protein diet (15% of National Research Council requirement) alone.
b) EN formula supplying 100% of protein requirement.
c) Continuing the low protein diet (15% of National Research Council requirement) with an additional supplement of N- acetylcysteine.

After 5 days of recovery, on day 15 of the study, piglets were euthanized with Euthanasol (750 mg sodium pentobarbital IV; Schering Plough Canada Inc, Pointe Claire, QC, CA). The piglets in the active control group were euthanized on day 10 with no recovery phase. See **Figure 1** and **Table 1** for study flow-chart and summary chart of 15-day trial of *in-vivo* measurements. Once euthanized, the right leg from each piglet (n=21) was removed in order to excise the femur and the lumbar spine (n=21). McGill University Animal Care Committee approved this study in accordance with the Canadian Council on Animal Care Guidelines.

NAC Supplement

NAC supplement (N-acetyl-(L)-cysteine; Fisher Scientific, Fair Lawn, NJ, USA) dosage is based on studies in malnourished children recovering from protein energy malnutrition. In the study by *Jahoor et al*, a 3 g protein per kg body weight per day was used to promote rapid growth recovery in severe malnourished children, where the protein intake was 2.5 times the normal recommendation [79]. This factor of 2.5 was adapted into formulating the NAC supplement for piglets. In piglets under 5 kg, normal protein requirement is 14.4 g/(kg·day). The protein mix ingredient used in the piglet diet provides on average 0.311 g/(kg·day). Since supplementing NAC for recovery and anti-inflammatory response is the focus, a total intake of cysteine can be extrapolated and calculated based on protein and cysteine levels described in Jahoor's study. Therefore, a total cysteine intake for recovery and anti-inflammatory response was calculated by

multiplying normal cysteine intake by the 2.5 factor obtained from Jahoor's study, which gives 0.778 g/(kg·day). By subtracting this total cysteine intake by the amount of cysteine in the diet, the amount of cysteine supplement is calculated to be 0.731 g/(kg·day), which is equivalent to 0.984 g/(kg·day) NAC based on molecular weights of cysteine and NAC. 7.872 g of NAC was used to make 500 mL NAC solution (concentration: 1.57%). NAC solution was infused with a separate pump from the protein deficient diet. The fluid content of the protein deficient diet used in the NAC group was adjusted to account for the fluid in the NAC supplement [80].

Diet Regimen

All diets including NAC supplement were infused using enteral pumps (Compat Enteral Delivery System with Dose Limit, Enteral Delivery Pump Set with Inline "Y" Adaptor and 1000 mL Vinyl Bag; Nestlé Healthcare Nutrition Inc., Minnetonka, MN, USA). The total daily volume of formula was calculated based on the piglet's daily weight. Diet was made regularly and not kept for more than 2 days. Due to the use of continuous feeding, total daily formula volume was divided into two and added in the morning and the afternoon to minimize bacterial growth at room temperature. Enteral feeding equipment was washed or changed daily. In addition, both total volume of diet added and pump reading was recorded in order to accurately calculate total nutrient intake.

Post-surgery diet adaptation was obtained by providing a rate of 60% of targeted rate on day 1, followed by rate of 80% of targeted rate on day 2, and reaching 100% target rate on the morning of day 3. Adequate protein diet change adaptation was done by gradually switching (25% increase per 12 hours) protein deficient diet to adequate protein diet within 2 days.

Growth Measurements:

In-Vivo:

Weight was obtained daily, whereas chest circumference and snout-to-rump length was measured under anesthesia on day 1, 9, 14 and 15. Body composition was also measured.

Piglets underwent DXA scans (whole body, femur, & lumbar spine) (QDR 11.2 4500A series; Hologic Inc., Waltham, MA, USA) on day 1, 9, 14 while under anesthesia. DXA scans were analyzed using the infant whole-body and lumbar spine software, with whole body, femur and lumbar spines scans (L1-L4, L3). Blood was taken, on average at 8 am, through the jugular catheter for bone markers at day 3, 10, and 15, and stored at -80° for within group comparison as well as progression along the study.

Ex-Vivo:

1. DXA

Once the piglets were euthanized, the right leg and lumbar spine region were removed and frozen at -20°. Bones (femur and lumbar spine) were cleaned and covered in saline soaked gauze and refrozen until they underwent DXA scans using standardized positioning. Scans were captured and analyzed using the "small animal" software (Hologic Inc., Waltham, Massachusetts, USA). Quality Control scans using Spine Phantom and Step Phantom were performed daily. Each bone was placed in a water bath where the water raised 1 cm above the most prominent part of the bone. Femur positioning was standardized with the lateral condyle of bone being dorsal whereas spine was apical lateral. Data collected included bone area (BA; expressed as cm^2), bone mineral content (BMC; expressed as grams) and bone mineral density (BMD; expressed as g/cm^2).

2. µCT Analysis

Each bone was scanned *ex-vivo* using a Latheta LCT-200 µCT unit and analyzed using the standard analysis software (Hitachi-Aloka Medical Ltd., Tokyo, Japan). Each spine analyzed was placed in a holder size of 120 mm in diameter while each femur was placed in a 60 mm holder size. Positioning was standardized with spines in the dorsal position: the distal end of the spine and femur were positioned facing the opening of the unit (specimen poster will be determined as "face down and head front"). Scan conditions were set on isolated bone for spines as follows: pixel size - 120 µm, slice thickness - 120 µm, slice pitch - 120 µm, speed - fast, rotation angle - 360°, x-ray voltage - high. High-resolution scans were conducted on L3 of the lumbar spine. L3 of the lumbar vertebrae is

studied, as it is the larger and most representative of the L1-L4 region. Porcine lumbar spines are claimed to be the most representative animal model for spinal research [81]. The mid-section splices (20) of L3 were then analyzed. Focus was put on the vertebral body, excluding spinal processes. Parameters measured at midpoint included focus on trabecular analysis: trabecular area (cm^2), trabecular total volume (BV/TV – cm^3), and trabecular volumetric BMD (vBMD – mg/cm^3). Before positioning the femurs, the length of each bone was measured with a digital calliper (Fisherbrand Traceable Digital Calliper, Fisher Scientific). Femurs were measured from the tip of the femoral head (at the proximal end) to the tip of the condyle (at the distal end). Scan conditions for femurs were set as follows: pixel size - 60 μm, slice thickness - 120 μm, slice pitch – 180 μm, speed – fast, rotation angle - 360°, x-ray voltage – low, and number of slices – 450. The setting recognition was also altered (1200 to 400) to better recognize differences in cortical versus trabecular bone. The position of the mid-diaphysis (i.e. slice number) was determined first identifying the slice number where the bone began (i.e. femoral head) and subtracting from the slice number of where the bone ended (condyle) then dividing by 2. The mid-diaphysis slice was then analyzed for the moment of inertia by using the measuring tool and recording the values of x, x', y and y' as per *Ferretti et al.* [82]. Parameters measured of total femur included: bone length (mm), total bone volume (cm^3), total BMC (mg), and total vBMD (mg/cm^3). Focus on cortical bone was determined at mid-diaphysis (5 slices on each side for a total of 10 slices), and included analysis of total cortical volume (BV/TV – cm^3), and cortical vBMD (mg/cm^3). Focus on trabecular bone was determined at the metaphysis (15 slices in from landmark of epiphyseal plate for a total of 25 slices), and included analysis of total trabecular volume (BV/TV – cm^3), and vBMD (mg/cm^3).

3. Bone Strength

Bone strength was examined using *ex-vivo* biomechanical testing and the 3-point flexure method (Instron 5544, Canton, MA, USA). Breaking strength is defined as the amount of force that is required to break the bone when it is held in a horizontal position. Bones were balanced between the 2 fixed supports in the supine position so that the load descends in the anterior to posterior direction. Mid-diaphysis was determined by dividing

the length of the bone (as previously measured by calipers) by 2. A black felt tip marker was used to mark the midpoint where one loading point will apply pressure to the bone. A test speed of 1 mm/min was used for piglet femurs, as determined from practice on two test femurs. The femurs were loaded until failure. The time to break, on average, was 5.09 minutes. Load extension curves (N vs. mm) were constructed for each bone by the standard software (Bluehill v3; Instron, Canton, MA, USA). Extrinsic parameters measured included: energy at break (mJ), ultimate force (N), ultimate displacement (mm) and work to failure (kJ). Intrinsic parameters measured included: stress (MPa), strain (no units), Young's modulus (MPa), and modulus of toughness (mJ/mm^3).

5. Bone Biomarkers & Pro-inflammatory Cytokines

Bone markers and pro-inflammatory cytokines in serum (collected on days 3, 10, and 15) were measured by enzyme immunoassay (EIA), specifically for the porcine species, using diluted samples. In order to obtain functional results for bone markers of interest, samples of serum were diluted at varying ratios (1:1, 1:4, 1:5, 1:8, 1:10). All bone biomarkers were analyzed with a plate reader by Bio-Tek Instruments (Winooski, Vermont) using KC4 ver 2.6 software (Bio-Tek Instruments, Winooski, Vermont). Bone markers assessed included BAP (EIA 8012, Quidel Corp., Athens, USA), OC (EIA 8002, Quidel Corp., San Diego, CA), and NTx (EIA 9021, Alere Scarborough Inc., ME, USA). The investigation of pro-inflammatory cytokines was previously analyzed which included IL-1-beta, IL-6, and TNF-α.

STATISTICAL ANALYSIS:

A sample size of 10 per group was previously determined (for the study overall) to detect a 30% difference of plasma albumin fractional synthesis rates between two groups. However, due to limited resources, only 21 piglets formed the study. All statistical analysis was done using SAS 9.4 (Statistical Analysis System Institute Inc. Cary, North Carolina, US). Normality was tested using PROC. UNIVARIATE function and Shapiro-Wilk's Test. Data were transformed if it was not normally distributed and/or had heterogeneous variances. For objective 1 of the study, Pearson Correlation was generated

to test relationships between bone outcome measurements and pro-inflammatory cytokines. For objective 2, first, homogeneity of variances was tested using Bayesian Criteria. A mixed model analysis of variance (ANOVA); PROC MIXED was performed to test if there were significant differences between the groups in relation to bone accretion, bone geometry, and biomarkers values and multiple comparisons were adjusted using Tukey. All analyses were interpreted using a 5% probability level.

Figure 1. Study flow-chart of the 15 days study including active colitis phase and recovery phase

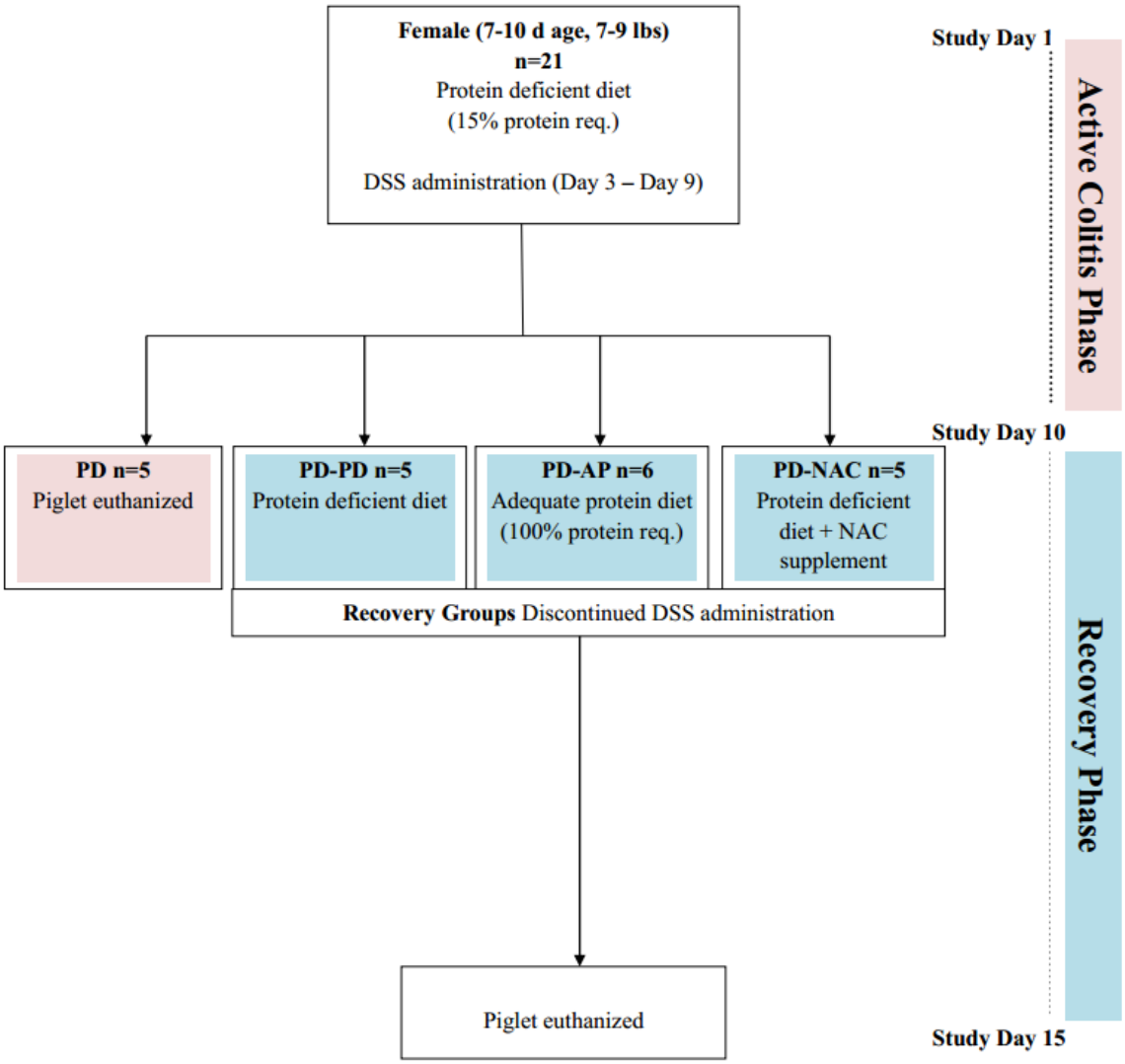


TABLE 1: Study protocol of the 15-day study of *in-vivo* measurements:

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Body weight	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Linear growth - Snout-to-rump - Chest circumference	x								x					x	x
Body composition DXA - Whole Body - Femur - Lumbar spine (L1-L4, L3)	x								x					x	
Blood sampling Bone markers: - BAP - OC - NTx - Cytokines			x							x					x

TABLE 2: Diet in each study group of piglets with DSS-induced colitis

	ACT	REC	AP	NAC
Colitis Induction Phase	PD diet*	PD diet	PD diet	PD diet
Recovery Phase	N/A***	PD diet	AP diet**	PD diet + NAC****

* PD diet: 15% protein deficient diet
** AP diet: adequate protein diet
*** Active Control group will be euthanized at the end of colitis induction phase
**** NAC: 15% protein deficient diet + NAC supplementation

TABLE 3: Energy percentage of liquid diets administered to piglets with DSS-induced colitis

	Diet					
	PD			AP		
	g/(kg·d)	kcal/(kg·d)	kcal%	g/(kg·d)	kcal/(kg·d)	kcal%
Protein	2.2	8.8	4.1	14.4	57.6	26.9
Carbohydrate	26.0	103.9	48.0	19.6	78.3	36.6
Lipids	11.5	103.9	48.0	8.7	78.3	36.6
Total Energy		216.5	100		214.2	100

TABLE 4: Liquid diet composition administered to piglets with DSS-induced colitis

	Diet	
Components in liquid diet as fed	**PD**	**AP**
Egg White g/L[1]	5.34	34.95
Whey Protein Concentrate (WPC) g/L[2]	5.50	36.00
Lactose g/L[2]	86.63	56.73
Maltodextrin g/L[1]	22.15	22.15
Corn Oil g/L[3]	24.52	18.49
Canola Oil g/L[3]	21.64	16.31
Mineral Mix g/L[1]	13.70	10.80
Vitamin mix g/L[1]	0.38	0.38

1-Obtained from Harlan Laboratories in Indianapolis USA
2-Product from Quadra Ingredients in Vaudreuil-Dorion, CA
3-Product of Compliments in CA

TABLE 5: Lipid components and n-6 to n-3 fatty acids ratio of liquid diet administered to piglets with DSS-induced colitis

Lipid	n-6 %	n-3 %	g/L	n-6 (g)	n-3 (g)	n-6:n-3 ratio
Canola Oil	16.67%	8.89%	4.08	0.680	0.363	
Corn Oil	55.56%	1.11%	4.62	2.567	0.051	
Total			8.7	3.247	0.414	7.842

Canola oil and corn oil are both from Compliments, Sobeys Inc., Canada

TABLE 6: Amino acid composition in g/(kg.d) in relation to NRC requirement of the protein deficient and adequate protein diets					
		Diet			
Amino Acids		**PD**		**AP**	
	Req't g/(kg.d)	**Intake g/(kg.d)**	**% Req't**	**Intake g/(kg.d)**	**% Req't**
Arginine	0.34	0.093	27	0.621	183
Histidine	0.26	0.046	18	0.309	119
Isoleucine	0.44	0.121	27	0.805	183
Leucine	0.83	0.201	24	1.337	161
Lysine	0.82	0.163	20	1.089	133
Cysteine/Cystine*	0.25	0.047	19	0.311	124
Methionine	0.22	0.062	28	0.416	189
Methionine + cysteine	0.46	0.109	24	0.727	158
Tyrosine**	0.28	0.071	26	0.476	170
Phenylalanine	0.48	0.104	22	0.691	144
Phenylalanine + tyrosine	0.77	0.175	23	1.167	152
Threonine	0.51	0.104	20	0.696	137
Tryptophan	0.15	0.040	26	0.265	177
Valine	0.55	0.135	25	0.901	164
Alanine	n/a	0.117	n/a	0.781	n/a
Aspartic acid	n/a	0.205	n/a	1.369	n/a
Glutamic acid	n/a	0.352	n/a	2.345	n/a
Glycine	n/a	0.060	n/a	0.399	n/a
Proline	n/a	0.108	n/a	0.720	n/a
Serine	n/a	0.130	n/a	0.868	n/a

*: Values calculated by subtracting methionine from methionine + cysteine

**: Values calculated by subtracting phenylalanine from phenylalanine + tyrosine

Amino acid intake composition is based on a 50:50 mixture of spray dried egg white and whey protein concentrate.

Amino acid requirement (g/(kg.d)) is based on the average body weight requirement for growing piglets of 3-5kg and 5-10kg provided by NRC (1998)

TABLE 7: Mineral intake in liquid diets as fed in relation to NRC requirement as a percentage					
Minerals		**PD**		**AP**	
	Req't Amt/(kg.d)	**Intake Amt/(kg.d)**	**% Req't**	**Intake Amt/(kg.d)**	**% Req't**
Calcium (g)	0.55	0.66	120	0.66	120
Phosphorus (g)	0.44	0.52	120	0.52	120
Sodium (g)*	0.21	0.25	120	0.25	120
Chloride (g)**	0.27	0.32	≥120	0.32	≥120
Magnesium (g)	0.03	0.03	120	0.03	120
Potassium (g)	0.19	0.22	120	0.22	120
Copper (mg)	0.39	0.47	120	0.47	120
Iodine (mg)	0.01	0.01	120	0.01	120
Iron (mg)	6.46	7.75	120	7.75	120
Manganese (mg)	0.26	0.31	120	0.31	120
Selenium (mg)	0.02	0.02	120	0.02	120
Zinc (mg)	6.46	7.75	120	7.75	120

Mineral requirement (g/(kg.d)) is based on the average body weight requirement for growing piglets of 3-5kg and 5-10kg provided by NRC (1998).

*: Na and Cl requirement is based on the average body weight requirement for growing piglets of 5-7kg provided by NRC (2012)

**: Cl requirement is based on the average body weight requirement for growing piglets of 5-7kg provided by NRC (2012) and is slightly higher than 120% of requirement because of the presence of Chloride from spray dried egg white solids that is not provided by the company or indicated in CNF.

TABLE 8: Vitamin intake in liquid diets as fed in relation to NRC requirement as a percentage					
Vitamins		PD		AP	
	Req't g/(kg.d)	**Intake g/(kg.d)**	**% Req't**	**Intake g/(kg.d)**	**% Req't**
Vitamin A (IU)	142.08	284.17	200	284.17	200
Vitamin D (IU)	14.21	142.08	1000	142.08	1000
Vitamin E (IU)	1.03	2.07	200	2.07	200
Vitamin K (mg)	0.03	0.07	200	0.07	200
Biotin (mg)	0.005	0.02	400	0.06	1422
Choline (g)	0.04	0.07	200	0.07	200
Folacin (mg)	0.02	0.04	200	0.04	200
Niacin (mg)*	1.60	3.19	200	3.19	200
Pantothenic acid (mg)	0.71	1.42	200	1.42	200
Riboflavin (mg)	0.24	0.61	254	1.35	558
Thiamin (mg)	0.08	0.16	200	0.16	200
Vitamin B6 (mg)*	0.37	0.74	200	0.74	200
Vitamin B12 (µg)	1.21	2.42	200	2.42	200

Vitamin requirement (g/(kg.d)) is based on the average body weight requirement for growing piglets of 3-5kg and 5-10kg provided by NRC (1998).
*Niacin and Vitamin B6 requirement is based on the average body weight requirement for growing piglets of 5-7kg provided by NRC (2012)

RESULTS:

General Records of Study Previously Analyzed

Clinical aspects of this piglet study were previously conducted and analyzed. The piglets utilized were overall healthy upon arrival. Piglets in block 2 demonstrated the lowest weights (< 3kg) in comparison to other blocks obtained. Successful surgeries were carried out and piglets were able to adapt to their target feeding rates by day 3 of the study. Randomization of piglets into groups at day 10 was performed with positive adaptation of diet changes. Intake was monitored daily for all piglets and it was determined that energy intake was not different among groups. Over the entire study, piglets were able to obtain 89% of the targeted energy intake of 217 kcal/ (kg·day). DSS induced colitis was confirmed in the majority of pigs by testing positive for occult blood by Hemoccult Sensa after study day 8. This however, was later than expected based on previous studies [61].

The average weight of the piglets upon arrival was 3.29 kg and gained 15% of their initial weight throughout the active phase of the study, while receiving the low protein diet [83]. Piglets in the recovery phase receiving an adequate protein diet (AP) had an average weight gain that was 2.5 times more than the protein inadequate (REC) group (p= 0.0002) and 3.5 times more than the piglets receiving the low protein diet with NAC supplementation (NAC) (p = 0.0003). It was noted that the rate of weight gain tripled in the recovery phase compared to the active colitis phase for the AP group (p < 0.0001), however this was not the case for the piglets that remained on the low protein diets. The piglets overall were able to gain more than 40% of their initial weight in the AP group, while REC and NAC groups only gained 25% of their initial weights. Previous findings for this study also noted that there were no differences noted in snout-to-rump and chest circumference among any of the groups at any time points of the study. An overall gain of 13-18% of their initial length and about 8% in chest circumference was detected. Snout-to-rump growth rate (cm/day) in the recovery phase almost doubled compared to the active colitis phase in all groups [83].

Body composition was also previously analyzed for the piglets. DXA scans were preformed in-vivo throughout the study on day 1, 9, and 15. Initial body composition of piglets was 93.5% lean, 4.5% fat and 2% bone, and no significant changes were noted throughout the study. It was interesting to note that piglets in the active phase gained on average 402 g lean, 157 g fat, and 5 g bone mass despite receiving a low protein diet. In the recovery phase, however, AP piglets had 2 times more lean mass gain than REC piglets (p = 0.0019) and 3 times more than NAC piglets (p = 0.0022). Lastly, when the researchers compared the active phase to the recovery phase, the recovery phase demonstrated that the lean to fat gain ratio increased 14 times in the AP group, 2 times in the NAC group, and no change was noted in the REC group [83]. The data found was clearly able to demonstrate that piglets in the AP group had greater lean mass gains and a greater total mass than piglets that remained on the low protein diets.

In-vivo Measurements

DXA:

Femurs and spines were examined separately in addition to whole body compositions that have been previously analyzed to emphasize bone growth. DXA scans were analyzed at day 1, day 9, and day 14 of the study with focus on BA (cm^2), BMC (g), and BMD (g/cm^2) (**Tables 9 & 10**). For femurs, no significant differences between groups throughout the different time points for BA ($p > 0.05$) were noted, however, there was a significant effect of time noted (i.e. from day 1 to day 9 and from day 9 to day 14, $p < 0.0001$) (**Figure 2**). This significance over time demonstrated that all bones continued to grow over time despite receiving low protein diets and DSS induced colitis. For BMC, no significant differences between groups were seen at day 1, 9, or 14 (p = 0.5413), however, a significant increasing effect over time in BMC was noted (p = 0.0001) (**Figure 3**). When looking at BMD, again there were no significant differences between groups at the different time points, however, a decreasing effect over time in BMD was noted (p = 0.0001) (**Figure 4**).

For lumbar spine BA, there were no significant differences between groups in the active colitis phase (p >0.005), however significant differences in all groups were noted over

time from day 1 to day 9 while still in the active phase (p <0.0001), demonstrating that bone growth continued despite receiving inadequate protein and DSS induced colitis. In the recovery phase, piglets in the AP group had a significantly greater BA than piglets receiving a low protein diet (REC) (p = 0.0015), while no significance was detected in piglets receiving the NAC supplementation (p = 0.7363). Both piglets receiving adequate protein and NAC supplementation had increased in BA from day 9 (active phase) to day 14 (recovery phase) (p <0.0001), while there was no increase in BA for the piglets just receiving the low protein diet from day 9 to day 14 (p = 0.4127) (**Figure 5**). For BMC and BMD there were no differences detected between groups or over time for spinal analysis (p > 0.05) (**Figures 6 & 7**). While a possible trend of increasing BMC is noted over time, a decreasing p-value is noted for BMD, demonstrating similar results to that of the femurs.

Ex –vivo Measurements

µCT:

Whole bone (Right femur)

Piglet femurs and spines were examined ex-vivo. Piglets in the recovery phase, receiving AP, had significantly longer femurs than piglets in the active phase receiving a low protein diet (ACT) (p = 0.0178). Although bones in the active phase (day 9) were younger than bones in the recovery phase (day 15), it was interesting to note that there were no differences in length from day 10 to day 15 with REC and NAC piglet bones (**Figure 8**).

Further analysis of femur whole bone included focus on total volume (cm^3), BMC (mg), and vBMD (mg/cm^3). No significant differences were noted between groups for total bone volume or BMC (**Figures 9 and 10**). Significant differences were noted however for vBMD, with piglets in the AP group having a lower vBMD than piglets in the ACT group (p = 0.0312). No differences were noted in comparison to REC and NAC group (**Figure 11**)**.**

Cortical Analysis (femur): Diaphysis

Piglets receiving adequate protein (AP group) in the recovery phase were shown to have significantly lower cortical volume than piglets on the low protein diet (ACT) in the active colitis phase ($p = 0.0321$) (**Figure 12**). There were no differences, however, noted between the REC and NAC groups in the recovery phase compared to other groups. When looking at vBMD, AP piglets in the recovery phase were significantly lower than all other groups ($p < 0.005$) (**Figure 13**).

Trabecular Analysis (femur): Metaphysis

To further investigate trabecular bone, focus was put on the metaphysis of the femur where trabecular bone is at its highest content. At this point trabecular volume (BV/TV) and trabecular vBMD were analyzed. It was noted that there were no significant differences between any of the groups for trabecular volume (**Figure 14**). Piglets in the AP group had significantly lower trabecular vBMD than piglets in the active phase (ACT) and piglets in the recovery phase receiving NAC ($p < 0.05$). There were no differences seen in the REC group compared to other groups (**Figure 15**).

Trabecular Analysis (Spine)

The spines of all piglets, excluding one piglet in the REC group and one piglet in the AP group due to improper extraction, were analyzed with focus on L3. Trabecular area (cm^3), trabecular volume, and trabecular vBMD were investigated. Piglets receiving adequate protein in the recovery phase (AP) were shown to have a significantly lower trabecular area, as well as, trabecular volume in comparison to all other groups ($p = 0.05$) (**Figure 16 & 17**). There were no differences noted among any other groups. In contrast to the trabecular vBMD of the femur, there were no significant differences between any of the groups noted (**Figure 18**).

Bone Markers and Cytokines

Bone biomarkers from day 10 and day 15 were analyzed to get the best representation of the active and recovery phase. Bone markers of interest included BAP, OC, and NTx. BAP is a precise early marker of the bone forming activity of osteoblasts. Although no

significant differences were noted between any of the groups, there was significance over time, showing a decrease in BAP at day 15 in the recovery phase compared to day 10 in the active colitis phase (p = 0.0057) (**Figure 19**)**.** OC is also synthesized by osteoblasts and concentrated in the bone matrix. OC levels correlate with bone formation rates and serum concentrations of OC reflect the portion of newly synthesized protein that does not bind to the mineral phase of the bone but is released directly into the circulation [71]. As similar to BAP, there were no significant differences between groups at day 10 or day 15, however, there was an overall significance in time noted (**Figure 20**)**.** OC decreased from day 10 to day 15 overall (p = 0.0022). Lastly, NTx was measured as a marker of bone resorption. There were neither significant differences noted between groups at either time point (p = 0.4746), nor any differences in the marker over time (p = 0.0628) (**Figure 21**)**.**

Inflammatory cytokines were also analyzed at day 10 and day 15 to determine whether there were any differences between the active and recovery phase of colitis. Here, IL-1β, IL-6, and TNF-α were of focus. Neither IL-1β nor IL-6 showed any significant differences between groups or over time (**Figures 22 and 23**). As for TNF-α, no significant differences were noted between groups nor overtime, however, there is a trend noted of TNF-α decreasing in the recovery phase (p = 0.0694) as is expected with the termination of DSS (**Figure 24**).

In addition to analyzing bone markers and cytokines separately, determining whether they correlate was important. Since inflammatory cytokines such as the ones studied are known to be elevated in IBD and how these diseases can interrupt bone growth in children, correlations were analyzed between each bone marker and cytokine at day 10 in the active colitis phase. No correlations were noted for either of the comparisons (**Figure 25**).

Bone Strength

There were no differences noted between groups for the amount of energy needed to break the bones (p >0.05), however, piglets receiving an adequate protein diet in the

recovery phase had significantly lower work to failure needed (p = <0.05) (**Figures 26 and 27**). Work to failure is the amount of energy necessary to break the bone [84].

Intrinsic parameters examined, which are independent of the size and shape of the bone, included stress (MPa), strain (no units), Young's modulus (MPa), as well as modulus of toughness (mJ/mm^3). Piglets in the AP group had a significantly lower stress max, the load per unit area, than all other groups (p = <0.05) (**Figure 28**). Strain max, however, showed no differences between groups, which indicates the fractional change in length of a loaded body (**Figure 29**). Young's modulus is determined by an experiment where the bone sample is subjected to a load, and the strain and stress are determined concurrently. It is the slope of the linear portion of the stress-strain curve and represents the stiffness of the material [84]. The higher its value, the stiffer the material, therefore, more force is needed to produce the same strain when compared to a less stiff material. As for the piglet bones tested, piglets receiving adequate protein again where significantly lower for Young's modulus than piglets receiving a low protein diet in the active phase (ACT) as well as in the recovery phase (REC) (p <0.0202) (**Figure 30**). In addition, there was no difference noted with the NAC group compared to all other groups. Lastly, modulus of toughness was examined. This is the measure of energy per unit volume that is absorbed by the bone when subject to impact, up to the point of fracture [84]. Again, piglets receiving the high protein diet had a significantly less toughness than all other groups (p <0.05) (**Figure31**).

TABLE 9: Study day 1, 9 and 14 DXA (right femur) *in-vivo* measurements of piglets with DSS induced colitis consuming a protein deficient diet (ACT) from study day 1 to day 10, and then either continue protein deficient diet (REC), or consuming adequate protein diet (AP), or continue protein deficient diet but supplemented with N-acetylcysteine (NAC) from day 10 to day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

		ACT	REC	AP	NAC
Day 1	BA (cm^2)	5.510 ± 0.093	5.504 ± 0.251	5.438 ± 0.327	5.544 ± 0.257
	BMC (g)	1.618 ± 0.139	1.522 ± 0.325	1.427 ± 0.198	1.638 ± 0.233
	BMD (g/cm^2)	0.293 ± 0.021	0.275 ± 0.047	0.262 ± 0.035	0.294 ± 0.029
Day 9	BA (cm^2)	6.320 ± 0.221	6.250 ± 0.228	6.393 ± 0.216	6.360 ± 0.087
	BMC (g)	1.718 ± 0.148	1.748 ± 0.555	1.570 ± 0.215	1.798 ± 0.328
	BMD (g/cm^2)	0.272 ± 0.028	0.277 ± 0.081	0.245 ± 0.029	0.282± 0.048
Day 14	BA (cm^2)	-	6.978 ± 0.450	7.357 ± 0.296	7.276 ± 0.273
	BMC (g)	-	1.788 ± 0.472	1.670 ± 0.200	2.016 ± 0.342
	BMD (g/cm^2)	-	0.254 ± 0.054	0.227 ± 0.021	0.277 ± 0.040

FIGURE 2: BA of right femur of piglets determined by in-vivo DXA with DSS induced colitis consuming a protein deficient diet (ACT) from study day 1 to day 9, and then either continue on protein deficient diet (REC), or switching to adequate protein diet (AP), or continue on protein deficient diet with N-acetylcysteine supplementation (NAC) from day 9 to day 14. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p < 0.05$.

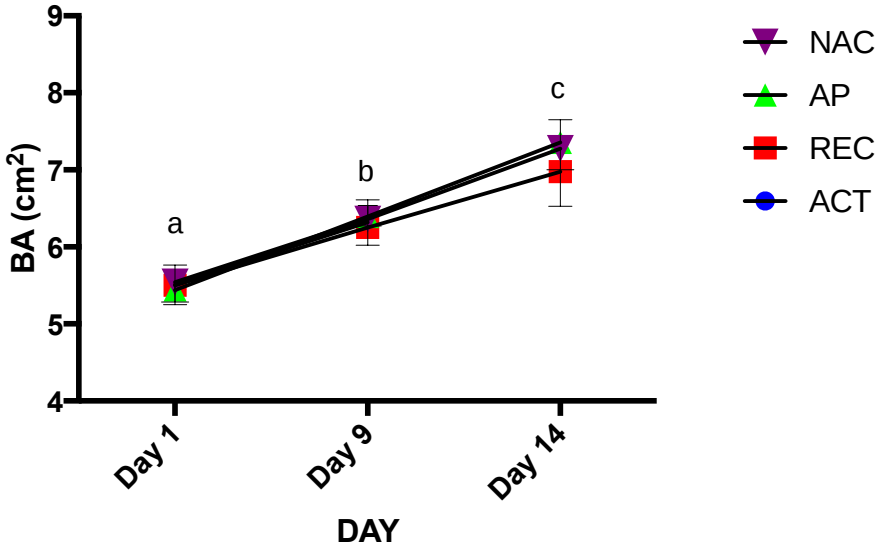


FIGURE 3: BMC of right femur of piglets determined by in-vivo DXA with DSS induced colitis consuming a protein deficient diet (ACT) from study day 1 to day 9, and then either continue on protein deficient diet (REC), or switching to adequate protein diet (AP), or continue on protein deficient diet with N-acetylcysteine supplementation (NAC) from day 9 to day 14. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p < 0.05$.

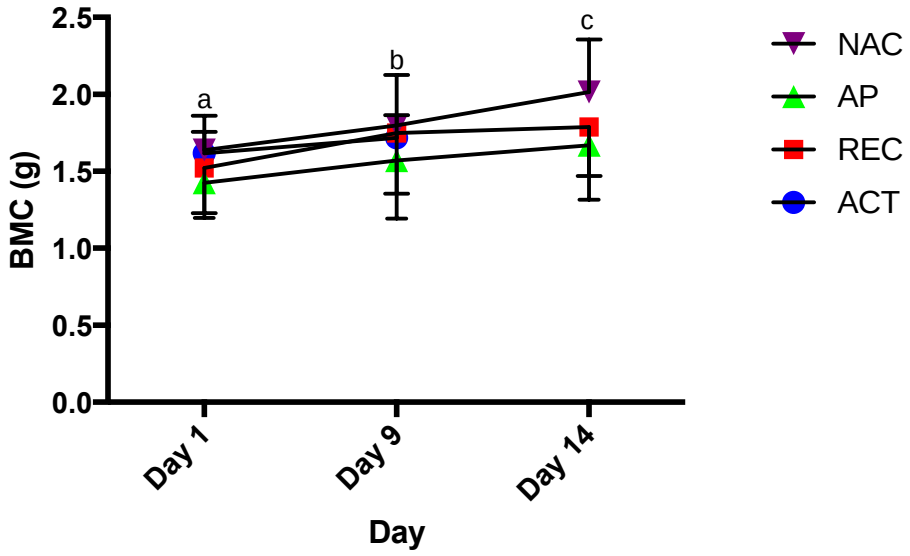


FIGURE 4: BMD of right femur of piglets determined by *in-vivo* with DSS induced colitis consuming a protein deficient diet (ACT) from study day 1 to day 9, and then either continue on protein deficient diet (REC), or switching to adequate protein diet (AP), or continue on protein deficient diet with N-acetylcysteine supplementation (NAC) from day 9 to day 14. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p < 0.05$.

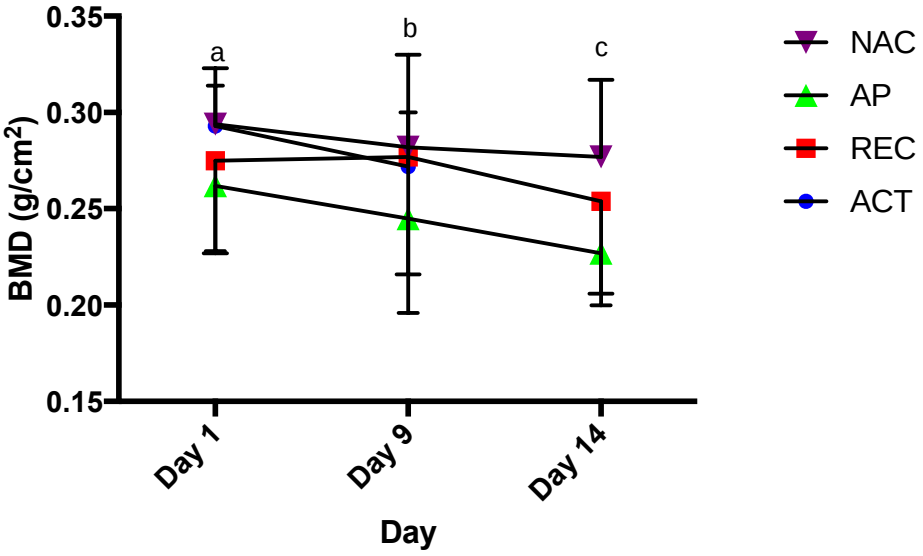


TABLE 10: Study day 1, 9 and 14 DXA (spine) *in-vivo* measurements of piglets with DSS induced colitis consuming a protein deficient diet (ACT) from study day 1 to day 10, and then either continue protein deficient diet (REC), or consuming adequate protein diet (AP), or continue protein deficient diet but supplemented with N-acetylcysteine (NAC) from day 10 to day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

		ACT	REC	AP	NAC
Day 1	BA (cm^2)	6.976 ± 0.331	7.004 ± 0.587	7.080 ± 0.195	7.002 ± 0.325
	BMC (g)	1.832 ± 0.124	1.718 ± 0.329	1.747 ± 0.203	1.868 ± 0.202
	BMD (g/cm^2)	0.263 ± 0.021	0.244 ± 0.031	0.247 ± 0.029	0.266 ± 0.019
Day 9	BA (cm^2)	7.892 ± 0.507	7.844 ± 0.332	7.925 ± 0.258	7.882 ± 0.258
	BMC (g)	1.798 ± 0.287	1.958 ± 0.140	1.843 ± 0.129	2.134 ± 0.358
	BMD (g/cm^2)	0.230 ± 0.036	0.249 ± 0.008	0.233 ± 0.015	0.271± 0.050
Day 14	BA (cm^2)	-	8.192 ± 0.213^{a}	9.032 ± 0.258^{b}	8.770 ± 0.220^{ab}
	BMC (g)	-	1.962 ± 0.151	1.863 ± 0.240	2.248 ± 0.388
	BMD (g/cm^2)	-	0.239 ± 0.015	0.207 ± 0.029	0.256 ± 0.040

FIGURE 5: BA of spine (L1-L4) of piglets determined by *in-vivo* DXA with DSS induced colitis consuming a protein deficient diet (ACT) from study day 1 to day 9, and then either continue on protein deficient diet (REC), or switching to adequate protein diet (AP), or continue on protein deficient diet with N-acetylcysteine supplementation (NAC) from day 9 to day 14. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

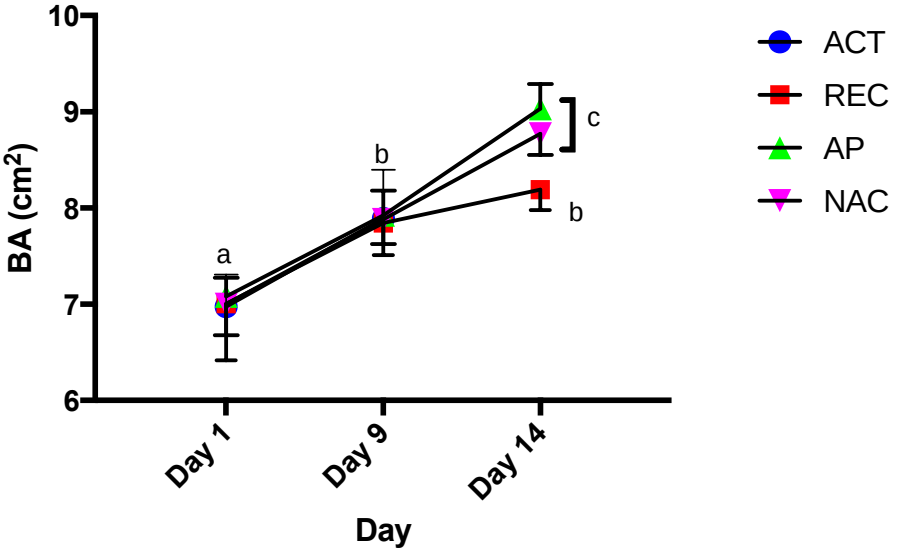


FIGURE 6: BMC of spine (L1-L4) of piglets determined by *in-vivo* DXA with DSS induced colitis consuming a protein deficient diet (ACT) from study day 1 to day 9, and then either continue on protein deficient diet (REC), or switching to AP diet, or continue on protein deficient diet with NAC supplementation (NAC) from day 9 to day 14. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

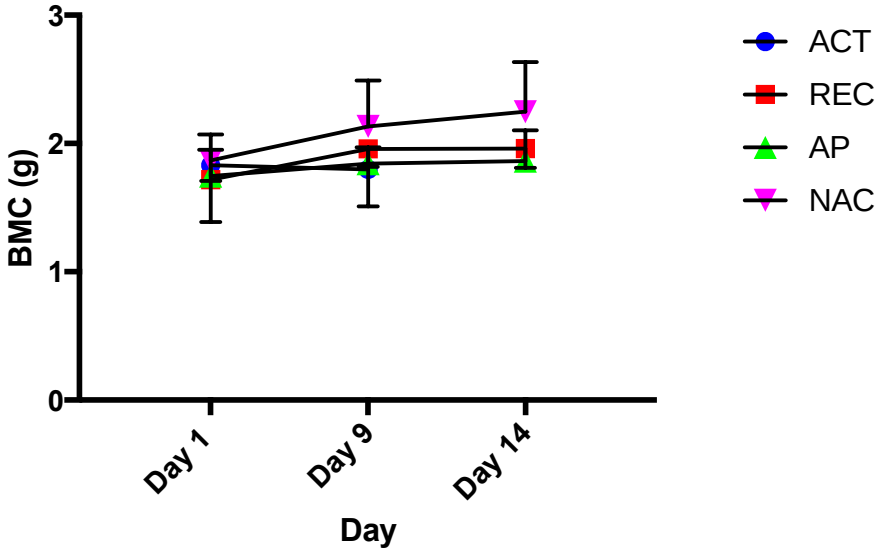


FIGURE 7: BMD of spine (L1-L4) of piglets determined by *in-vivo* DXA with DSS induced colitis consuming a protein deficient diet (ACT) from study day 1 to day 9, and then either continue on protein deficient diet (REC), or switching to AP diet, or continue on protein deficient diet with NAC supplementation (NAC) from day 9 to day 14. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

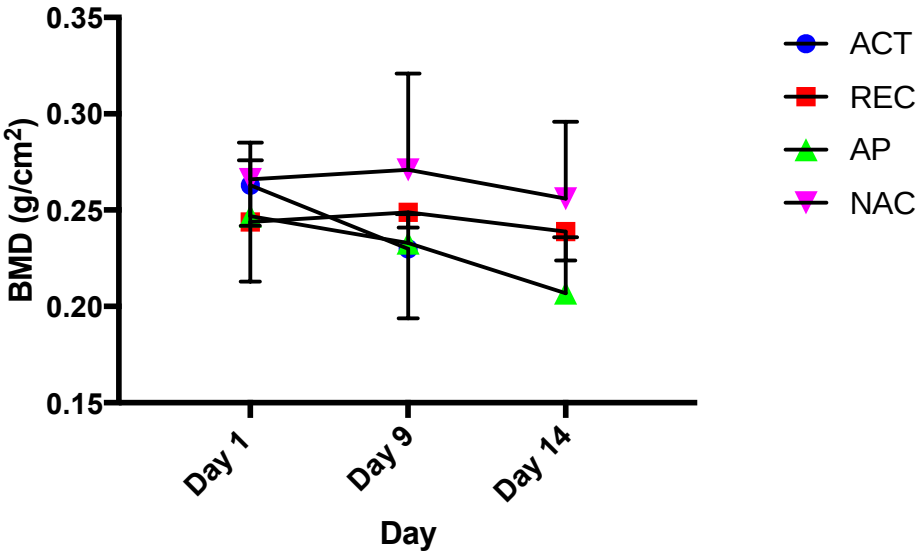


FIGURE 8: Right femur length in piglets with colitis in the active phase (ACT) at day 10 and recovery phase (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

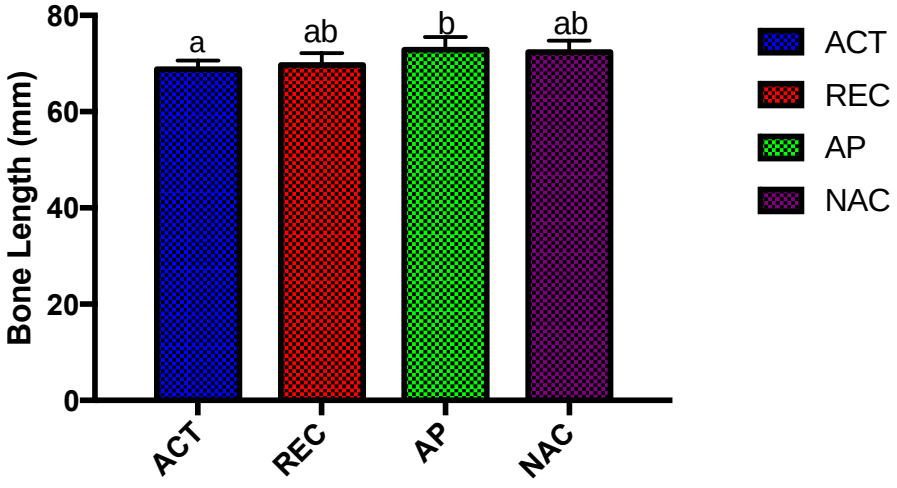


FIGURE 9: Whole bone total volume of right femur determined µCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phase (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$. Data was non-homogeneous and log transformed to obtain homogeneous data.

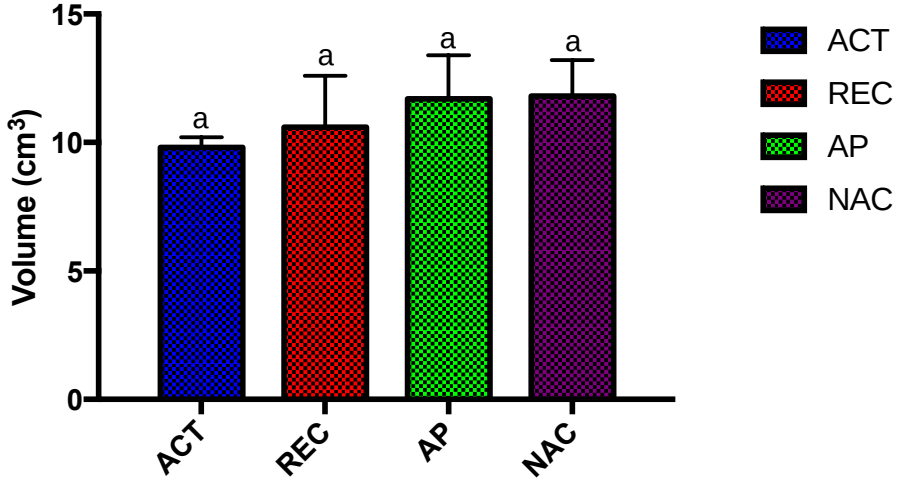


FIGURE 10: Whole bone BMC of right femur determined by µCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phase (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

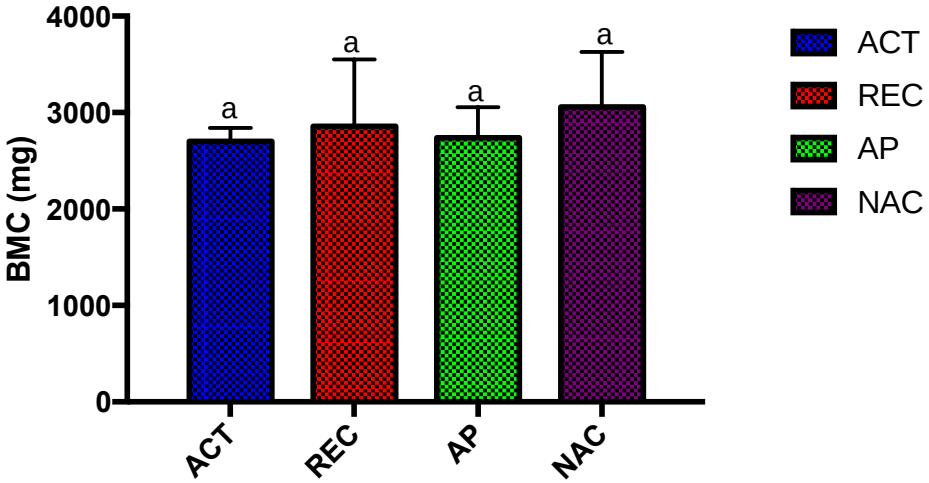


FIGURE 11: Whole bone vBMD of right femur determined by µCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phase (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

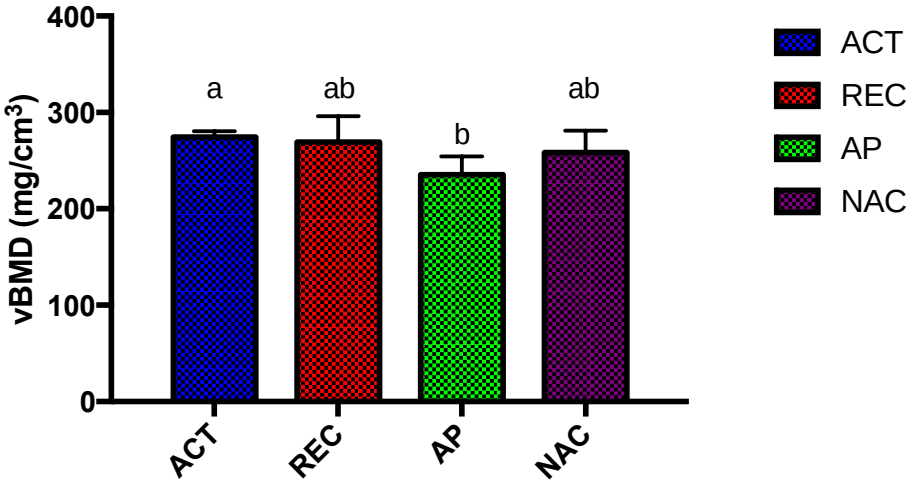


FIGURE 12: Cortical volume at diaphysis of right femur determined by µCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phase (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

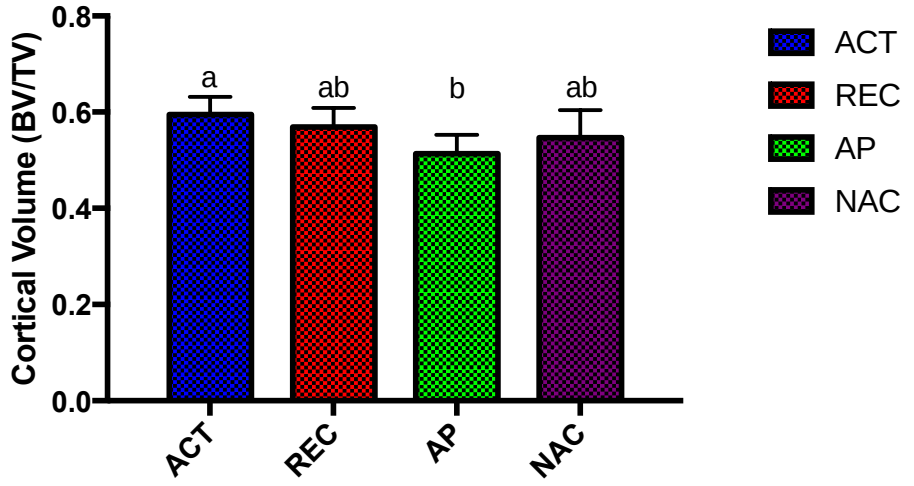


FIGURE 13: Cortical vBMD at diaphysis of right femur determined by μCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, p<0.05.

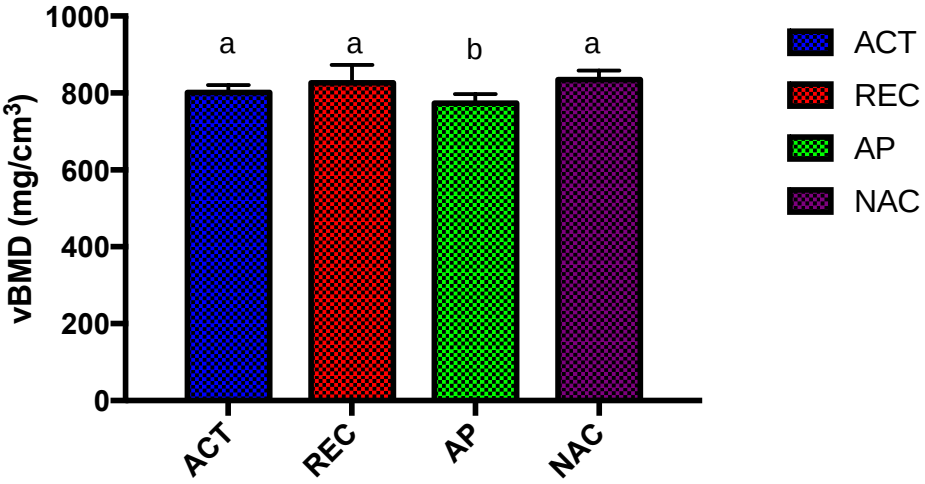


FIGURE 14: Trabecular volume at metaphysis of right femur determined by μCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, p<0.05.

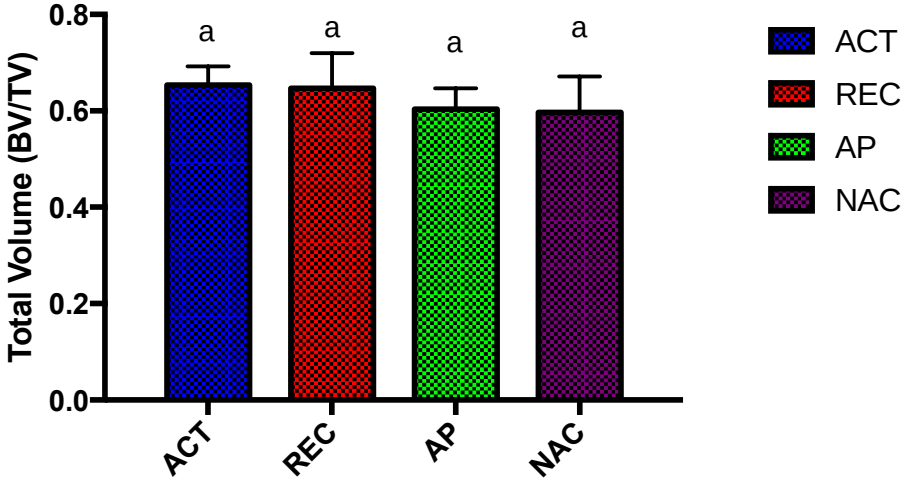


FIGURE 15: Trabecular vBMD at metaphysis of right femur determined by µCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

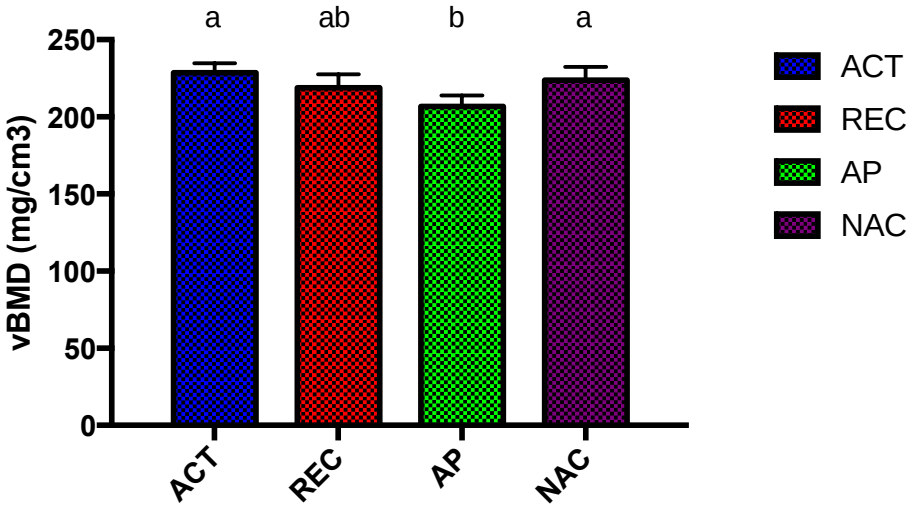


FIGURE 16: Trabecular area of spine at L3 determined by µCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 4; AP, n = 5; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

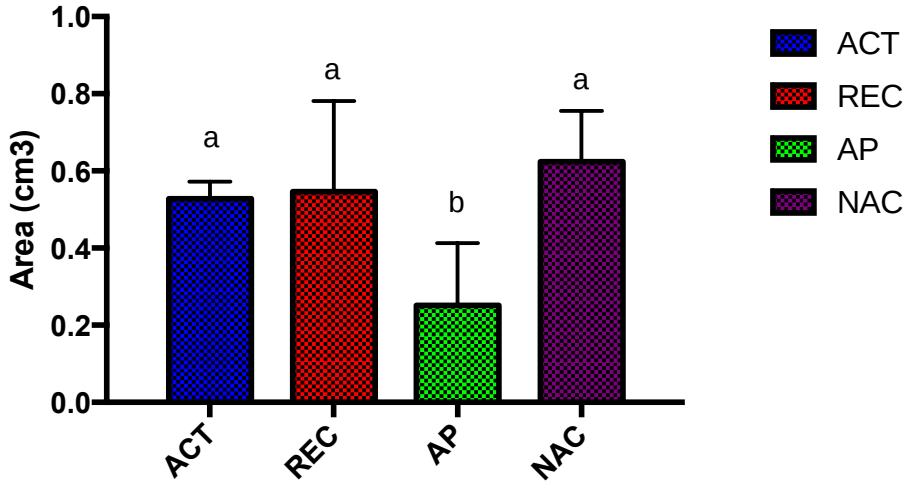


FIGURE 17: Trabecular volume of spine at L3 determined by µCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 4; AP, n = 5; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

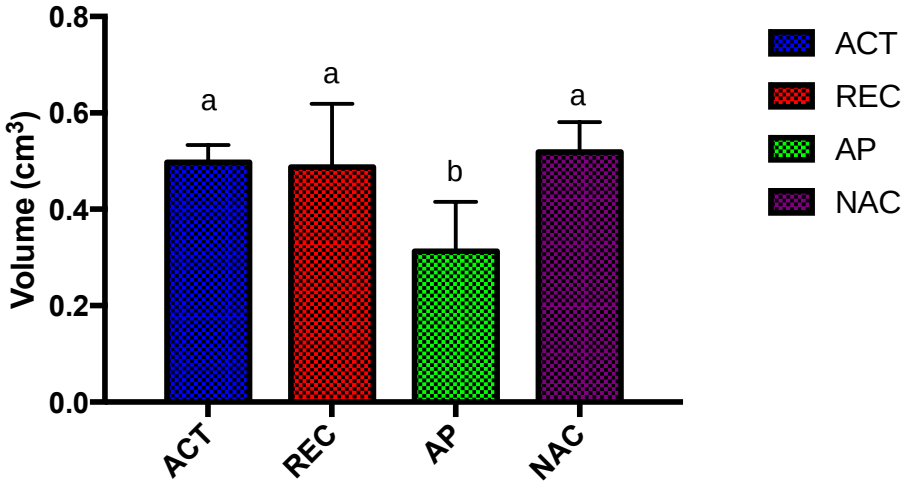


FIGURE 18: Trabecular vBMD of spine at L3 determined by µCT in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 4; AP, n = 5; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

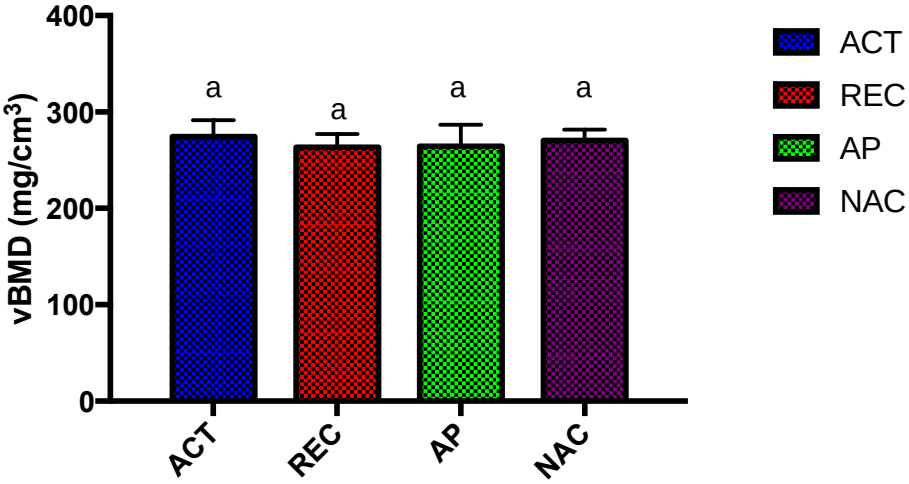


FIGURE 19: Serum BAP concentrations in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 4 per group; REC, n = 5; AP, n = 6; NAC, n = 5.

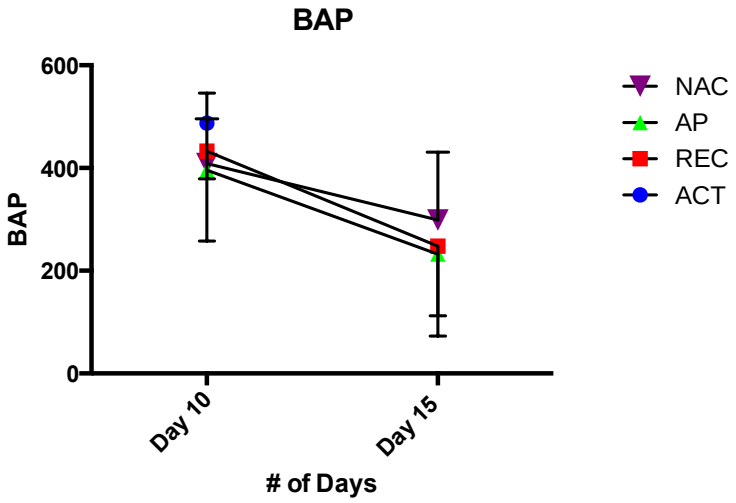


FIGURE 20: Serum OC concentrations in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 3 per group; REC, n = 4; AP, n = 4; NAC, n = 5.

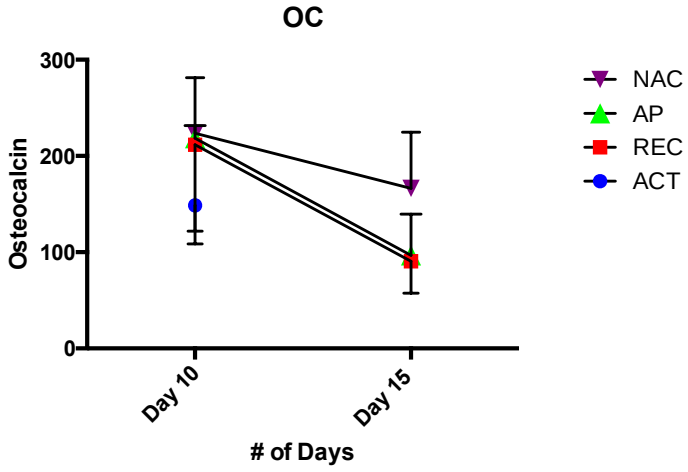


FIGURE 21: Serum NTx concentrations in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 4; AP, n = 6; NAC, n = 5.

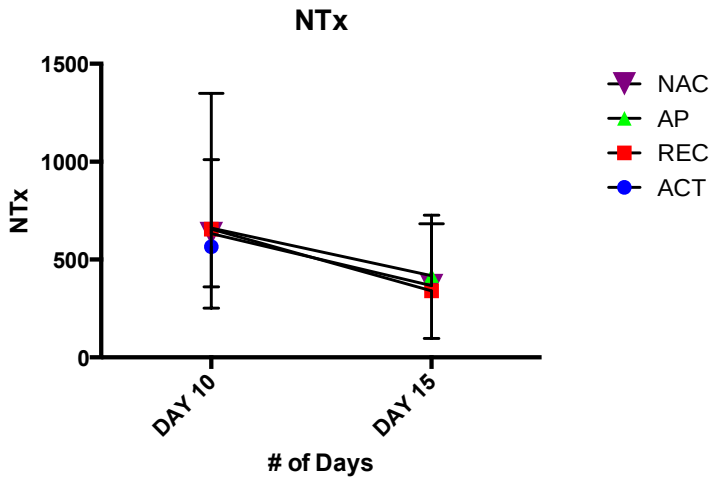


FIGURE 22: Serum IL-1β concentrations in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 5; NAC, n = 5.

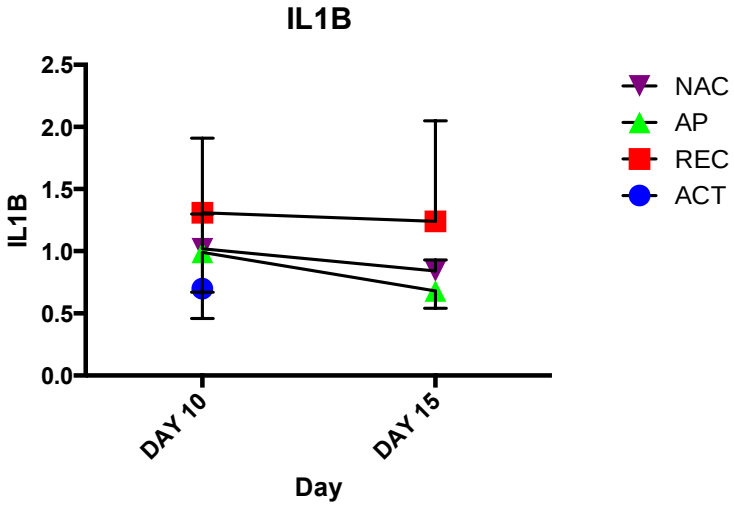


FIGURE 23: Serum IL-6 concentrations in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 5; NAC, n = 5.

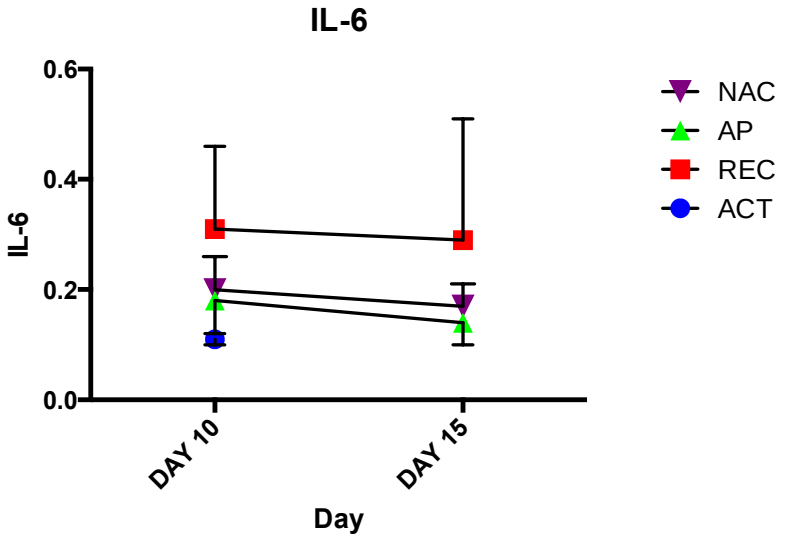


FIGURE 24: Serum TNF-α concentrations in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 5; NAC, n = 5.

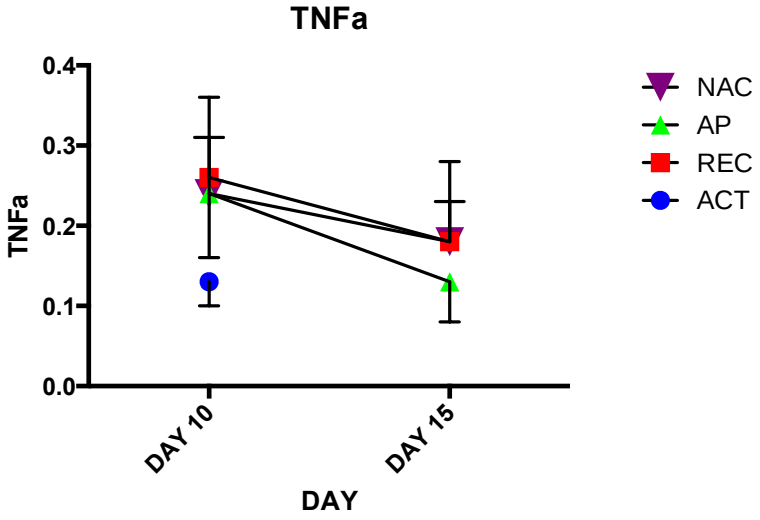


FIGURE 25: Correlation graphs of bone markers (BAP, NTx) and pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) in piglets with DSS induced colitis in the active phase (ACT) at day 10. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 5; NAC, n = 5.

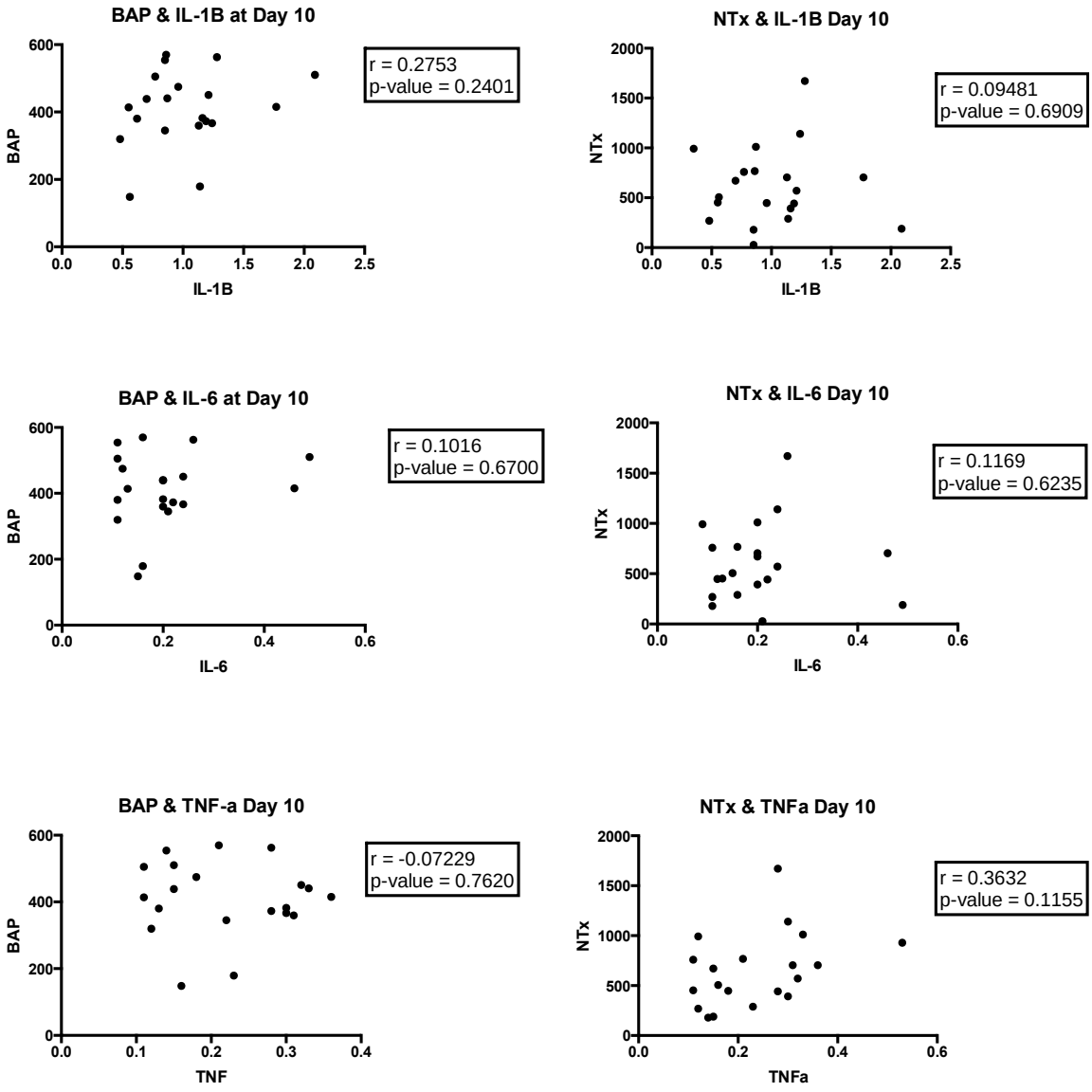


FIGURE 26: Energy at Break of right femur determined by INSTRON in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, p<0.05.

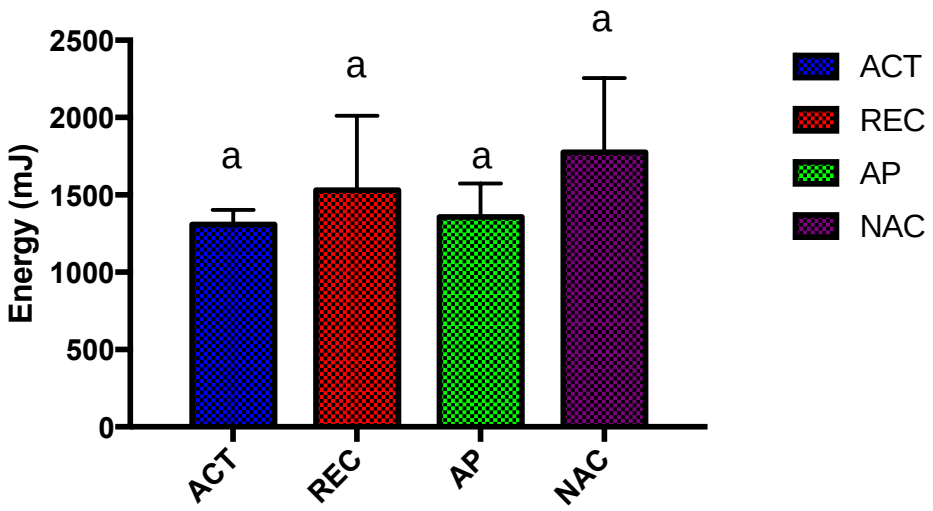


FIGURE 27: Work to Failure of right femur determined by INSTRON in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, p<0.05.

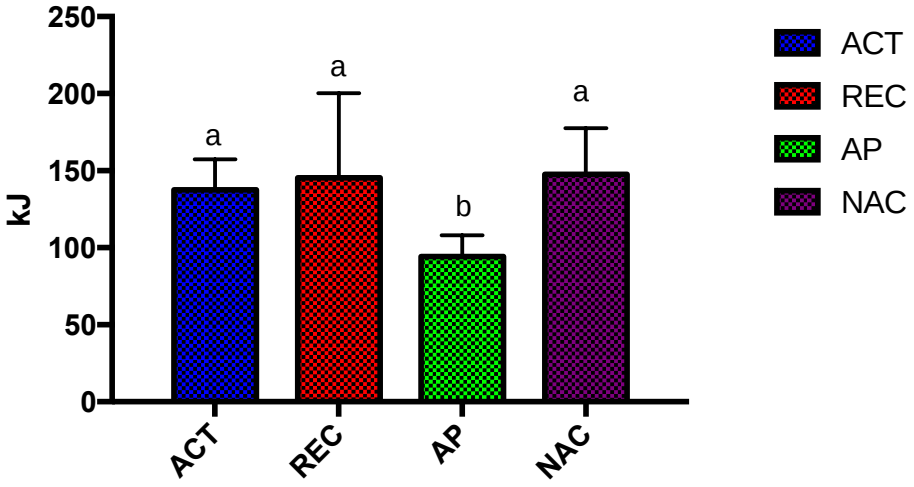


FIGURE 28: Stress Max of right femur determined by INSTRON in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

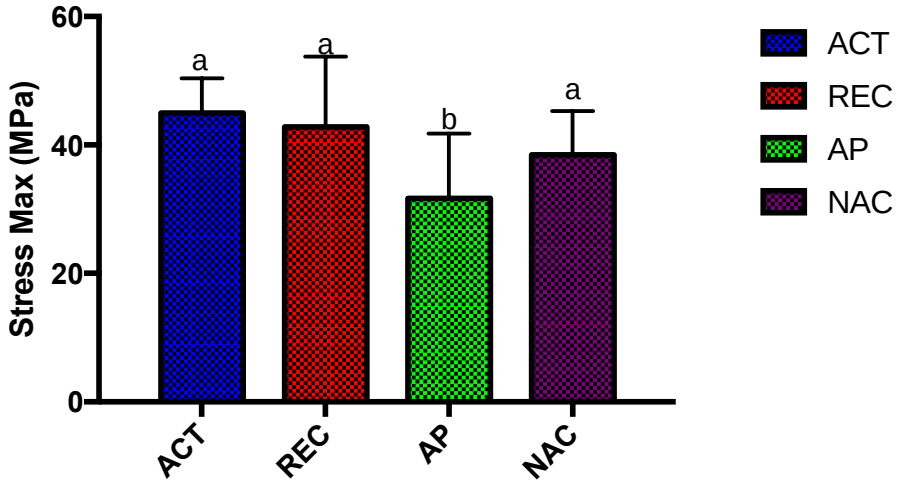


FIGURE 29: Strain Max of right femur determined by INSTRON in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

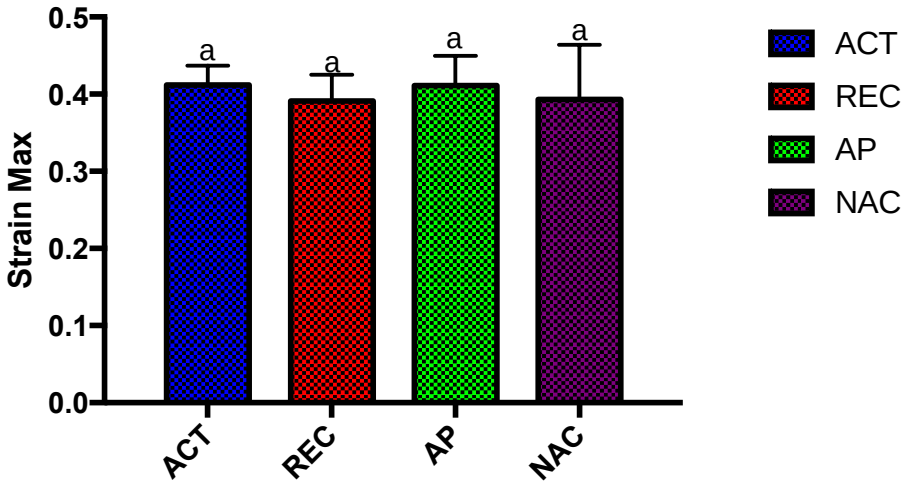


FIGURE 30: Young's Modulus of right femur determined by INSTRON in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.

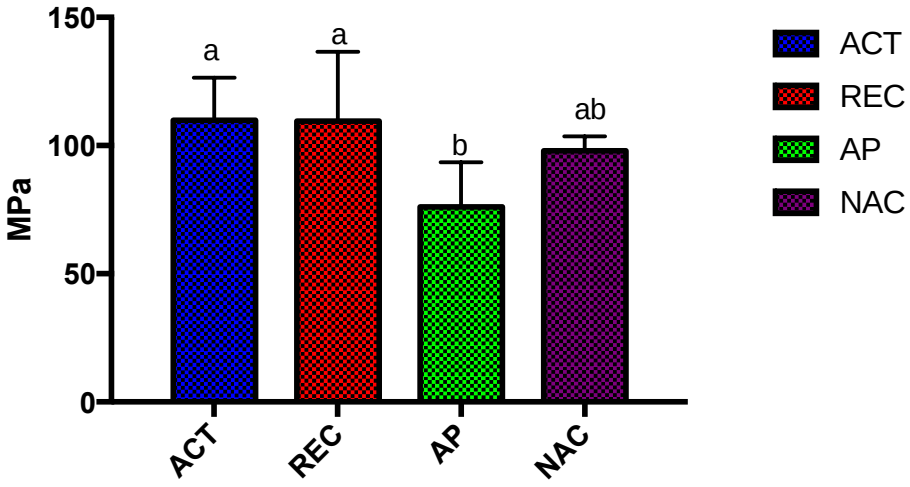
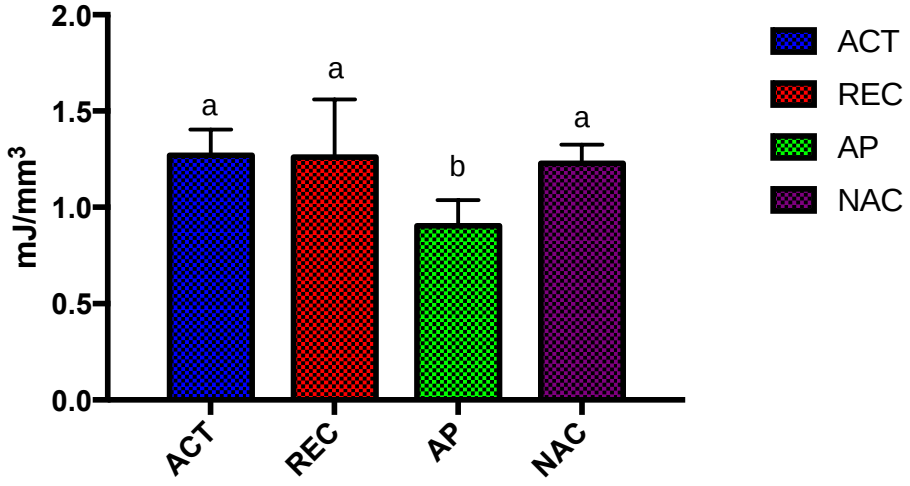


FIGURE 31: Modulus of Toughness of right femur determined by INSTRON in piglets with DSS induced colitis in the active phase (ACT) at day 10 and recovery phases (REC, AP, NAC) at day 15. Values are mean + SD, ACT n = 5 per group; REC, n = 5; AP, n = 6; NAC, n = 5. Means without a common superscript differ, $p<0.05$.



DISCUSSION:

General

Young piglets act as a useful model for the growing child with IBD due to their rapid growth rates and sensitivity to dietary interventions. This model enables us to closely study the interaction of malnutrition and the inflammation of colitis. While the majority of studies on IBD in children focus solely on the active phase of IBD, further exploration of the recovery phase is also imperative to examine the effects of recurrent malnutrition that is typically seen in pediatric IBD. More specifically, how this malnutrition and in particular protein malnutrition and inflammation effects bone health outcomes in children with IBD. This early phase of recovery presents an opportunity for clinical nutrition interventions. This study has been the first to study this early recovery period in the piglet model. Previously analyzed data of this piglet study determined that despite the co-existence of severe protein deficiency and inflammation, the piglets continued to grow throughout the study. Once DSS was discontinued, the rate of linear growth was faster and the rate of weight gain tended to be greater in the recovery phase compared to the DSS phase, however accretion of lean mass was not different. It was determined that piglets receiving the adequate protein in the recovery phase had the greatest rate of weight gain, and lean mass gain compared to other recovery groups. Piglets receiving NAC supplementation as the anti-inflammatory intervention did not have any impact on growth parameters in the recovery phase compared to the control [83].

The first specific aim for this study was to characterize bone mineral accretion and biomarkers of bone growth and bone metabolism in a protein deficient piglet model with active colitis and, in addition, their association with inflammation as indicated by pro-inflammatory cytokines. Bone mineral accretion was determined by *in-vivo* DXA. Despite a low protein diet and induced colitis, BA increased over time in the active phase for both the femur and the spine. BMC also increased in the active phase at the femur, however, only an increasing trend was noted at the spine. BMD for the femur decreased in the active phase and a decreasing trend was also seen at the spine site. As hypothesized, a decrease in BMD was expected. As for bone markers, they were only

analyzed at the end of the active phase and end of recovery phase, therefore; only a comparison of the two points could be made. Biomarkers of bone growth, such as BAP and OC, were higher than expected at the end of the active phase (in comparison at the recovery phase) while bone resorption markers, such as NTx, was higher at the end of the active phase, which was predicted. It was also predicted that pro-inflammatory cytokines would be higher in the active colitis phase, however, no differences were noted in the different phases. Lastly, concentrations of bone markers and pro-inflammatory cytokines were not correlated.

The second aim of the study was to determine the impact of anabolic nutrition intervention or anti-inflammatory NAC supplementation in the early recovery phase of colitis by assessing bone mineral accretion, bone geometry, biomarkers of growth and bone metabolism and pro-inflammatory cytokines. While piglets receiving an adequate protein diet or NAC supplementation in the recovery phase demonstrated a further increase in BA and BMC of the femur and spine over time, neither treatment was shown to be any different at the end of the recovery phase than piglets just receiving the low protein diet in the recovery phase. BMD continued to decrease in the recovery phase in the femurs (and a decreasing trend noted in the spine), which was opposite of that hypothesized. When further analysis was completed ex-vivo, μCT demonstrated that AP piglets in the recovery phase had longer femurs than those in the active phase, however, no differences were seen with NAC supplementation or the continued low protein diet in the recovery phase. Neither intervention made any impact on the total volume or BMC of the whole femur; however, piglets receiving the adequate protein in recovery had a lower vBMD than those pigs in the active stage. While the total cortical volume at the diaphysis demonstrated no differences in interventions, AP piglets in the recovery phase were significantly lower than piglets in the active phase. The cortical vBMD was lowest in piglets receiving adequate protein in the recovery phase. Trabecular volume and trabecular vBMD at metaphysis showed no differences in either intervention as well in the recovery phase; however, AP group was significantly lower than the active phase. The spine, in contrast to the femur, did demonstrate lower trabecular volume in the AP piglets, though no changes were seen in trabecular vBMD amongst interventions.

Overall, NAC supplementation had no effect on bone mineral accretion in comparison to adequate protein in the recovery phase.

Biomarkers of bone growth, BAP and OC, both decreased in the recovery phase which was opposite of what was expected. NTx indicated that there was no difference in the recovery phase, however, a decreasing trend was noted, which is what was expected. Cytokines, IL-1β, IL-6, and TNF-α, were also unchanged in the recovery phase, though a decreasing trend was noted for TNF-α. Here it was expected that once DSS was discontinued, pro-inflammatory cytokines would decrease and even more so in piglets receiving adequate protein.

Lastly, for the second aim, bone strength was examined for the recovery phase. Although neither intervention showed any differences in energy at break, work to failure was lowest in piglets receiving adequate protein, which was opposite, of what was expected. Stress max and modulus of toughness was also lowest in piglets in the AP group with no impact noted with NAC supplementation. In addition, while AP piglets demonstrated the lowest stiffness through Young's modulus compared to a low protein diet, NAC piglets were neither different from the REC piglets or AP piglets. Through examination of multiple bone health measurements, the results of study confirmed significant results of using an anabolic nutrition intervention, however, the ant-inflammatory intervention of NAC in the recovery phase did not have an impact on bone health outcomes.

Active Colitis Phase

The risk of growth failure in children with IBD is of major concern. Difficulties are faced to meet nutritional requirements in the active colitis phase, which can lead to issues of malnutrition, including delays in skeletal maturation, onset of menarche, and epiphyseal fusion in long bones [25]. Permanent growth failure can result in colitis if delayed puberty occurs, in contrast to healthy children. As growth failure can be very insidious, it is crucial that these issues are addressed early in the disease to prevent permanent effects on height and maturation. Due to the difficulties in studying children with IBD, a piglet model is a beneficial model given their physiological and metabolic similarities [57].

Using a piglet model, limiting nutrients is possible to mimic the active phase of colitis with protein deficiency, which resembles a case of late, or undiagnosed IBD where protein accretion is low. Simulating this active phase served as a control and initial state for the recovery phase in order to characterize growth under different circumstances. Overall, the piglets successfully developed colitis and severe protein deficiency by the end of the active phase, which enabled examination of how this active phase affects bone health outcomes. A decrease in BMD is commonly seen due to inadequate nutrition and the inflammatory process from the disease itself. Despite the low protein diet and DSS dose the piglets received in the active phase, BA and BMC increased over the 7 days. BMC did however increase at a slower rate then the BA, which, in turn, established a decrease in BMD over time for all piglets. With BMC increasing less than BA on proportion, this makes sense that BMD decreased overtime as it shows that the bones were not mineralizing as quickly. Growth and maturation of bone is normally accompanied by an increase in bone density. In piglets, growth in length predominates over growth in thickness and density, whereas as they get older and weight further increases, bone growth is appositional [85]. For BMD to be increasing it is implied that bone mineral deposition is occurring at a greater rate than a dimensional increase in the bone. In studies that focus on bone growth in healthy piglets, 3 to 138 kg, bone mineral was being deposited faster than the bone area was increasing. In addition, research shows that the increase in BMD seems to be proportional to the BMC in various body regions including the back legs (femurs) and spine in healthy pigs [85]. As seen with the piglets in the active phase of colitis, bone growth is clearly disrupted. This risk of having a low BMD is associated with increased fracture risk and development of osteoporosis and osteopenia and this low BMD is common in children with IBD [29].

Osteoblasts originate from mesenchymal stem cells in bone marrow. In an effort to decipher the cellular mechanisms in bone formation in piglets in the active colitis phase, bone markers were examined. Bone biomarkers of growth were higher than expected in the active phase. Due to the dose of DSS and protein insufficient diet, higher inflammation, as seen by pro-inflammatory cytokines would be expected in the active phase, which in turn, would decrease bone formation biomarkers. In contrast, both BAP

and OC at the end of the active phase were higher than at the end of the recovery phase. In addition, bone resorption markers, such as NTx - a collagen telopeptide breakdown product - showed no differences from the active to recovery phase, however, a decreasing trend was noted. This suggests that the short duration time of the study or a possible delay in DSS was noted in the active phase. Having a high BAP, despite a low protein diet and DSS dose, the higher BAP in the active phase reflects how much osteoblasteogenesis is going on at this immature stage as compared to BAP at the end of the recovery phase. Once the bone begins to mature, less BAP is noted, meaning bones have further matured and are now making collagen at day 15 of the recovery phase. In other words, differentiation of osteoblast precursors develops into mature osteoblasts [86]. OC also showed the same pattern as BAP. OC reflects the total osteoblast activity including matrix synthesis, mineralization and resorption. Newly released OC spills over into blood as does OC released from being imbedded in the matrix during resorption. A higher level, as seen in the active phase, most likely indicates greater bone turnover, while the decrease in the recovery phase suggests less bone turnover and further maturation of bones.

Recovery Phase

While there is no cure for colitis, therapy is directed at achieving and maintaining remission by minimizing inflammation and ensuring adequate nutrition for the maintenance of normal growth and development – the recovery phase. Administering DSS for six and half days followed by five and half days of recovery without DSS, it is possible to mimic acute recovery in colitis and explore the impact of nutrition interventions on bone accretion and inflammation during remission. The ideal situation is to achieve adequate nutrition, which in this study is presented by the adequate protein diet piglets (AP group). Due to the difficulties faced to meet optimal intake in children due to IBD associated complications as previously mentioned, NAC was given to examine its effects on reducing inflammation and eventually leading to improved disease severity despite receiving an inadequate protein diet.

Previously illustrated, AP piglets in the recovery phase demonstrated a trend of a more rapid growth rate than piglets receiving the low protein diet, however, significance was not detected [83]. Looking at further analysis of bone accretion, both AP piglets and NAC piglets in the recovery phase did demonstrate further increases in BA and BMC, however, treatments were shown to be no different than each other or than piglets receiving the low protein diet. If a larger sample size was used, a possible trend or significant results may have been identified. It has been calculated by previous researchers using the same group of piglets that a minimum sample size of 16 piglets per group is needed to reduce within group variance and obtain significant differences between the two phases [87]. BMD, as shown through DXA scans, surprisingly continued to decrease in the recovery phase for all groups. As previously discussed, this indicates that the bones continued to grow, however, were not mineralizing as quickly; this is common as mineralization always follows growth. A delay in DSS may have also altered the recovery phase here. As piglets continue to grow in this recovery phase, and most importantly with a well-nourished diet, a further increase in weight continued and bone growth is characterized by thickening and ossification [85]. Keeping children in remission of colitis, mineral deposition would eventually occur at a greater rate than dimensional increase in bone. Having a longer recovery period may have made the outcomes of this more visible.

The more in-depth μCT analysis and morphometry further backed up this rationale by showing greater bone lengths in AP piglets in the recovery phase compared to the active phase. Growth in length of AP piglets predominated over growth in thickness and density as seen by the significantly lower vBMD in the recovery phase. This lower vBMD in AP piglets was also noted at the diaphysis and metaphysis, stating bones were growing at a greater rate in BA than BMC, which established this decrease in BMD.

Using both extrinsic and intrinsic parameters, bone strength was analyzed using the 3-point flexure method. There are a number of biochemical parameters that can be used to determine the integrity of bone. An important relationship exists between the load applied to the bone and displacement in response to a load-displacement curve [84]. While the

slope of the curve represents the extrinsic factors such as stiffness or integrity, other properties such as work to failure (the area under the curve) represents the amount of energy necessary to break the bone. For example, bones that are osteoporotic are brittle and thus will display a reduced work to failure in comparison to a bone from a young child, which tends to be poorly mineralized and weak, but very ductile, resulting in an increased work to failure [84]. In contrast to the piglets receiving adequate protein in the recovery phase, where the bones were even more poorly mineralized compared to the other groups, work to failure was the lowest. When load is converted to stress and deformation converted to strain by engineering formulas, the relationship between stress and strain in bone follows a curve called the stress-strain curve [88]. This slope of the curve is called Young's modulus, which is a measure of the intrinsic stiffness of the bone. AP piglets demonstrated to have the lowest stiffness compared to ACT and REC, however no differences in comparison to NAC piglets. The area under the Young's modulus curve is a measure of the amount of energy needed to cause bone failure and this property is called modulus of toughness. Again, AP piglets demonstrated to have the lowest toughness.

Ultimate strength as determined by the stress-strain curve is an intrinsic factor of bone meaning it is dependent on the size and shape of the bone. While strength and stiffness are used to define the health of the bone, it is the amount of energy required to cause a fracture in the bone that predicts the risk of fractures [84]. BMD is highly correlated with strength and stiffness; however, there is an inverse relationship between bone stiffness (Young's modulus) and ultimate strain. For example, a deer antler, which is poorly mineralized, has a very high strain (ductility) but low Young's modulus, which is what AP piglets demonstrate [84]. This combination of properties supposed to make the bone much harder to break, however, the opposite was shown with piglets receiving adequate protein. This again, may be due to the already poor quality bones increasing at a greater rate in length and area than BMC. In comparison to a bone that is highly mineralized and it has high Young's modulus, it is much more brittle and easier to break. It was interesting to note that the AP piglet bones demonstrated more bend in the bone before they broke and the break was longitudinal on the underside of the bone where stress was

placed as opposed to a clean straight down break noted in the ACT, REC, and majority of NAC piglet bones.

Bone strength is closely associated with the risk of fracture. Having a higher peak bone mass, as defined by the highest level of BMD or BMC reached during life, could aid in reducing the risk of fractures [89]. Several qualities affect bone strength including the degree of mineralization, micro-damage accumulation, bone size, collagen crosslinks formation, and bone turnover [84]. Various nutritional components also influence the growth of bone mass and of particular interest in this study, protein, which is a component of bone matrix that is essential for increasing bone mass strength. Having adequate protein intake is positively related to sufficient BMD or BMC during the growth period. As previously discussed, protein malnutrition can affect the secretion of IGF-1, preventing normal growth of bone mass and linear growth [35, 36]. Previous research has clearly demonstrated that a low protein diet during growth produced a lower BMD. In this study, piglets receiving adequate protein in the recovery phase have the lowest BMD compared to the piglets in the active phase only because the bone length and BA are significantly larger; therefore, they are growing larger however not yet mineralizing as quickly in this short study period. Bone strength depends 70% on its density and 30% depends on its quality [89]. In this case, we would expect the bones with the lowest BMD to have the most decrease in strength. Due to the fact that the AP piglets had the lowest BMD and poor bone quality (previously on a protein insufficient diet plus receiving DSS) may be the reason for the lower energy needed to break bone. In a study by Takeda et al., the study demonstrated that a low protein diet in growing rats, suppressed acquisition of bone mass and increasing bone strength during growth periods. It was also interesting to note that the rats fed a moderate protein diet had a better effect on bone mass or strength in comparison to a high protein diet [89]. In summary, this study demonstrated that adequate protein greatly affected bone strength; however, NAC seemed to have little impact.

CONCLUSION:

This study investigates the impact of protein deficiency in piglets during the active and recovery phase from colitis on bone health, as well as the separate impacts of an anabolic adequate protein diet and of an anti-inflammatory NAC supplementation during recovery. Use of a piglet model demonstrated that despite having active colitis and a protein deficient diet, growth was still positive, however, growth was significantly increased in AP piglets in the recovery phase. Further exploration of bone growth confirmed that piglets receiving an adequate protein diet in the recovery phase grew the longest length, however, had the lowest BMD. Keeping children in this recovery phase with an adequate diet, mineral deposition would eventually occur at a greater rate than dimensional increase in bone. Having a longer recovery period, greater than 5 days, is needed in piglets with pre-existing protein deficiency to demonstrate these outcomes.

Examining bone strength also revealed that the young bones are poorly mineralized however are ductile. AP piglets demonstrated lowest toughness and stiffness compared to ACT and REC, however, not NAC. Although NAC appeared to be ineffective in bone growth through DXA and μCT analysis, piglets receiving NAC were independent for examination of stiffness of the bones. This may back up its function as an osteogenesis enhancing molecule, activating osteoblastic differentiation, thus improving bone growth and a greater recovery period may have also revealed this. In other studies using NAC supplementation, NAC appeared to be effective in promoting better bone growth and disease severity when adequate nutrition was also present, suggesting that a low protein diet may affect cellular levels of GSH which increases stimulation of osteoblastic differentiation [54, 56]. Studies have successfully linked NAC with decreases in pro-inflammatory cytokines, which would result in an improved remission for UC; however, this was not revealed in this study.

In general, both protein malnutrition and inflammation contribute to growth failure in IBD, therefore, a combination of adequate protein and NAC supplementation may be beneficial to maximize the body's full potential to resume normal growth and bone

mineralization as well to decrease inflammation to further prevent growth failure in children with IBD. Early investigation of bone health is crucial in pediatric IBD to ensure adequate skeletal development of growth is achieved.

Study Limitations

Limitations are present in this study and are important to take into consideration when interpreting the results obtained. Although using piglets have been shown to be a good representation for pediatric patients, differences in the characteristics of colitis still exist. DSS is a toxic agent that induces inflammation in the intestine to mimic that of colitis, however IBD is still idiopathic. Therefore, a gene-disrupted model of spontaneously occurring colitis would be more advantageous in studying colitis in children. This study also had the limitation of not having a positive control group of piglets without having colitis, which made it difficult, as there was no reference to compare the different phases and interventions to. In addition, originally a sample size of 10 piglets per group was previously determined, however, due to complications not related to colitis, only 21 piglets formed the study - leaving only 5-6 piglets in each group. This low sample size and heterogeneous groups of piglets may have concealed further significant results of the study. The short duration of the recovery phase of the study was also a limitation and may have masked significant results of bone health outcomes. The low dose of DSS given to the piglets in the active colitis phase may also have complicated interpretation and whether a delay in DSS occurred, consequently, not representing a true early recovery phase. Other limitations of the study, which were non-design related included the pre-existing health of the piglets obtained from the farm that may have obscured the inconsistency in response to stress. Piglets were indeed exposed to high levels of stress, such as, transportation from the original farm to the lab, surgery completed on the same day, and an overall new environment, which increased their risk of infections and/or exposure to bacteria.

Implications of Research

This study provides a model of IBD in growing children using malnourished piglets with induced colitis to further investigate the effect of IBD on pediatric bone health. Research

in the pediatric population is limited with regard to the effect nutritional interventions can have on bone growth and mineral accrual. This study investigates bone health outcomes in both the active and early recovery phase of colitis. Not only did this study use DXA and μCT analysis to assess cortical and trabecular bone compartments and bone geometry, but further examination of bone biomarkers for indicators of bone quality was assessed. Bone biomarkers are proven to be helpful in monitoring the response to nutritional interventions and have the advantage over BMD and BMC in that they provide information about mechanism of effect and changes are often observed much more rapidly. Both malnutrition and inflammation as seen in pediatric IBD leads to growth failure. By investigating nutritional therapeutic strategies to either provide an adequate protein diet to target an anabolic response or to control inflammation (NAC) can help provide bases for nutrition interventions in children with IBD. By providing strategies to reach adequate protein intakes in children with IBD it is possible to help prevent growth failure and promote disease recovery. In situations where achieving optimal protein intake is challenging, providing NAC to control inflammation in disease recovery can be further studied. In addition, due to the important roles they both play on the inflammatory response and bone growth, a combination of adequate protein intake and NAC supplementation may be considered as a treatment approach for IBD. These nutritional interventions during recovery in IBD can provide indication for further studies on the strategies to optimize bone health outcomes and decrease disease severity in pediatric IBD.

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