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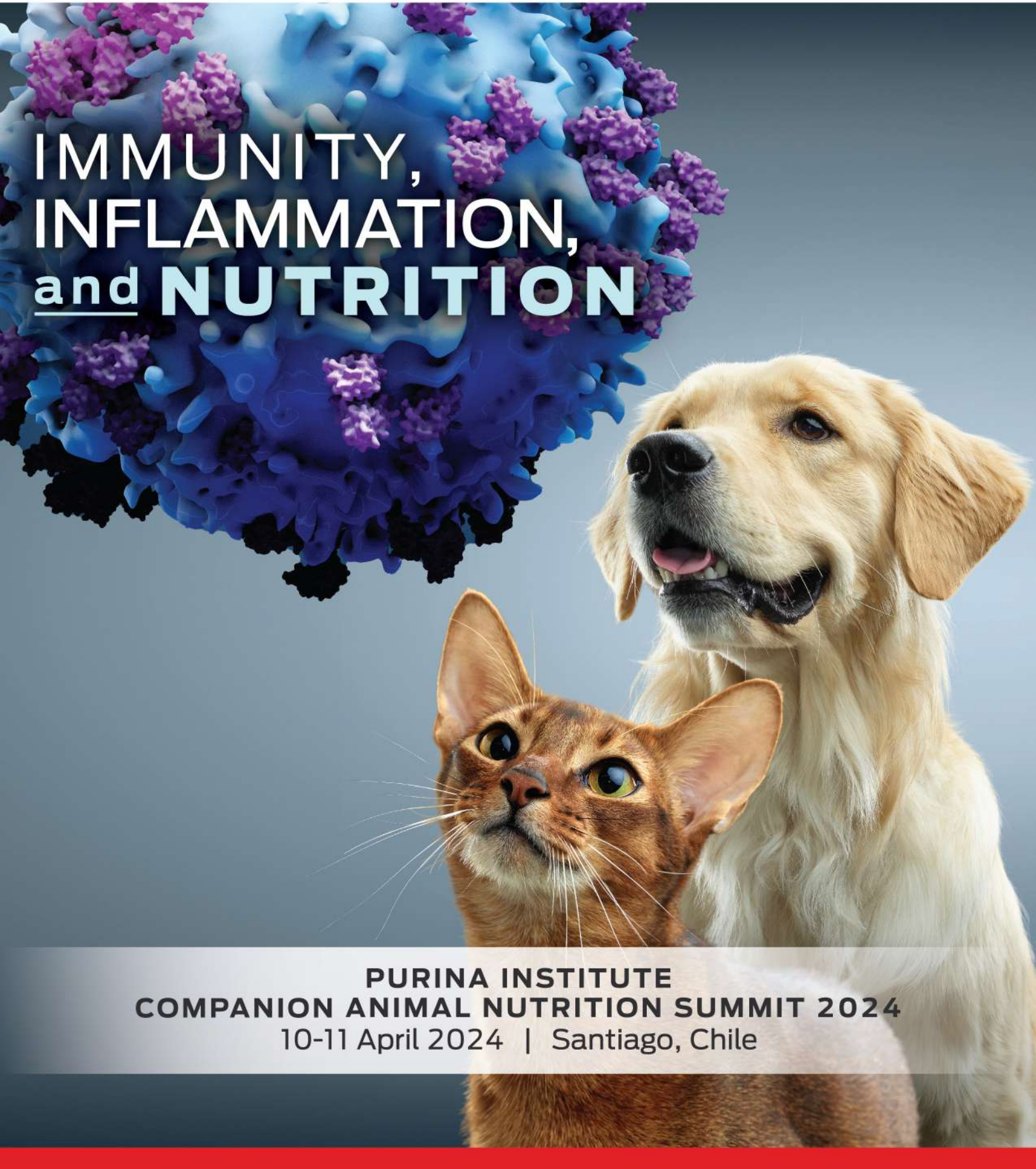

IMMUNITY, INFLAMMATION, and NUTRITION

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Immunity, Inflammation, and Nutrition

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Nutritional modulation of immune and inflammatory responses

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Abstract

This presentation delves into the complex relationship between nutrition and the immune system. It outlines how immune and inflammatory responses, critical for defending against pathogens, can be influenced by various factors, including aging and nutritional status, both under- and over-nutrition (obesity). Nutritional strategies to improve immune and inflammatory responses to increase resistance to infection and chronic disorders will be discussed.

Glossary of abbreviations

F&V Fruits and vegetables

Introduction/Background

The immune response, particularly cell-mediated immunity, is essential for combating pathogens and tumors. Inflammatory responses are also needed to aid the cell-mediated immunity in their fight against pathogens and tumors and in the recovery phase.[1] When the immune and inflammatory responses are functioning at an optimal level, once the pathogen has been disarmed, the inflammatory responses will subside and go back to their pre-pathogen exposure levels. However, if the inflammatory response is not controlled, they can damage nearby cells and result in morbidity and even mortality such as is observed in COVID-19. Further, a continued chronic inflammatory response can result in chronic diseases, including Alzheimer's, diabetes, cancer, and cardiovascular diseases. Conversely, an underperforming cell-mediated immune response can lead to conditions like cancer, autoimmune diseases, infections, and arthritis.[1]

Many factors can influence regulation of immune and inflammatory responses, key among them is aging and nutritional status.

Aging and Immunity

The number of older adults is increasing world-wide. According to WHO between 2015 and 2050, the proportion of the world's population over 60 years will nearly double from 12% to 22%,[2] these numbers are much higher in Western countries. As the population ages so does their companion animals. It is estimated that 25% of dogs and cats in US are over the age of 8, i.e. are considered seniors.[3]

Aging is associated with dysregulation of immune and inflammatory responses across all species. It is consistently observed that cell-mediated function, particularly T cell mediated function declines with age resulting in increased susceptibility to and morbidity and mortality from infectious diseases including respiratory and gastrointestinal infections as demonstrated by the recent pandemic of COVID-19.[1,4] Pneumonia and influenza combined are the 4th leading cause of morbidity and mortality among older adults. On the other hand, as we age there is increase in chronic inflammation (referred to as inflammaging),[1,4] which is considered a key contributor to

higher incidence of and morbidity from age-associated chronic diseases. It is interesting to note that there is heterogeneity in dysregulation of immune and inflammatory response i.e. not all older adults or senior cats and dogs exhibit these changes at a certain age, and some may not exhibit them at all.[5-7] This suggest that dysregulation of the immune and inflammatory responses is not an inevitable consequence of aging and could be influenced by environmental factors such as nutrition.

Impact of nutrition on immune and inflammatory responses and resistance to diseases

Nutrition plays a key role in regulation of immune and inflammatory responses. Both under- and over- nutrition (obesity) can result in impairment of cell-mediated immunity, on one hand, and increase inflammatory responses, on the other hand. Deficiency of all nutrients except for carbohydrates, impairs cell mediated function and increases production of inflammatory cytokines and oxidative stress.[8] Obesity exerts similar effects,[9] in both cases resulting in decrease resistance to infections and increased risk of chronic diseases. Thirteen percent of the global population is obese, and this number is much higher i.e. 40-50% in Western countries.[10] Similarly, 30-40% of dogs and cats in US are considered obese.[11] Of interest, exposure to pathogens can impact the nutritional status of the host enhancing morbidity from diseases.[12,13]

In recent years there has been an increase interest in the impact of supplementation with higher than recommended level of nutrient on immune response to enhance immune response, control inflammation, and enhance resistance to diseases.[14,15] However, few interventions have been successful, mainly due to methodological limitation of the studies. Some examples are briefly discussed below.

Vitamin E, for instance, has been shown to improve cell-mediated immune responses in humans and several species of animals including rodents, chickens, pigs and calves. Several studies have been conducted in the elderly and have shown vitamin E supplementation above recommended level to improve T cell mediated functions including response to vaccines and reduce inflammation. Further, a 1-year supplementation with 200 IU/day of vitamin E supplementation was shown to reduce the risk for respiratory infections, particularly upper respiratory infections.[16,17]

Zinc deficiency is associated with increased risk of pneumonia, particularly in children and the elderly, while zinc supplementation has been shown to improve immune function.[14,18] There is controversy regarding the effectiveness of vitamin C supplementation in improving the immune response while vitamin D seems to be effective in controlling inflammation and autoimmune disorders.[14,15]

Recently there has been an increase in investigating the dietary patterns particularly, plant-based diets on immunity and health in general. We recently conducted 3 Preclinical studies with a mixture of 24 fruits, vegetables, and legumes (VF-24) consumed by most Americans. Mice fed a normal or a high fat diet supplemented with VF-24 showed reduced inflammation, improved T cell function, and mitigated disease conditions such as non-alcoholic fatty liver disease (NAFLD)[19] and atherosclerosis.[20] Further, a life-long study in which mice were supplemented with and without VF-24 from when they were 1 month old till

the first diet group reached median life span, showed that increased consumption of fruits and vegetables can significantly reduce cancer incidence and overall mortality.[21] The beneficial effect of F&V is mediated through reduced inflammation, and compositional changes in gut microbiome. The findings suggest that a balanced diet rich in fruits and vegetables can enhance immune function, reduce inflammation, and potentially extend lifespan and reduce disease incidence. The relationship between nutrition, immune response, and disease underscores the importance of dietary choices in maintaining health and preventing chronic conditions.

Conclusions

Immune and inflammatory responses play an important role in maintaining normal bodily function and protecting against diseases. Normal immune and inflammatory responses are influenced by many factors such as aging and nutrition. Both under and over-nutrition can result in dysregulation of immune and inflammatory responses. Proper nutrition intervention can optimize the trajectory of immune and inflammatory diseases leading to longer and healthier life.

Conflicts of Interest

Simin Meydani is a co-inventor on a Tufts's university patent focused on the mixture of fruits and vegetables mentioned in the above studies.

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Associations and causal relationships between early bioactive nutrient intake, health, and disease

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Abstract

This discussion will focus on the developing gastrointestinal tract, which is more than simply a digestive absorptive organ. It is the largest immune organ of the body, contains large quantity of neural tissue, and is an important exocrine and endocrine organ as well. We will focus on the developmental timeline of the intestinal microbial ecosystem that has numerous effects on the development of intestinal mucosal immunity as well as some of the metabolites that regulate intestinal function. Permeability an immune function will be emphasized. We will also discuss new developments in the fields of artificial intelligence, multi omic integrations and how these will provide for personalized, precision nutrition in the future.

Introduction

This review will provide a brief overview of the developing gastrointestinal tract especially in terms of the mucosal immunity and how this develops in relation to the microbial ecosystem of the developing gastrointestinal tract. It is highly pertinent to most mammalian species. The relationship of the infant's own mother's milk, the various bio active factors found in the milk and how these relate to the developing gastrointestinal tract are of major relevance as to how we promote health, prevent disease, diagnose, and treat various illnesses in the developing mammal.

It is important to recognize that the developing gastrointestinal tract is more than simply a digestive absorptive organ. It is also the largest immune organ of the body. Inflammatory responses that are initiated in the gastrointestinal tract may have consequences for the entire organism. Systemic inflammatory responses seen in sepsis and various types of injury such as trauma, may have their origin in the gastrointestinal tract. Foods that are placed into the gastrointestinal tract interact with the microorganisms. These in turn regulate the intestinal responses of the immune system and or microbial metabolite production, which in turn regulates intestinal barrier function. It also has an effect on production of metabolites that are critical for normal neurologic function that may control both intestinal motility as well as various interactions the gut brain axis.

The innate intestinal barrier function

There are several components to the innate immune system. These are components that do not require significant "education" or time to develop. For example, the intestine is lined by a single layer of epithelial cells which harbor microvilli and constitute one of the largest surface areas of the body. This large surface area interacts with a microbial ecosystem containing bacteria, viruses

and fungi which may play either protective or pathologic roles in the gastrointestinal tract. Protective roles involve low-grade stimulation of toll like receptors. Such low-grade stimulation of toll like receptors has been found to be highly protective to the intestinal barrier function.

The intestinal epithelial cells are also connected by junctional complexes such as the tight junctions found at the apical surface of the intestinal epithelium. When these tight junctions break down, there is increased permeability or "leakiness" of the gastrointestinal tract. Abnormally high permeability has been associated with translocation of bacteria as well as numerous other potentially immunoreactive compounds that can induce an inflammatory response that is present not only in the intestinal tract but may extend well beyond to other organ systems in the form of a systemic inflammatory response if this is not controlled. Thus, the barrier function of the gastrointestinal tract is a very important component of this innate immune system.

Adaptive immunity

The adaptive immune system is component of the immune system that takes time to develop and classically is mediated by various types of immunoglobulins and effector or tolerizing T cells. The mucosal surface is closely associated with dendritic cells which have the capability to extend their cell wall components into the intestinal lumen, sense components in the intestinal lumen such as microbes or various other antigenic materials and transduce specific signals to undifferentiated T cells in the subepithelium These will differentiate into effector T cells or tolerizing T cells depending on the stimulus that is provided by the dendritic cell. These will then interact with other cell types such as plasma cells to have a memory of these previous interactions and will be senescent until their next exposure to a similar stimulus that they were previously exposed to.

Intestinal microbiome and relation to intestinal functions

The developing microbial ecosystem responds to the environment. Microbes in the pregnant mother's gastrointestinal tract produce many metabolites that are transferred to the fetus. The composition of these metabolites is highly dependent on the types of microbes in the maternal gastrointestinal tract. The mother' diet have a major effect on the developing gastrointestinal tract especially the mucosal immune system.

There is an aboral gradient of microbes in the gastrointestinal tract which develops over time. Meconium appears to be very low in microbial composition, but this rapidly increases over the first couple of weeks after birth especially if there is exposure to maternal milk, which contains significant number of microbes. The composition of microbes in maternal milk maybe very important and have a personalized composition for that mothers own infant. There are factors present in milk such as oligosaccharides that may play a role certain microbe. Bifidobacteria, for example, are highly reliant on these oligosaccharides' metabolomic role. The production of various metabolites such as short chain fatty acids which can play a very important role in development of the gastrointestinal tract for example one of the short chain fatty acids produced by the interaction of bifidobacteria and milk oligosaccharides is butyric acid, which is critical in maintenance of intestinal epithelial junctional integrity. Without butyric acid the intestinal

epithelium becomes highly permeable and thus may be prone to translocation of microbes into the subepithelium where they induce an inflammatory response and cause intestinal injury. This has been hypothesized to be one of the mechanisms for various forms of enterocolitis seen in humans, especially premature infants.

Select immunonutrients including probiotics

Various nutrients they play a major role in direct interaction with the gastrointestinal tract or indirect interaction via the microbial ecosystem where the microbes "bioreactor" role breaks down nutrients into various metabolites such as the F were mentioned short chain fatty acids which play a major role in intestinal epithelial integrity as well as an immunologic role. Glutamine, arginine, omega-3 versus omega-6 fatty acids, various vitamins concluding vitamin D, E, ascorbic acid, niacin, and others play important roles.

Probiotics are live microorganisms that play a putative role in promotion of health of the individual. There's been a lot of hype about probiotics in the promotion of health. There's also been considerable emphasis placed on the possibility of probiotics playing a role in prevention of disease. One example is prevention of necrotizing enterocolitis in human premature infants. Several studies that are underpowered for a primary outcome of necrotizing enterocolitis suggested benefits, as have meta-analysis. However, the only large adequately powered study has not shown efficacy. More studies are currently underway but some of the advisory bodies such as American Academy of Pediatrics have not supported the use of these agents largely because definitive prospective randomized trials that show safety and efficacy with a pharmaceutical grade agent have not yet been completed.

The future: precision nutrition

In the field of human neonatology, numerous guidelines for providing nutrition to premature infants have been developed. These have been very helpful in terms of guiding therapeutic strategies and providing uniformity in terms of nutritional approaches to these infants. However, infants are very different from one another. For example, a 23-week preterm infant has very different nutritional requirements compared to a 32-week premature infant. This is not accounted for in these guidelines. Newly developed technologies such as machine learning in artificial intelligence and integration of multiple forms of omics are highly promising in terms of helping us understand how to best approach the individual infant. This is a very exciting new area of investigation.

Conflicts of interest:

Josef Neu, MD is a member of the Scientific Advisory Board of the Nestle Nutrition Institute

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Role of immunonutrients in critical care

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NOTE: The following proceedings were prepared by the Purina Institute staff based on the slides and transcript of Dr. Ochoa Gautier's recorded presentation.

Glossary of abbreviations

ADMA Asymmetric dimethyl arginine
ADS Arginine Deficiency Syndrome
ART Arginine Replacement Therapy
MDSC Myeloid-derived suppressor cell
NO Nitric oxide

Introduction

Arginine is an amino acid that is conditionally essential for humans and rodents and essential in dogs, cats, birds, and fish.[1-3] It is a component of multiple proteins and is generated through endogenous production, protein turnover (which accounts for 80% or more of arginine availability), and dietary intake.[1] Glutamine and citrulline serve as sources of arginine: the kidney is the principle source of its production from citrulline.[1] Glutamine is converted to ornithine, then citrulline before it is converted to arginine.[1]

Arginine is utilized for protein translation, the urea cycle, and nitric oxide production.[1] Arginine availability is generally sufficient to maintain normal physiologic functions, but it may become deficient in certain disease conditions such as cancer, surgery, trauma, infections, and hemolytic diseases.[1]

Arginine Deficiency Syndrome (ADS)

A syndrome is really a group of signs and symptoms that occur together to characterize a particular abnormality or condition. Our team described Arginine Deficiency Syndrome in 2007 as a common set of signs and symptoms that can be attributed to decreased arginine.[4]

The anabolic process is limited by the amino acid with the lowest concentration (the rate-limiting amino acid) in the cellular environment. Arginine is central to new protein formation (anabolism) and if arginine levels are insufficient, anabolism will cease and catabolism will dominate as cells are forced to oxidize other amino acids or destroy them to generate energy instead of new protein. This affects a number of downstream metabolic processes which alter organ physiology and manifest as clinical illness.[1,5]

Arginine may become depleted through non-specific or specific mechanisms. Non-specific mechanisms also deplete other amino acids and include malnutrition due to low dietary protein intake and sarcopenia caused by illness or poor dietary protein intake. Specific mechanisms include systemic release of arginase through hemolysis (in humans only), hepatic necrosis, or from myeloid-derived suppressor cell (MDSC) activity associated with cancer or surgery;[1,6,7] and competitive inhibition by asymmetric dimethyl arginine (ADMA), such as that seen in chronic illness (e.g., chronic kidney disease, diabetes) and cardiovascular disease such as atherosclerosis.

Myeloid-derived suppressor cells and arginine depletion

Arginine depletion by MDSC expressing arginase is increasingly recognized as a mechanism of tumor evasion contributing to poor prognosis in cancer.[8-11]

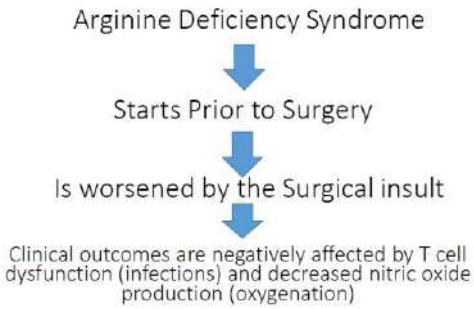
Physiologic and clinical consequences of ADS

There are physiologic impairments downstream from the lack of arginine as a substrate for particular metabolic processes, and this leads to a clinical manifestation of disease.[4,12] Examples include renal cell carcinoma, trauma, and sickle cell disease.[7,13,14]

Arginine deficiency is characterized by the following:[4,5,12]

- Reduced protein translation and T lymphocyte function, which compromises cell-mediated immunity and increases the risk of infection
- Reduced nitric oxide levels induce vasoconstriction, which reduces tissue oxygenation and increases the risk of ischemia and necrosis[14,15]
- Reduced hydroxyproline levels impair collagen formation, leading to impaired wound healing and increased risk of wound breakdown and surgical site dehiscence

Nitric oxide (NO) is an important regulator of vascular tone: excessive NO induces vasodilation and may induce hypotension, while insufficient NO results in vasoconstriction and hypertension.[14] Primary sepsis is often associated with the former, while trauma is associated with depleted NO. Interestingly, sepsis following trauma is also associated with NO depletion.[14]



Arginine Replacement Therapy (ART)

By restoring arginine as a substrate, we can restore function, correct the physiologic impairments, and improve clinical outcomes. For example, improving nitric oxide-mediated vascular flow results in improved tissue oxygenation, which in turn improves anastomotic healing and reduces the likelihood of dehiscence.

Other names used for ART include immunonutrition, immune-enhancing diets, and immune-modulating diets, but these terms are inexact and may be misleading; for this reason, the author prefers ART. ART is generally delivered as an oral nutritional supplement, but it can be administered enterally (via feeding tube) or parenterally.

A foundational study performed by Evans *et al.*[16] evaluated the dose-response in healthy subjects for increasing doses of arginine and demonstrated that a dose between 9 and 21 grams per day significantly increased plasma arginine levels. However, arginine is not given alone, but must be complemented with a balanced diet containing additional protein, optimized micronutrients, and

omega-3 fatty acids. Up to almost 90% of the healthy population over 50 years of age have severe omega-3 fatty acid deficits.[17]

Scharwtz *et al.*[18] observed that altering the levels of other macronutrients (e.g., fats, carbohydrates) affected the mean plasma arginine levels: an enteral supplement with lower carbohydrate and fat levels, but equal protein and arginine levels, significantly increased arginine plasma levels (and, therefore, pericellular concentrations) for up to 6 hours.

ART for gastrointestinal cancer surgery

Pre- and perioperative ART (12.5gm/day arginine for 5-7 days prior to surgery *or* prior to and after surgery) better preserved T lymphocyte function in non-malnourished patients undergoing colorectal resection for cancer compared to patients who received an isonitrogenous, isocaloric diet.[19]

"Pulses" of ART are recommended as opposed to continuous administration of arginine. A standard protocol for elective surgeries begins ART 5 days preoperatively and continues it for 5 days postoperatively if feasible.[20-24] Preoperative administration "preloads" arginine, prepares T cells to respond to surgery-induced activation, and improves T cell survival.[25]

Preoperative ART in well-nourished patients undergoing colorectal surgery (n=200) significantly reduced postoperative infection (12% vs 32% in controls) and anastomotic leakage (3% vs 6% in controls) as well as length of hospital stay (9.5 days vs 12 days).[19] Significantly lower rates of surgical site infection, venous thromboembolism, and hospital readmission (by almost 50%) within the first 6 postoperative months, as well as a trend toward shorter hospital stays, were associated with ART for patients undergoing colorectal surgery.[26]

Pre- or perioperative ART significantly reduced length of hospital stay by 2-3 days in malnourished patients undergoing surgery for colon cancer.[27] Although preoperative ART reduced postoperative complication rates (28% vs 42% in controls), only perioperative ART produced significantly lowered complication rates (18% vs 42%) compared to controls.[27]

Postoperative ART significantly reduced anastomotic leaks (by approximately 50%) and infectious complications (by approximately two-thirds) in patients undergoing total gastrectomy for treatment of gastric cancer.[28] Similarly, early postoperative ART in gastrectomy patients (n=66) significantly reduced postoperative infections (6.7% vs 30% controls) and was associated with no wound complications (compared to 26.7% in controls).[29] Hydroxyproline levels were significantly increased in patients receiving ART (59.7 nmol/cm vs 28.0 nmol/cm); the higher the concentration of hydroxyproline, the faster the wound healing rate.[29]

A meta-analysis comparing ART to standard nutrition for cancer patients observed significantly reduced risk ratios for postoperative infections, anastomotic leak, and length of hospital stay with ART.[30]

ART for other cancers

ART including omega-3 supplementation was associated with a nearly 6-fold reduction in oro-cutaneous fistula formation in patients undergoing surgery for head and neck cancer.[31] Perioperative ART in high-risk head and neck cancer patients (n=177) significantly reduced

postoperative complication rates (24% vs 44% in controls) and reduced the length of hospital stay by 3.2 days on average.[32] ART increased the overall postoperative survival time of severely malnourished patients with head and neck cancer.[33] Three "pulses" of ART also significantly improved tumor-free survival associated with adjuvant chemoradiotherapy in head and neck cancer patients.[34]

Indications and contraindications for ART

Arginine replacement therapy in surgical patients is not for treating malnutrition, it is for treating arginine replacement therapy, and therefore it should be given to every patient with potential arginine deficiency. Any patient undergoing gastrointestinal surgery is likely to be arginine-deficient, and therefore likely to benefit from ART. No matter the location in the GI tract, perioperative ART is associated with a 30-50% reduction in postoperative infections.[21] There is a clear benefit in patients undergoing surgery for gastrointestinal cancer, and ART should be standard of care for these patients.[26,30,35,36]

However, patients undergoing non-gastrointestinal surgery may also benefit from ART. In a systematic review of perioperative ART studies in GI and non-GI applications, Drover *et al.*[21] concluded the use of ART was beneficial for reducing infection rates, with perioperative administration more effective than preoperative or early postoperative administration.

Extrapolation to non-human species

Arginine metabolism is central to the evolution of different species, and different species utilize arginine in very different ways. Therefore, it is important to avoid extrapolation to other species based on the information presented at this conference.

Conclusion

Perioperative ART should be standard of care in elective surgical patients with gastrointestinal cancer as supported by evidence-based science and public health and economic benefits. There is increasing evidence of benefit for perioperative ART during chemotherapy, radiotherapy, and immunotherapy.

Conflicts of interest

The author has no conflicts of interest to declare regarding the material presented.

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Immunonutrition – Companion animal perspective

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Abstract

Nutritional immunology explores the interplay between diet and the immune system, shifting from its initial focus on malnutrition-related immune deficiencies to addressing the impact of life-stage and stress on immune function. Unlike malnutrition, which can be addressed by correcting underlying nutritional issues, immune deficiencies resulting from life-stage or stress are more intricate and lead to compromised immune responses, increasing susceptibility to infections, autoimmune diseases, and cancers. The gut, housing a significant portion of immune cells, serves as a primary target for dietary modulation, with nutrients influencing immune function at various levels. From providing essential nutrients to fine-tuning immune responses through components like probiotics, omega-3 fatty acids, and polyphenols, dietary interventions provide opportunities for optimizing immune health and combatting immune-related disorders.

Glossary of abbreviations

APC	Antigen presenting cell
LPS	Lipopolysaccharide
Th	T_{helper} [cell]

Introduction

Nutrition and immunity are intricately linked, with both essential for survival and having co-developed organ systems and signaling pathways throughout evolution.[1] For example, in the fruit fly *Drosophila melanogaster*, immune and metabolic responses are controlled by the same organ. While higher animals have distinct immune and metabolic organ systems, their proximity and interaction are evident, particularly in tissues like adipose and liver, where immune cells such as macrophages reside. Molecularly, cells involved in metabolism and immunity share pathways, responding similarly to bacterial products like lipopolysaccharide (LPS). Chronic nutrient imbalances can disrupt this relationship, leading to adverse health effects; for instance, obesity-associated adipose tissue produces elevated levels of proinflammatory cytokines, contributing to low-grade inflammation and metabolic disorders like insulin resistance, type 2 diabetes, and atherosclerosis.[2] However, understanding this relationship presents opportunities to proactively enhance immune health.

Ensuring immune health is vital not only for protecting against infections but also for maintaining overall metabolic balance and influencing various body systems, including cognition and brain aging. A healthy immune system serves as the first line of defense, preventing pathogens from establishing active infections. However, compromised immunity weakens this barrier, leading to disease. While the immune response is crucial for neutralizing pathogens, it comes at a metabolic cost, consuming significant energy and resources.[3] This can impact vital processes like reproduction, lactation, and growth, as the body prioritizes protection over other functions.

Moreover, repeated immune activation can lead to increased oxidative stress, which can be particularly harmful in aging individuals. Recent research indicates that poor immune health may negatively affect cognition and brain aging.[4] Thus, maintaining immune health has far-reaching metabolic implications beyond disease prevention, emphasizing its importance for overall well-being and quality of life.

What impacts immune health?

In the absence of disease, age and stress play crucial roles in influencing immune status. Neonates and older individuals typically exhibit less vigorous immune responses compared to adults, rendering them more susceptible to infections. Aging is associated with chronic low-level inflammation,[5] contributing to a decline in the immune system's ability to respond and regulate immune reactions. Additionally, chronic stress has been shown to have a significant negative impact on the immune system,[6] regardless of the individual's age. These factors underscore the importance of addressing age-related changes and managing stress to maintain optimal immune function and overall health. Underweight and overweight/obese conditions affect immunity. Malnutrition and protein deficiency result in immune defects and can increase the risk of infection, while overweight and obesity can induce chronic low-grade inflammation and immunosenescence.[7,8]

Immune response in neonates

Neonatal immune responses are typically weaker compared to adults, as evidenced by lower mitogenic and lymphocyte proliferation activity in young animals.[9] Additionally, neonates tend to exhibit a Th2 bias in their immune responses, characterized by anti-inflammatory cytokines and less effectiveness in combating microbial infections, rendering them more susceptible to diseases. Several cellular and molecular factors contribute to this Th2 bias: neonatal antigen-presenting cells (APCs) have reduced efficiency in antigen presentation due to decreased expression of crucial co-stimulatory molecules;[10] the feto-placental environment is immunosuppressive and Th2 biased; neonatal B cells exhibit altered signaling, dampening their response and class-switching ability; and neonatal Th1 cells undergo apoptosis due to unique receptor characteristics, leading to a dominance of Th2 responses.[11] However, as neonates age, the accumulation of appropriate dendritic cells, particularly in the spleen, enables the production of IL-12, which rescues Th1 cells from apoptosis and promotes a balanced immune response. This highlights the importance of cytokines like IL-12 in initiating a Th1-biased immune response in neonates.[12]

Immune response as animals age

Aging brings about significant changes in both humoral and cellular immune responses, characterized by defects in hematopoietic bone marrow function, lymphocyte migration, maturation, and overall function.[5] Additionally, aging involves thymic involution,[13-15] contributing to the loss of immune function over time. One notable consequence of aging is the reduced plasticity of the immune system,[16] resulting in a diminished response to danger signals such as pathogens, tissue damage, and oxidative stress, as well as a reduced ability to return to a quiescent state afterward. Chronic metabolic stress associated with aging further exacerbates this decline in immune plasticity, leading to a condition known as 'immunosenescence,' which

increases the risk of age-related diseases like cancer and infections.[17] Declining immune plasticity also contributes to cell death or necrosis induced by oxidative stress. Thus, considering nutritional strategies to support immune system effectiveness becomes crucial, especially in addressing the impact of age on immune status.

Stress

Stress, whether physical or mental, exerts a significant negative impact on the immune system, regardless of age. Both major and minor stressful events have been shown to influence immune responses, leading to increased oxidative stress and a gradual erosion of immune plasticity. The field of psychoneuroimmunology has emerged to study the intricate interaction between psychological processes, the nervous system, and the immune system. Research using vaccine responses as indicators of immune status has demonstrated that stress can lower vaccine responses, thereby increasing susceptibility to pathogens.[18-21] On a molecular level, stress delays inflammation, decreases immune surveillance efficiency, IFN-γ secretion, and antigen presentation efficiency, thus impairing immune responses to vaccination.[22,23] Hormones play a crucial role in the effects of stress on the immune system, with chronic stress leading to sustained activation of stress responses, including the release of glucocorticoid and catecholamine hormones, which negatively impact immune function.[24]

Age and stress together can compromise immune status, leading to increased vulnerability to diseases, infections, malignancies, and autoimmune disorders. Therefore, there is a critical need to evaluate immune status and address deviations to enhance the quality of life and overall health.

How can nutrition impact the immune system?

Besides serving as the gateway for nutrient intake, the gut is the largest immune organ in the body, housing over 65% of all immune cells and over 90% of immunoglobulin-producing cells.[25,26] In adults, the intestine contains three times more Ig-producing cells compared to the bone marrow, with approximately 6 x 10^{10} Ig-producing cells in the gut.[27] It is estimated that around 3-5 grams of secretory IgA is secreted daily into the intestinal lumen of an adult human.[27,28] Therefore, the immune system is exposed to everything that passes through the gastrointestinal tract, and anything ingested may affect the immune system. This highlights the significant interaction between the immune system and dietary intake, emphasizing the importance of diet in supporting immune health for both humans and pets.

Gut Associated Immune Tissue [GALT] plays an important role in development of the immune system

Research conducted with germ-free animals has highlighted the crucial role of environmental antigens and the gut microbiota in the development of a healthy immune system. Germ-free animals exhibit underdeveloped immune systems, emphasizing the importance of symbiotic microbiota and environmental antigens. The GALT offers a unique opportunity for immunomodulation via diet, as it is constantly exposed to a diverse array of antigens from food and commensal microorganisms. This exposure remains quiescent until encountering a threat, such as a pathogen, which initiates immune response through Pathogen Associated Molecular Patterns (PAMPs) expressed by microbial pathogens.

Efficient antigen presentation by antigen-presenting cells (APCs) like macrophages is essential for an effective immune response. APCs play a central role in the altered immune response observed in neonates, aging immune systems, and during stress, characterized by the lack of immune-potentiating cytokines like IL-1 and IL-12. Strategies to address this deficiency involve providing the necessary signaling to APCs, primarily targeting receptors on immune cells in the gut with PAMPs. Examples include yeast b-glucans,[29] yeast mannans,[30] nucleic acids,[31] and probiotics, which initiate local pro-inflammatory cytokine secretion, activating local APCs to efficiently present antigens to T lymphocytes. This enhanced immune activity in the GALT spreads to the entire immune system through the trafficking of activated lymphocytes and cytokines, facilitating a more robust immune response. Overall, dietary immune response modifiers in the GALT play a crucial role in enhancing immune function and protecting against pathogens.

Nutrition and Immune System

The interaction between nutrition and the immune system occurs at multiple levels, which can be broadly categorized into four stages:

Stage I: Complete Nutrition
This stage involves providing the immune system with essential nutrients such as dietary energy, protein, vitamins (A, C, E), and minerals (e.g., Zn, Mg, Fe). These nutrients are crucial for immune function and maintaining overall health. A complete and balanced diet meets, and may exceed, this level.

Stage II: Optimizing Macro & Micronutrients
In this stage, the focus is on optimizing key nutrients critical for immune cell function above basic levels. Providing higher levels of these nutrients can enhance immune function. For example, higher quality proteins, vitamins, and minerals support the structural components and processes of the immune system.

Addressing oxidative stress is another aspect of this stage. Oxidative stress, which increases with aging and environmental stressors, can damage cellular DNA and impair immune function. Strategies such as caloric restriction, dietary antioxidants, prebiotics, and postbiotics can help mitigate oxidative damage and support immune health.

Stage III: Active Modulation of the Immune System
In this stage, active interaction with the immune system is emphasized to modulate its function towards specific goals. Examples include reversing Th2 bias and restoring Th1 response by promoting efficient antigen presentation. This can be achieved using probiotics, prebiotics, and other immune response modifiers in the diet.

Dietary omega-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) provide anti-inflammatory support.

Bovine colostrum provides active immunomodulation. The inclusion of colostrum in the diet of sled dogs increased fecal IgA, enhanced distemper vaccine response without inducing an

overactive immune response, and stabilized the gut microbial populations.[32] Similarly, colostrum increased fecal IgA and enhanced the immune response to rabies vaccination without excessive immune stimulation in post-weaning kittens.[33] *Enterococcus faecium* SF68 (NCIMB 10415) (SF68) is an example of a probiotic with immunomodulatory activity. When administered daily to puppies for 44 weeks, SF68 enhanced the puppies' immune response to distemper vaccination and increased overall fecal IgA.[34]

Stage IV: 'Personalized' Nutrition

The concept of personalized nutrition involves tailoring dietary strategies based on individual genetic makeup and disease susceptibility. Biomarkers play a crucial role in identifying susceptibility to disease and guiding personalized dietary interventions. The goal is to modify physiology through personalized dietary regimens to prevent or delay the onset of disease and enhance quality of life.[35]

In summary, as our understanding of the complex interplay between nutrition and the immune system advances, various dietary approaches to support immune health will become available. These approaches not only provide basic nutrition but also actively modulate immune function, offering the promise of improved health and well-being for both humans and pets.

Conclusions

Nutritional immunology investigates the intricate interplay between diet and the immune system, evolving from a focus on correcting malnutrition-related immune deficiencies to addressing broader factors like life-stage and stress. While malnutrition-related immunodeficiency can be remedied by addressing nutritional gaps, immune challenges arising from life-stage or stress demand multifaceted approaches. The gut, housing a significant proportion of immune cells, serves as a primary target for dietary modulation, with nutrients and bioactive compounds influencing immune function at multiple levels. From providing essential nutrients to fine-tuning immune responses through probiotics, omega-3 fatty acids, and polyphenols, dietary interventions hold promise in optimizing immune health and mitigating susceptibility to infections, autoimmune diseases, and cancers. Nutritional immunology offers insights into tailored dietary strategies for promoting immune resilience across different life stages and stress conditions, with implications for both human and companion animal health.

Conflicts of interest

Dr. Satyaraj is an employee of Nestlé Purina PetCare.

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Pathophysiology of early and progressive CKD in cats

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Abstract

Chronic kidney disease (CKD) is highly prevalent and an important cause of morbidity and mortality in adult and senior cats. Histologically, CKD is characterized by interstitial inflammation, tubular atrophy, and fibrosis in most cats, with interstitial fibrosis present even in those in the early disease stages. Several factors promote ongoing renal inflammation and the development of fibrosis in cats with CKD, playing a crucial role in the pathophysiology of disease progression. Among others not discussed in this lecture and associated materials, these factors include single-nephron adaptations to loss of functioning renal mass, activation of the renin-angiotensin-aldosterone system, systemic arterial hypertension, proteinuria, interstitial lipid accumulation, CKD-mineral and bone disorder, hypoxia, and oxidative stress.

Glossary of abbreviations

BP	Blood pressure
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease-mineral and bone disorder
GFR	Glomerular filtration rate
HIF	Hypoxia-inducible factor
RAS	Renin-angiotensin system
RAAS	Renin-angiotensin-aldosterone system
SAH	Systemic arterial hypertension
TGF-β1	Transforming growth factor-β1

Introduction

Chronic kidney disease (CKD) is broadly defined as an abnormality of kidney structure or function that has been present for a minimum of three months, with implications for health.[1] Cats are particularly susceptible to CKD, with the reported disease prevalence ranging up to ~4% of the general cat population,[2] and between 15 and 80% of cats over 15 years of age.[3,4] Importantly, CKD is an important source of morbidity and mortality in domestic cats, with renal disorders being the leading cause of death for cats aged 5 years or older.[5]

The term CKD applies to heterogeneous disorders with varying causes, pathology, severity, and rate of progression.[6] Several primary kidney diseases affect cats (e.g., amyloidosis, polycystic kidney disease, lymphoma, pyelonephritis, and acute kidney injury of various causes); however, an etiologic diagnosis is not apparent in a majority of those affected by CKD.[7,8] Regardless of the inciting cause, the most common histopathologic renal lesion in cats with CKD is tubulointerstitial inflammation and fibrosis[8] – a finding that is strongly correlated to functional impairment.[9] Importantly, this pattern of histologic change is present even in the early CKD stages in cats.[10,11]

Traditionally, CKD is viewed as an inherently progressive and self-perpetuating disease in which adaptive changes in renal structure and function eventually become maladaptive.[12] Although the self-progressive nature of CKD is well established, disease progression is highly variable in cats, with a subset of individuals experiencing slow- to non-progressing disease, while others rapidly progress through the CKD stages.[13] Numerous factors have been implicated in the progression of CKD in cats.[12,14] Of these, single-nephron adaptations to loss of functioning renal mass, activation of the renin-angiotensin-aldosterone system (RAAS), systemic arterial hypertension (SAH), proteinuria, interstitial lipid accumulation, CKD-mineral and bone disorder, hypoxia, and oxidative stress will be reviewed below.

Single-nephron adaptations to loss of functioning renal mass

Work performed in models of CKD models, such as the remnant kidney model, demonstrates that reducing the number of functioning nephrons triggers a sequence of events that results in compensatory growth of the remaining, intact nephrons, as well as functional adaptations, which include increased renal blood flow and intracapillary glomerular pressure, with consequently increased single-nephron glomerular filtration rate (GFR), and net reabsorption of solutes and water.[15,16] These adaptations allow the kidneys to partially compensate for nephron loss by minimizing the global decrease in GFR; however, they eventually become maladaptive, leading to further nephron injury, and perpetuating a vicious cycle that ultimately results in progressive kidney disease.[17,18] Such single-nephron adaptations have been well characterized in cats, who develop increases in single-nephron GFR, glomerular capillary hypertrophy, mesangial matrix expansion, and renal proteinuria after partial renal ablation.[19]

The renin-angiotensin-aldosterone system

The RAAS is a master regulator of extracellular fluid volume, systemic arterial blood pressure (BP), and sodium and potassium balance. Despite its critical homeostatic role in health, chronic, sustained overactivation of this system contributes to accelerated renal damage in CKD.[20] The deleterious effects of RAAS activation in CKD include increased systemic and intraglomerular pressure, and consequent increase in glomerular filtration of plasma proteins; [20] reduced blood flow through the peritubular capillaries, promoting a hypoxic environment to the renal tubules;[21] increased oxidative stress via activation of NADPH oxidase;[21] and expression and/or transcription of growth factors and proliferative cytokines, including transforming growth factor-β1 (TGF-β1), thereby stimulating inflammation, fibrosis, and cellular growth.[21-24]

Our understanding of the RAAS has expanded substantially over the past few decades with the recognition of both a classical and an alternative, counterbalancing pathway of the circulating system,[25] and the existence of independently regulated tissular renin-angiotensin systems (RAS) in various organs, including the kidney.[26] Mixed evidence on activation of the RAAS has been presented by different studies of the classical circulating RAAS in experimentally induced[27,28] and naturally occurring CKD in cats.[29-31] However, the alternative circulating RAAS pathway and the intrarenal RAS, which is proposed to have a central role in the pathophysiology of kidney diseases,[32,33] have been less frequently studied. Importantly, data obtained from rodent models

reveal that regulation of the intrarenal RAS is independent of that of its circulating counterpart.[33] The available, small studies evaluating renal tissue angiotensin II immunoreactivity[34,35] or angiotensinogen mRNA levels in cats with CKD[36] support a role of the intrarenal RAS in CKD in this species; however, large-scale studies have not been performed.

Systemic arterial hypertension and renal proteinuria

Renal sodium and fluid retention, impaired baroreceptor sensitivity, sympathetic and RAAS overactivity, and endothelial dysfunction promote the development of SAH in individuals with CKD.[37] The relationship between SAH and CKD is complex and bidirectional, as SAH accelerates renal damage. Consequently, increased systemic BP is a well-documented risk factor for CKD progression in people[38] and dogs.[39] Proposed mechanisms for hypertensive renal damage include glomerular hyperfiltration and the development of renal proteinuria.[40] Proteinuria is both a marker and a mediator of renal damage. Proximal tubular cells can be activated by an increase in the concentration of proteins in the tubular fluid, which are thought to partially accumulate in the cytoplasm during the process of tubular reabsorption.[41] The activated proximal tubular cells synthesize proinflammatory mediators, especially monocyte chemoattractant molecules (e.g., the chemokine regulated on activation, normal T cell expressed and secreted [RANTES], monocyte chemoattractant protein-1, fractalkine, and complement component 3) and profibrotic factors (e.g., endothelin, angiotensin II, and TGF-β).[42,43]

While SAH is common in cats with CKD,[44,45] a direct link between BP elevation and worse outcomes has not been established in spontaneous CKD in this species, possibly because of the use of antihypertensive therapy in the hypertensive cats evaluated.[46,47] Importantly, hypertensive cats with CKD are more likely to be proteinuric than those who are normotensive,[46,47] and antihypertensive therapy with the calcium channel blocker amlodipine can reduce the magnitude of proteinuria[48] – a strong predictor of survival in cats with CKD.[46,49]

Treatment with the angiotensin-converting enzyme inhibitor benazepril decreased the magnitude of proteinuria but failed to provide a significant survival benefit to cats with CKD.[50] Whether treatment with angiotensin receptor blockers, such as telmisartan, which more completely blocks the RAAS in cats,[51] might extend survival in cats with proteinuric CKD is presently undetermined. Antihypertensive treatment with telmisartan has been studied in two large clinical trials in cats, which documented its antihypertensive efficacy but did not evaluate its effects on CKD progression or survival.[52,53]

In addition to RAAS antagonist therapy, current guidelines recommend feeding a “clinical renal diet” to cats with proteinuric CKD, regardless of disease stage.[54] Among other nutrient modifications, these diets are typically protein- and phosphate-restricted. Although feeding protein-restricted diets to cats with CKD is subject to debate,[55,56] feeding a renal diet was associated with improved survival in cats.[57,58]

Hypoxia and oxidative stress

Renal hypoxia is additionally proposed to contribute to CKD progression.[59] Several factors contribute to renal hypoxia in CKD, including decreased peritubular capillary blood flow due to an increase in vasoconstricting factors (such as angiotensin II), loss of capillary integrity, increase in oxygen demand from compensatory hyperfiltration and tubular hypertrophy, and increased oxygen diffusion distance between peritubular capillaries and tubular and interstitial cells due to accumulating extracellular matrix.[60,61] The increased oxygen consumption enhances the production of reactive oxygen species and oxidative stress, further stimulating inflammation and fibrosis.[60] It is now believed that the progression of many forms of CKD is caused, at least in part, by a vicious cycle of hypoxia, renal inflammation, and fibrosis, which ultimately leads to renal failure.[62,63]

The hypoxia-inducible factor (HIF) family plays a major role in the regulation of adaptive responses to renal hypoxia.[64] While the role of HIFs in CKD remains to be fully elucidated, increased renal HIF-1α expression is associated with tubulointerstitial injury in the tissues from human patients with CKD.[65] Similarly, transcription of HIF-1α gene was increased in renal tissue samples from cats with spontaneous CKD,[66] and was positively correlated with worsening pathologic changes in those from an ischemic model of CKD in cats.[67]

Anemia, a common consequence of CKD, can further exacerbate renal hypoxia and is a known modifiable risk factor for CKD progression in cats.[13]

Renal lipid accumulation

In people, obesity and hyperlipidemia are independent risk factors for CKD, and lipid accumulation in the renal parenchyma is thought to damage podocytes and tubular and interstitial cells through various mechanisms, including stimulation of the production of reactive oxygen species and lipid peroxidation, and promotion of mitochondrial damage and tissue inflammation.[68] Though a link between these and obesity has not been examined, renal tubular and interstitial lipid deposits are observed in healthy cats and those with experimentally induced and spontaneous CKD, with increasing frequency with advancing age.[69-71] It has been hypothesized that, following renal injury, rupture of tubular cells might release lipid into the interstitium and trigger chronic granulomatous inflammation.[7] In support of this, interstitial lipid deposits were associated with focal inflammation in recent studies in cats.[69,70]

Chronic kidney disease-mineral and bone disorder

Alterations in mineral and bone metabolism, collectively termed CKD–mineral and bone disorder (CKD-MBD), are prevalent in individuals with CKD and are associated with increased morbidity and mortality.[72] Renal phosphate retention that develops as a consequence of a global reduction in GFR is believed to initiate and drive many of the other disturbances in CKD-MBD.[73]

Hyperphosphatemia is a well-established risk factor for CKD progression in cats,[13] and feeding phosphate- and protein-restricted diets has been shown effective in reducing morbidity and mortality in azotemic CKD in this species.[57,58] Importantly, cats fed highly phosphate-restricted

diets are at risk for developing hypercalcemia,[74] and attenuation of phosphate restriction can alleviate hypercalcemia in these cats.[75] Moderate protein and phosphate restriction might be more appropriate for cats in the earlier CKD stages.

Conclusions

Chronic tubulointerstitial nephritis and fibrosis are the most common histologic features of CKD in cats. Numerous factors with synergistic relationships between them are likely implicated in the maintenance of a proinflammatory and profibrotic renal milieu that favors the progression of CKD in cats. As the pathophysiology of CKD progression is multifactorial, treatment aimed at delaying its progression should be multimodal. Nutritional and pharmaceutical strategies are available to address many of the progression factors presently known. Nonetheless, a subset of cats experiences worsening of the disease despite seemingly optimal management.

Conflicts of Interest

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Immune complex-mediated glomerular disease in cats and dogs

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Abstract

Immune complex-mediated glomerulonephritis (ICGN) refers to a pattern of disease in which antigen-antibody complexes are deposited in the glomeruli of the kidneys, inciting complement activation, inflammation, and kidney damage. While the immunological and pathophysiological mechanisms of ICGN are best understood in humans and rodent models, veterinary medicine has continued to advance its understanding of the underlying causes, pathophysiology, and diagnostic and clinical implications of ICGN in domestic species. This session will review our current understanding of the prevalence, causes, and pathogenesis of ICGN as it occurs in dogs and cats, the requirement for comprehensive renal biopsy for accurate diagnosis and the diagnostic features on biopsy, and possible promising non-invasive biomarkers of ICGN.

Glossary of abbreviations

ANCA Antineutrophil cytoplasmic autoantibody
EVRPS European Veterinary Renal Pathology Service
GN Glomerulonephritis
GBM Glomerular basement membrane
HE Hematoxylin and eosin
IC Immune complex(es)
ICGN Immune complex-mediated glomerulonephritis
IF Immunofluorescence
IVRPS International Veterinary Renal Pathology Service
JMS Jones Methanamine Silver
LM Light microscopy
MAC Membrane attack complex
MGN Membranous glomerulonephritis
miR MicroRNA
MPGN Membranoproliferative glomerulonephritis
MT Masson's Trichrome
PAS Periodic Acid Schiff
TEM Transmission electron microscopy

Introduction

Glomerular diseases are a significant cause of proteinuria, and accurate diagnosis of the specific form of glomerular disease is important for guidance of therapy. In human medicine, glomerular diseases are extensively classified based on disease pathogenesis, biomarkers, and comprehensive pathologic evaluation. In veterinary medicine, performance of comprehensive renal biopsy for diagnosis of glomerular disease has become more common in the last several decades, particularly

in dogs. In general, glomerular lesions in dogs cluster into 2 broad categories: those that are mediated by immune complexes (IC), termed immune complex-mediated glomerulonephritis (ICGN), and those are not (non– ICGN). The non-ICGN category includes amyloidosis, diseases of podocyte injury such as focal segmental glomerulosclerosis, and diseases of the glomerular basement membrane (GBM).[1]

While both ICGN and non-ICGN glomerular diseases cause proteinuria and management often involves various antiproteinuric therapies, immunosuppressive therapy is often administered in companion animals with definitively diagnosed ICGN. Therefore, accurate diagnosis of ICGN and non-ICGN glomerular diseases is necessary to avoid inappropriate and unnecessary use of immunosuppressive therapy. Accurate diagnosis of ICGN requires comprehensive renal biopsy, as evaluation by light microscopy (LM) alone results in frequent misclassification of glomerular diseases (e.g., ICGN misclassified as non-ICGN or non-ICGN misclassified as ICGN), risking incorrect medical management.[1]

Types and prevalence of ICGN in dogs and cats

Glomerulonephritis (GN) is a general term that refers to inflammation of the glomerulus. Four major categories of GN in people are mediated by immunoglobulin, complement, or both. These include ICGN, anti-glomerular basement membrane mediated GN, antineutrophil cytoplasmic autoantibody (ANCA) mediated GN, and complement factor 3 glomerulopathy mediated by complement dysregulation. Anti-GBM and ANCA mediated glomerulonephritis are not recognized in veterinary medicine. Furthermore, multiple subtypes of ICGN are recognized in people, including membranous glomerulonephritis (MGN), immune complex-mediated membranoproliferative GN (IC-MPGN), Lupus nephritis, immunoglobulin A (IgA) nephropathy, cryoglobulinemic GN, and GN with monoclonal immunoglobulin.[2,3] The most common subtypes of ICGN in dogs and cats are MGN and MPGN, while other forms described in people are exceedingly rare.[2] Finally, 2 forms of MGN are recognized in human medicine: primary MGN, due to autoantibody development against podocyte proteins (e.g., PLA2R), and secondary MGN, due to release of circulating antigens from an underlying disease. A causative podocyte antigen has not been identified in veterinary medicine; rather, MGN in companion animals is typically secondary to an underlying disease, such as an infection.[2]

The importance of comprehensive renal biopsy for definitive diagnosis of ICGN in dogs and cats has been increasingly recognized. Of 501 dogs from North America and 162 dogs from Europe and the United Kingdom, 48.1% and 50.6%, respectively, were diagnosed with ICGN via comprehensive renal biopsy for suspected glomerular disease.[4,5] A smaller proportion, 27%, of 62 dogs biopsied for glomerular disease in the UK alone were diagnosed with ICGN.[6] The lower proportion of ICGN in the UK alone may be related to differences in infectious disease prevalence. In all studies, differentiation between ICGN and non-ICGN glomerular diseases based on clinical and laboratory data alone was not possible due to frequent overlapping of clinicopathologic values.

Renal proteinuria due to glomerular diseases and performance of renal biopsy are less common in cats than dogs, but as with dogs, assumptions about glomerular disease diagnosis cannot be made based on clinicopathologic values alone. Of 58 feline renal biopsies and autopsies submitted to the International Veterinary Renal Pathology Service (IVRPS) for evaluation of renal proteinuria, 58% were diagnosed with ICGN.[7] MGN and MPGN make up the majority (>70%) of ICGN subtypes in cats, and similar to dogs, clinicopathologic values cannot accurately distinguish between ICGN and non-ICGN glomerular diseases.[7,8] Indeed, Rayhel *et al.* found that the median UPC of non-ICGN cats was greater than that of ICGN cats, while the opposite was found by Rossi et al.[7,8] There does appear to be a male predilection for MGN in cats,[7,8] and cats with ICGN were more frequently positive for retroviral infections than those with non-ICGN diseases.[8]

General pathogenesis of ICGN

Formation of IC deposits

Immune complexes, composed of antigen + antibody with or without complement, may originate as circulating ICs which are deposited within glomeruli, or they can form in situ.[9,10] Inciting antigens are rarely definitively identified but are often suspected to be secondary to infectious, inflammatory, or neoplastic diseases, or even drugs, and thorough workup is often undertaken to seek the inciting cause.[9,11] Lists of reported causes of ICGN in domestic animals can be found in other resources.[11-13] The deposition of circulating ICs in glomeruli likely occurs when antigen and antibody concentrations are in relative equivalence or when there is a slight excess of antigen. In situ formation may occur when an antigen penetrates the GBM and subsequently binds antibodies. This penetration of the GBM may be aided by inflammatory products, such as IgE-mediated histamine and serotonin from platelets and basophils, which increase GBM permeability.[9,11]

Once deposited within the GBM, ICs may be eliminated or become larger. Elimination occurs via solubilization, phagocytosis, or degradation. If the inciting antigen can be eliminated, ICs may be cleared, possibly resolving the GN and resulting proteinuria. Enlargement of ICs may occur if they combine with other antigens, antibodies, ICs, and complement components. This is particularly recognized in the presence of continued antigenemia which may occur with chronic microbial and parasitic infections.[11]

Mechanisms of glomerular damage

Once ICs have formed in situ or are deposited in glomeruli, there are several mechanisms that can cause glomerular injury. One of the best-established mechanisms of glomerular injury is mediated by the complement cascade. The presence of ICs activates complement fixation and formation of multiple complement components, including C3a, C5a, and C567.[11,14] C3a and C5a are anaphylatoxins that induce histamine release from mast cells and basophils, increasing vascular permeability. Increased permeability of glomerular capillaries allows further IC deposition to occur.[14] Further, these anaphylatoxins are chemotactic for neutrophils, particularly in highly inflammatory forms of ICGN, such as MPGN. Neutrophils function to ingest ICs, but in doing so, they release lysosomal enzymes, neutrophil proteinases, arachidonic acid metabolites, and oxygen derived free radicals, causing damage to the GBM. C567 assembles with C8 and C9

molecules, forming the terminal membrane attack complex (MAC) which forms a pore in plasma membranes, including tissue cell membranes. The MAC directly acts on and injures podocytes, endothelial cells, and mesangial cells by production of oxidants and proteases with subsequent cell detachment and protein leakage through the damaged GBM.[9,11,14]

Damaged cells within the glomeruli and tubulointerstitium release chemokines that further activate circulating leukocytes, which, once adhered to endothelial, mesangial, and interstitial cells undergo effector functions such as respiratory burst, phagocytosis of ICs, and release of hydrolases and removal of matrix, cell debris, and apoptotic cells. Further, these activated leukocytes release more chemokines and cytokines, thereby recruiting even more leukocytes, perpetuating inflammation and glomerular damage. Additionally, activation of coagulation can cause formation of fibrin thrombi and cause glomerular ischemia.[9,11,14]

The comprehensive renal biopsy

Definitive diagnosis of ICGN in companion animals requires a comprehensive renal biopsy, which involves light microscopy (LM) with special stains, immunofluorescence (IF) with a panel of markers, and transmission electron microscopy (TEM) of the glomerular capillary loops. The specifics of performing a renal biopsy and obtaining a diagnostic sample are beyond the scope of this session. Readers are directed to other literature on techniques, considerations, and contraindications for obtaining quality renal biopsies for evaluation of glomerular diseases.[15,16]

Light microscopy (LM)

While renal biopsy evaluation should include staining with hematoxylin and eosin (HE), HE is insufficient for comprehensive renal evaluation by itself. Additional required stains include Periodic Acid Schiff (PAS), Masson's Trichrome (MT), and Jones Methanamine Silver (JMS) which allow for evaluation of GBM thickening and remodeling, identification of hypercellularity and IC deposits, and presence of fibrosis. It is critical that tissues for these 4 stains (HE, PAS, MT, and JMS) are sectioned at 3 µm to enable identification of true hypercellularity and remodeling of the GBM. Additional special stains, such as Congo Red for confirmation of amyloidosis, may be needed as well.[17]

Immunofluorescence (IF)

IF allows identification of IC deposits in glomeruli and is particularly helpful for identifying early deposits before significant changes are seen on LM. Tissues for IF must be stored and transported to the diagnostic laboratory in chilled Michel's transport solution and processed within 5 days of renal biopsy. While other antibodies may be used, the IVRPS stains and evaluates canine samples with canine-specific IgA, IgG, and IgM, and C3 antibodies and feline-specific IgA, IgG, and IgG. Additionally, universal lambda light chain (LLC) cross reacts with both canine and feline samples.[17]

Transmission electron microscopy (LM)

TEM is used to identify electron dense IC deposits and their location in the glomeruli and to characterize other damage to the glomeruli. Similar to IF, TEM is especially useful in cases with

early IC deposits before significant changes are present on LM. TEM should ideally be performed on small (e.g., 1 to 2 mm^3) tissue sections that are either placed in 3% glutaraldehyde shortly after biopsy or on formalin fixed tissues that are postfixed in 3% glutaraldehyde.[15,17]

Diagnostic features of ICGN subtypes: MGN and MPGN

The most common subtypes, or patterns, of ICGN seen in dogs and cats are MGN and MPGN. In **MGN**, ICs form on the abluminal (subepithelial) side of the GBM and are composed of immunoglobulin (usually IgG) and antigen (***Figure 1***), although the inciting antigen is typically unknown. Complement components may also be present within ICs. As the IC deposits are located further away from circulation as compared with MPGN, inflammation is typically less severe in MGN. As such, on LM, glomeruli remain normocellular (i.e., hypercellularity is not a feature). In early MGN, there may be minimal, if any, lesions on LM and glomerular capillary loops, composed of fenestrated endothelium, GBM, and podocyte foot processes (***Figure 1***), may appear normal. This necessitates IF and TEM for diagnosis. In later stages of MGN, the GBM undergoes remodeling with formation of spikes on the abluminal surface and holes within a thickened capillary wall (seen on JMS stain), and subepithelial nodular deposits may be identified (MT stain).[3,18] IF and TEM are extremely helpful for identification of ICs, especially in early MGN when GBM remodeling may absent or minimal. A key feature of IC deposits on IF is granular ("lumpy-bumpy") staining along the capillary loops. On TEM, electron dense ICs are identified in subepithelial locations, and podocyte foot process effacement may be present.[18]

In **MPGN**, ICs form on the luminal (subendothelial) side of the GBM and sometimes within the mesangium (***Figure 1***) and are composed of immunoglobulin (usually IgG) and antigen. The source of antigen is often suspected to be exogenous and associated with an infectious process. Due to the proximity of the ICs to circulation and the immune system, complement activation elicits a strong inflammatory response, with recruitment of leukocytes. Endocapillary hypercellularity and thickening of glomerular capillaries by the ICs and GBM remodeling are key features on LM; periglomerular inflammation and fibrosis may be noted as well. Importantly, these features on LM identify a membranoproliferative *pattern* of glomerular damage but are not specific for IC-MPGN. Rather, other forms of glomerular injury can have a similar pattern on LM. Therefore, IF and TEM are needed to differentiate IC-MPGN. Positive IF staining reveals a granular ("lumpy-bumpy") pattern along capillary loops and in mesangial zones, and TEM reveals subendothelial and mesangial IC deposits.[3,18]

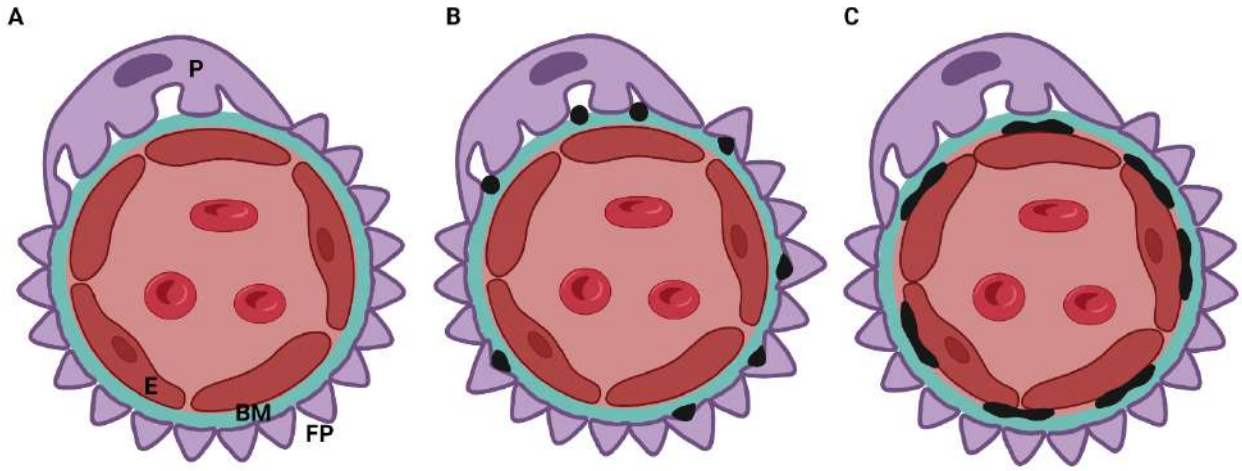

Figure 1. Normal glomerular capillary loop and immune complex deposit locations in membranous (MGN) and membranoproliferative (MPGN) glomerulonephritis.

A) The normal glomerular filtration barrier is composed of fenestrated glomerular capillary endothelium (E), the glomerular basement membrane (BM), and the podocyte (P) and their foot processes (FP).
B) In MGN, ICs (represented by black nodules) are located on the abluminal (subepithelial) side of the GBM.
C) In MPGN, ICs (represented by black globules) are located on the luminal (subendothelial) side of the GBM.

Biomarkers of ICGN

Initial clinical suspicion of ICGN in dogs and cats is typically based on the presence of one or more of the following: proteinuria, hypoalbuminemia, azotemia, hypertension, hypercholesterolemia, edema, or ascites, or a history of infection. As previously discussed, reliance on clinicopathologic values (e.g., magnitude of proteinuria by UPC) is inappropriate for accurate diagnosis of ICGN. Thus, comprehensive renal biopsy is still the gold standard for definitive diagnosis of ICGN in dogs and cats. However, due to risks of anesthesia and cost of renal biopsy, there is a need for minimally invasive biomarkers of ICGN in veterinary medicine.

Proteins lost into urine can be separated by gel electrophoresis (SDS-PAGE) into banding patterns that indicate primary glomerular damage, primary tubular damage, or a combination of both.[19] Early data (unpublished) has found that presence of multiple very large proteins (molecular weight >200 kDa) identified on SDS-PAGE from dogs had >90% specificity, but low sensitivity, for biopsy confirmed MPGN.

MicroRNAs (miRs) are small, noncoding RNAs that regulate gene expression and play roles in health and disease. Exploration of urine microRNAs in dogs with biopsy confirmed glomerular diseases found that miR-126 was highly sensitive (90%) and specific (92%) for ICGN compared with non-ICGN glomerular diseases.[20] Continued validation of these findings in a larger cohort of dogs and in those with non-renal diseases is underway at the IVRPS.

Conclusions

ICGN is due to deposition of ICs within glomeruli. In companion animals, ICGN is frequently considered to be secondary to an underlying disease, although a causative antigen is often not identified. MGN and MPGN are the most common subtypes in dogs and cats and are driven by complement mediated responses to the presence of ICs. Standard clinicopathologic findings are unreliable for diagnosis of ICGN, and a comprehensive renal biopsy processed and evaluated by an experienced pathologist is still the gold standard for definitive diagnosis of ICGN. Development and validation of minimally invasive biomarkers of ICGN in dogs and cats are underway. Promising biomarkers may include novel urine proteins and microRNAs.

Conflicts of Interest

The author is the director of the International Veterinary Renal Pathology Service (IVRPS).

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Inflammation in cats with lower urinary tract disease

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Abstract

Feline idiopathic (interstitial) cystitis (FIC) is a chronic painful condition that affects pet cats. Cats may have signs that include painful urination, bloody urine, and urinating outside their litter pan. While there are several animal models of IC, the naturally occurring model in cats has a similar clinical presentation and response to therapy as humans with painful bladder syndrome; FIC (and IC in humans) is confirmed by the absence of other conditions that could cause the lower urinary tract signs. Unfortunately, bladder-centric therapies have failed to result in long-term patient comfort. Clinical signs of FIC often wax and wane and are exacerbated by stressful events. Increased negative reactivity to daily threats is associated with increases in markers of inflammation. Inflammation is an important biological process by which the immune system defends the body from foreign organisms, but chronic, low-grade inflammation can promote the development of chronic primary painful conditions like FIC. Activation of this central threat response (CTRS) system plays a clinically important role in IC symptoms in humans, cats, and even rodent models. In both humans and cats with IC, a central sensitization theory (i.e., "top-down" approach) by the persistent perception of environmental threats, early adverse life experiences and/or a sensitized CTRS, leading to the activation of peripheral adrenocortical, autonomic, and immune systems, could result in systemic and local bladder abnormalities (e.g., inflammatory changes) and create a positive feedback loop that perpetuates the pain/inflammation of FIC. Multimodal Environmental Modification (MEMO),[1] is a tailored, patient specific plan that informs clients about the role of the CTRS in FIC and coaches them to create an environment that provides safety, predictability, and choice tailored to each cat's context. MEMO has become the standard of care in veterinary medicine for decreasing pain, the inflammatory response, and clinical signs in cats with this condition. Ideally, MEMO therapy should include items such as the addressing the number of litter pans, type of litter, addressing conflicts the cat perceives inside and outside of the household, and diet -allowing the cat to express its preferences for type and texture of food- as well as providing appropriate and safe feeding environments.

Abbreviations

BPS	Bladder pain syndrome
CTRA	Conserved transcriptional response to adversity
CTRS	Central threat response system
FIC	Feline idiopathic/interstitial cystitis
HPA	Hypothalamic-pituitary-adrenal
IC	Interstitial cystitis
LUTS	Lower urinary tract signs
SNS	Sympathetic nervous system
WAS	Water avoidance stress

Introduction

The prevalence of lower urinary tract signs (LUTS) in client-owned cats seen in small animal practice is approximately 1.5-4.5% of which ~55%-67%. are diagnosed with feline idiopathic/interstitial cystitis (FIC), making this the most common cause of LUTS for cats.[2] The clinical diagnosis of FIC is made based on the presence of waxing and waning LUTS, sterile urine, and the absence of other LUTS abnormalities on a CBC, serum biochemical panel, abdominal radiography, and/or ultrasonography.[3] Unfortunately, at the present time, there is no definitive test or biomarker to confirm the presence of FIC.[4] FIC is well documented naturally occurring model for bladder pain syndrome (BPS) in humans, with similar clinical presentations, bladder inflammation, and bladder histopathology lesions. Two subtypes of FIC (and BPS) have been reported, those associated with Hunner's ulcers (the "classic" or "ulcerative" form of FIC) and the non-Hunner ("non -ulcerative") form. The latter appears far more common in both cats and humans, resulting in the clinical signs of stranguria, pollakiuria, hematuria and dysuria that can become chronic and recurring in some cats.

Predisposing risk factors for cats with FIC include male sex,[5-7] neutered,[8] overweight [5,6,9,10] purebred,[5,10] middle-aged,[11] and having fearful or anxious behaviors.[6,12] Environmental risk factors include indoor confinement,[9,12-14] low litter-box-to-cat ratio,[7,9] and inadequate access to elevated resting areas or vantage points[6,8] Other risk factors include decreased activity and opportunities to engage in predatory behaviors,[9] and social conflict within and outside of the home.[5,6,8,9]

"Bottom up" Approach to FIC

Although the etiology of FIC is unknown, research on the bladder of cats with FIC (and humans with BPS) was the predominant area of investigation for decades. Biopsies are generally not necessary to confirm the presence of FIC in cats and lesions noted in most cat with FIC appear similar to humans with the non-ulcerative form of BPS. Histologic bladder lesions can include submucosal edema and hemorrhage, mild lymphoplasmacytic inflammation in the submucosa and, in severe cases, fibrosis.[2] Neutrophils are rarely noted, and degranulation of mast cells, which were noted in cats with FIC,[15] are thought to cause further inflammation. Although bladder mastocytosis is unlikely the cause of FIC, the increased sympathetic tone could contribute to bladder hypersensitivity, mast cell degranulation, further increasing bladder permeability and susceptibility to infection and inflammation.[16] The mucosal surface of the bladder should act effectively as a barrier to protect the urothelium from bladder content, but decreased urinary glycosominoglycans[17] among a number of other reasons can lead to increased urothelial permeability, a feature of FIC and IC. Abnormal urothelial permeability has been documented in cats with FIC,[18] resulting from and/or leading to the absorption of urine toxins (e.g., urea, potassium, and potentially an altered urinary microbiome) that results in increased nociceptive input to the central nervous system (i.e., "bottom- up" approach to the development of FIC).

The urothelium is comprised of three layers, the basal cell layer, which is attached to the basement member, the intermediate cell layers and the apical cell layer, which are large hexagonal cells also referred to as "umbrella" cells. As the bladder distends, these umbrella cells have apical

membrane proteins (e.g., uroplakins) that fuse with the surface and allow the umbrella cells to stretch and accommodate urine filling, while maintaining proper tight junctions between one another. In cats with FIC, these tight junctions are disrupted, there is a denuded epithelium, and in some cases, evidence of missing underlying intermediate cells layers.[19] When this permeability layer is disrupted, nociceptive c-fibers in the bladder wall are stimulated, leading to secondary inflammation, irritation and the clinical signs noted in FIC. Moreover, studies have also suggested that urothelial cells can function as primary transducers of some noxious stimuli through their close association with autonomic efferent and sensory bladder afferent nerves. Neuronal "sensor molecules" (e.g., purines, norepinephrine and acetylcholine) and urothelial mediators such as ATP, nitric oxide (NO), and various cytokines[20] can communicate with the bladder nerves, detrusor muscle, and inflammatory cells which exacerbate the inflammation. Activation of the SNS may increase epithelial permeability, permitting substances encountered in the environment greater contact with sensory afferent neurons; increased activation of sensory afferent neurons could subsequently lead to development of local inflammation.[21] Unfortunately, "bladder-centric" treatment aimed at restoring the abnormal urothelium, led to unsuccessful attempts to use pentosanpolysulfate, resiniferatoxin[22] and a number of other treatments to treat cats with both obstructive and non-obstructive FIC.[23-25]

"Top down" approach to FIC

Many risk factors for FIC have been well described and while certain predisposing risk factors cannot be controlled, such as genetics, sex, breed, and age, by obtaining a detailed environmental history, the clinician can help elucidate other environmental predisposing factors that could be altered to improve the cat's well-being. Environmental factors that can influence cats' perception of threat include the quality of human-cat relationships and interactions with others (including pets and humans within and outside of the household), cat's social environment and their perception of predictability and control of their surroundings.[26,27] Research has shown that perception of threat can activate the central threat response system (CTRS), leading to increased output of the sympathetic nervous system and altered response from the hypothalamic-pituitary-adrenal axis in cats with FIC.[18,28,29] These changes seemed to be driven by tonically increased hypothalamic corticotropin-releasing factor release[30] leading to increased sympathetic nervous system (SNS) activity and decreased hypothalamic-pituitary-adrenal (HPA) restraint, likely resulting from a developmental accident.[29] The changes are also associated with complex and variable abnormalities of the nervous, endocrine, and immune systems that affect more than just the urinary bladder of cats with FIC (ie, a "top-down" approach to FIC).[26,31,32] Some cats with FIC present with comorbidities besides chronic LUTS, including behavioral, endocrine, cardiovascular, and gastrointestinal problems,[33-35] and environmental stressors have been shown to result in increases in sickness behaviors (e.g., vomiting, lethargy, and inappetence) in these cats when other factors are controlled for.[32] Similarly, in humans with IC, only 25% of patients present solely with bladder/pelvic pain with many others having pain at 2 or more non-pelvic sites.[36]

Increased negative reactivity to daily stressors is associated with increases in markers of inflammation. Inflammation is an important biological process by which the immune system defends the body from foreign organisms, but chronic, low-grade inflammation can promote the development of chronic primary painful conditions like FIC.[37] In rodents, the water avoidance stress (WAS) is used as a model to evaluate the effects of chronic stress. Studies have reported increased bladder hypersensitivity and enhanced bladder nociceptive responses in the WAS-induced rodents. Histological changes in bladder tissue from these WAS-exposed rats included ulcerated areas, edema, vascular congestion, inflammatory cell infiltration, increased angiogenesis and mucosal mast cell numbers, and perhaps sensitized bladder C fibers.[38,39]

Although past research has revealed significant associations between negative reactivity and inflammation, less is known about the underlying biological mechanisms of these associations in painful conditions. Therefore, a detailed environmental history and complete physical examination, rather than restricting focus entirely on the urinary bladder, should be obtained from the owners of these cats to appreciate the complexity of some cases. This result led to the development of Multimodal Environmental Modification (MEMO).[1] The ideal MEMO approach is a tailored, patient specific plan that informs clients about the role of the CTRS in FIC and coaches them to create an environment that provides safety, predictability, and choice tailored to each cat's context. Through research and clinical trials, MEMO has become the standard of care in veterinary medicine for decreasing pain and clinical signs in cats with this condition.

MEMO therapy

Ideally, MEMO therapy should include items such as the number of litter pans, type of litter, diet (allowing the cat to express its preferences for type and texture), as well as appropriate and safe feeding environments, where the cats will not be startled by other animals, sudden movement, or activity of an air duct or appliance that may begin operation unexpectedly. Other forms of feeding enrichment can be incorporated such as puzzle feeders and incorporating ways for cats to express predatory behaviors. Lund, *et al.*[6] reported that frequent diet changes were found significantly more often in 70 cats with FIC in the final multivariate model of their matched, case-control study when compared to 95 control cats in Norway.[8]

A diet high in moisture (e.g., canned food) also may help prevent recurrences, but studies to evaluate this effect have not been conclusive to date. Although we have demonstrated that feeding wet food is not essential in enriched environments[1,32] it may be more beneficial in environments that are less enriched, although to our knowledge this has not been studied in a controlled trial. Cats may find wet food to be preferable due to the increased water content, whereas some cats seem to strongly to prefer dry foods. Obesity often is associated with FIC, so implementing a MEMO-based weight loss program also may be of benefit, if clinically indicated, but should be approached cautiously to avoid overwhelming the client with numerous recommendations at an initial visit. Diets containing additives that purportedly decrease anxiety (e.g., alpha-casozepine and L-tryptophan) in cats that are perceived to be “stressed” as well as those tailored for FIC management are marketed for cats, but the evidence for their effectiveness in management of FIC

have not been well investigated, and their general beneficial effects, if any, seem modest.[40,41] In most cases clients can choose whichever diet fits their and their cats' personal preferences, as long as the diet is balanced to meet all the nutritional needs of the cat and addresses concurrent sickness behaviors that might be present. This approach might help to minimize the effects of both client's and patient's perception of diet on the activation of their CTRS and subsequent inflammation.

Areas for investigation

Perception of threat can also lead to changes in gene transcription. Gene transcription responds to the needs of organisms' perception of their environment at any given moment, partly dependent on epigenetic modulation of gene expression.[42] Negative behavioral responses and increased inflammation are linked to the expression of genes that encode protein mediators of immune-related responses (e.g., inflammatory cytokines, antimicrobial molecules, etc.). Increases in these mediators are accompanied and preceded by increases in levels of gene expression that regulate inflammatory processes, such as the conserved transcriptional response to adversity (CTRA), which involves an up-regulation of genes involved in inflammation and a down-regulation of genes involved in type-1 interferon response and antibody production observed across distinct types of adversity in species ranging from fish to primates.[43] The CTRA is mediated via the sympathetic nervous system (beta-adrenergic signaling pathways) and could provide a genomic biomarker to help one understand the association between environmental stressors and inflammatory diseases.

Conclusions

Feline idiopathic/interstitial cystitis should no longer be considered an inflammatory disease of the bladder, but rather a chronic painful condition with bladder manifestations that are often associated with other sickness behaviors. The multidisciplinary approach to the study of chronic pelvic pain (MAPP) in humans also suggests that IC/PBS might not be a primary bladder disease, despite clinical urologic signs as the presenting complaint, and is often associated with other chronic debilitating comorbidities such as chronic fatigue syndrome, migraine, fibromyalgia among others. In both humans and cats with IC, a central sensitization theory (i.e., "top-down" approach) caused by the persistent perception of environmental stressors, early adverse life experiences and/or a sensitized CTRS, leading to the activation of peripheral adrenocortical, autonomic, and immune systems, could result in systemic and local bladder abnormalities (e.g., inflammatory changes) and create a positive feedback loop that perpetuates the pain/inflammation of FIC.

Conflicts of Interest

The author has no conflict of interests that pertain to the information presented in these proceedings.

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The Gut-Kidney Axis: CKD and constipation

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Glossary of abbreviations

BCFAs Branched-chain fatty acids
CKD Chronic kidney disease
GFR Glomerular filtration rate
IRIS International Renal Interest Society
SBAs Secondary bile acids
SCFAs Short-chain fatty acids
UDCA Ursodeoxycholic acid

Introduction

What is the effect of intestinal health on the kidney, and vice versa? In multiple species, a growing body of research supports the concept that there is significant connection between the gut and the kidney (aka Gut-Kidney Axis),[1] and that both systems have important influences upon the other with potential significant clinical implications. These interactions are particularly germane to the discussion of constipation. Constipation is a common clinical scenario in feline patients, and can lead to chronic frustration, poor quality of life and devastating outcomes with end stage disease. Constipation is defined as the infrequent or difficult evacuation of feces, whereas obstipation is intractable constipation. These conditions may progress to megacolon, an abnormal dilation of the colon which is typically associated with end-stage disease and permanent loss of colonic motility. Given the potential for progression and dire consequences, exploring pathophysiology and encouraging more proactive monitoring and patient assessments has the potential to improve outcomes.

Patient Assessment

Clinical history is very important in the assessment of constipation. Important questions include the frequency of defecation, time spent defecating, the degree of difficulty, straining, or vomiting associated with defecation as this information may not be volunteered by the caregiver. Repeated visits to the box or straining may be misinterpreted as straining to urinate or vice versa. Character of the stool should be discussed. Location and accessibility of litter boxes as well as box hygiene is also important information to capture. Fecal pellets left around the house may also indicate trouble with defecation. Importantly, decreased defecation frequency may be challenging to detect, and the caregiver may not notice an overt problem until a crisis occurs. Litter box monitor devices may provide a helpful assessment of defecation frequency. Cats with CKD with no history of overt constipation have been observed to have a significant decrease in defecation frequency when litter box monitoring devices are used to track litter box habits. Litter box monitors may provide the ability for early disease detection and intervention as well as monitoring response the therapy.

Hydration status is a crucial part of patient assessment and should be recorded in the medical record. Physical exam should include palpation of the colon and may reveal small hard feces in the descending

colon with a build-up of fecal material before the pelvic inlet, or, in more severe cases, a large amount of hard fecal material in the colon. Assessment for stifle, lumbosacral or hip osteoarthritis may also be helpful as significant arthritis may affect posturing make defecation more difficult. A rectal exam should be performed when possible to rule out abnormalities and the anal glands should be assessed to ensure they are not interfering with fecal evacuation.

Diagnostic labwork and imaging is recommended to assess the patient for underlying disease as constipation is almost always associated with an underlying condition or contributing factor such as CKD, osteoarthritis, gastrointestinal disease, endocrine disease, obesity, medications (e.g., anti-anxiety, phosphate binders, diuretics, opioids) and environmental stressors. Identifying and addressing underlying conditions is key to management. In a study assessing cats presenting to the emergency service for constipation, older, overweight cats and cats with CKD were found to have an increased risk of constipation.[2] Constipation is commonly associated with CKD and the etiology is likely a dysfunction of water balance. As the kidney fails to provide appropriate urine concentrating ability and the patient fights with chronic subclinical dehydration, water is reabsorbed from the colon to compensate. Additionally hypokalemia, the use of phosphate binders and an increase in uremic toxins may also contribute to constipation.[3-5] Diagnostic imaging including minimally abdominal radiography +/- abdominal ultrasound is key to assessing the fecal burden, the colon and the musculoskeletal structures.[6] The radiographic definition of megacolon is considered to be a colon that measures 1.5x the diameter of L5, in contrast normal patients would be expected to have a colonic diameter <1.3x the diameter of L5.[6]

Pathyphysiology

Gut Barrier Function

Gut barrier function is critical for maintaining the delicate balance between the GI tract and the body. The gut barrier consists of a single layer of epithelial cells, predominantly enterocytes interspersed with mucus-producing goblet cells and neuro-endocrine cells, joined by tight junctions and protected by a mucus bilayer.[7] Multiple factors can affect the health of the gut barrier. Aging results in a reduction in goblet cells and thinning of mucus layer.[8] Nutrition is key for gut barrier function and diets lacking in fiber are associated with decreased intestinal mucus and increased intestinal permeability and inflammation.[9] In CKD, increased intestinal permeability is evident, attributable to impaired tight junctions and an abnormal mucin layer, and increased translocation may contribute to systemic inflammation.[7,10]

Dysbiosis

A healthy bacterial microbiota and communication between host and bacterial metabolites is vital for the development and maintenance of a healthy immune system, assimilation of nutrients from the diet, maintenance of the gut barrier, nutrient synthesis, and protection against invading enteric pathogens.[11] Dysbiosis is defined as an imbalanced intestinal microbial community with alteration in the composition of the microbiota and their metabolic activities. In many diseases, dysbiosis is not just a marker of disease, but also actively contributes to pathology.[12] In people with CKD and rat models, intestinal dysbiosis has been extensively documented.[1] The uremia associated with CKD has been shown to negatively impact the gut microbiome in humans and rats causing intestinal dysbiosis, shifting the

intestinal microbiota from a more evenly distributed and complex community to one that is simpler and dominated by certain bacterial families.[12] Similar to humans, cats with CKD have been documented to have a fecal dysbiosis characterized by decreased fecal microbial diversity and richness based on 16S ribosomal rRNA gene sequencing.[13]

Uremic toxins

Uremic toxins are substances that build up in the blood stream as a result of decline in GFR. Of particularly interest are uremic toxins that are the waste products of protein catabolism (e.g., indoxyl sulfate, p-cresyl sulfate) as these are thought to not only have negative pathophysiologic effects, but also to contribute to the clinical syndrome of uremia. Uremic toxins that are the product of protein catabolism are produced in the colon via protein fermentation.[14-16] Indoles are produced by the metabolism of dietary tryptophan by tryptophanase in intestinal bacteria. P-cresol is generated via the partial breakdown of tyrosine and phenylalanine by many intestinal obligate or facultative anaerobes. Dysbiosis further contributes to the production of colonic-derived uremic toxins, initiating a vicious cycle.[15-17] Uremic toxins that accumulate as a results of dysbiosis and decreased renal elimination further induce oxidative stress and inflammation.[10]

Indoxyl sulfate is significantly elevated in feline CKD and is associated with disease progression.[18-20] Although p-cresyl sulfate concentrations did not significantly differ between healthy and CKD groups in one study, the highest concentrations were noted in CKD cats.[13] Interestingly even IRIS CKD Stage 2 cats have been documented to have uremic toxin concentrations that are significantly higher than control cats, implying this imbalance occurs relatively early in the disease process.

Constipation may exacerbate production of uremic toxins due to extended time for protein fermentation and absorption. Human patients with CKD and constipation (defined by an abnormal Bristol Stool Score) have higher concentrations of serum uremic toxins that patients with normal fecal scores, and in experimental studies in rodents uremic toxins have negative effects on gastrointestinal motility.[21,22] A rodent model of CKD demonstrated significant improvement in uremic toxins, creatinine and even kidney histopathology subsequent to a regimen of lactulose.[23]

Fecal fatty acids

Fecal fatty acids are another type of metabolite of colonic microbiota disrupted by intestinal dysbiosis. The short-chain fatty acids (SCFA) produced by the colonic microbiota consist of the straight-chain SCFAs acetic acid, propionic acid, butyric acid, valeric acid, and the branched-chain (BCFA) SCFAs isovaleric acid and isobutyric acid. Straight-chain SCFAs are major end-products of saccharolytic fermentation of fiber, and promote a healthy mucin layer and are essential nutrients vital for both intestinal and host-health.[24] They have several beneficial local and systemic effects including promotion of colonic motility, and anti-inflammatory properties.[25] In rodents, decreased SCFA are associated with constipation and delayed transit time.[26] In humans, dysbiosis in CKD is associated with a decrease in microbiota that produce SCFAs, specifically butyrate, which may contribute to constipation.[27] BCFAs are produced when protein passes through the small intestine unabsorbed and protein-derived BCFA are fermented by microbiota in the colon.[24] BCFAs and other products of protein fermentation in the colon are considered deleterious to the gut, and may serve as an instigator of inflammation as well as have negative effects on

motility.[24] Cats with CKD have not been demonstrated to have decreased fecal SCFA, but do have increased fecal isovaleric acid, in particular in IRIS CKD Stage 3&4 cats, in comparison to normal controls.[13]

Bile acids

Primary bile acids secreted by the host are transformed into secondary bile acids (SBAs) by gut microbiota. SBA play a role in gut motility and decreased SBAs are associated with constipation and delayed transit time.[26,28] Cats with CKD have decrease SBAs; specifically a decrease in UDCA is the most discriminating difference in comparison to normal controls.[29] In humans, treatment with a bile acid transporter inhibitor improved clinical signs of constipation, fecal score, and was associated with increased colonic SBAs (3.8 fold increase from baseline).[28] Our understanding of bile acid dysmetabolism is incomplete, but is an intriguing future direction.

Management

In order to choose the best management techniques for the patient, it is critical to have an understanding of the contributing disease factors at play, the chronicity of disease and the health of the colon.

Nutritional Management

Constipation associated with dehydration is most commonly seen as a manifestation of CKD but can occur with other disease processes. As the kidney becomes diseased, the ability to concentrate the urine is lost due to a reduced number of functioning nephrons. Hydration is not only important for promoting appropriate stool density but it is also vital for mucin layer which promotes motility. Decreased transit time is associated with drier and harder stools due to extended time for water reabsorption.[10] Therefore addressing hydration status is the key primary component to medical management. Feeding canned food instead of dry, adding water to food, changing viscosity of water (e.g., with a nutrient-enriched flavored water supplement) are other ways to potentially increased water consumption. Paying special attention to water sources in the house – fresh, accessible, water fountains etc., is also key. If possible, supplementation with free water (orally or with a feeding tube) is preferred to avoid the sodium load that comes with the electrolyte solutions available for subcutaneous use. However maintaining hydration by administering subcutaneous balanced electrolyte solutions appears to anecdotally improve appetite, activity and quality of life and reduce constipation in CKD patients, although no clinical trials have been performed.[30,31] Hydration should be assessed in all patients and corrected as needed in order to facilitate the efficacy of other treatments (e.g., dietary fiber and laxatives).

A combination of soluble (easily fermentable) and insoluble (poorly fermentable, bulking) is typically recommended for management of constipation, however few studies have been done in cats. Insoluble fiber adds bulk, draws water and stimulates colonic motility. Soluble, fermentable fibers are associated with production of SCFA by colonic bacteria, an important nutrient for intestinal epithelial cells.[32] However this is a generalization and not all soluble fibers are fermentable nor are all insoluble fibers unfermentable, and there is a range of fermentability for each fiber type. A psyllium-enriched diet was found to be helpful in management of feline constipation (not associated with CKD) in a previous open field trial and led to a decrease in the concurrent use of laxatives and promotility agents.[33] However, in CKD patients a renal diet would be the preferred therapeutic diet of choice, therefore fiber sources

(psyllium 1-4 tsp daily) are commonly added to canned food. The efficacy of these types of fiber on the management of constipation has not been assessed in patients with CKD. In general trial and error for types of diet and fiber that might benefit an individual patient is anticipated. Importantly, high amounts of insoluble fiber may be contraindicated in patients with advanced disease where colonic motility is compromised.[32] In these patients, a highly digestible strategy is more appropriate to decrease the volume of feces produced.

Medical Management

- Hypokalemia should be identified and addressed. Although the efficacy of potassium supplementation has not been evaluated in patients with constipation, potassium is necessary for both smooth and skeletal muscle function and hypokalemia has been identified as a risk factor in cats presenting for constipation.[2] A reasonable therapeutic goal is maintaining serum potassium >4 meq/L.
- Medical management of osteoarthritis may also be an important adjunctive treatment for constipation in elderly patients to facilitate appropriate posturing during defecation. Environmental management that includes accessible yet private litter boxes is key.
- *After* correction of hydration imbalance and hypokalemia, oral osmotic stool softeners are often a major part of management of constipation. Polyethylene glycol 3350 laxative is an osmotic that has been assessed in normal cats and found to be effective for softening stool.[34] It is commonly prescribed (1/8 – 1/4 tsp q 12-24 hrs titrated to effect) and is thought by some to be more effective than lactulose which is processed by intestinal microflora and may lead to bloating.[34] Another important consideration is that polyethylene glycol comes as a powder that appears to be well tolerated (and more likely to end up in the patient as opposed to all over it).
- Promotility agents are a common form of medical management for constipation, particularly for idiopathic presentations where colonic motility is questionable. The degree to which inherent colonic motility is affected has not been assessed in veterinary CKD patients but may be considered a second-tier option. Cisapride (2.5-5 mg/cat q 8 hr) is the most common promotility medication used. Although supported by feline-specific pharmacokinetics and a demonstrated positive effect on contractility in normal and abnormal feline colonic smooth muscle *in vitro*,[35,36] it has not been assessed in clinical trials.
- Probiotics may be a helpful adjunctive therapy. In one uncontrolled clinical trial assessing probiotic use in medically refractory idiopathic constipation, 7/10 cats had clinical improvement in fecal consistency.[37]
- Acupuncture may be a helpful adjunctive therapy.[38]

Conclusion

The gut-kidney axis and its clinical implications are a fascinating area of current research. Prompt identification of constipation is key to improving outcomes. Improved monitoring to allow earlier detection of disease and a better understanding of pathophysiology will help tailor management.

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Mechanisms of developing food allergy: Gut-skin crosstalk

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Abstract

The immune cellular players that promote TH2 polarisation, IgE production and mast cell degranulation in food allergic patients have been well described. However, the environmental factors that influence inappropriate immune reactions and atopic sensitisation via the gut and the skin are less well understood. One of the missing links are the microbes that inhabit body surfaces. In this lecture I will present some of our recent research that identifies specific microbes and microbial metabolic processes that mediate gut-skin interactions, which contribute to food allergen sensitisation.

Glossary of abbreviations

AD Atopic dermatitis
AhR Aryl hydrocarbon receptor
FXR Farnesoid X receptor
GPCRs G protein-coupled receptors
PRRs Pattern recognition receptors – PRRs
SCFAs Short chain fatty acids - SCFAs

Introduction

Epithelial and immune cells in the skin and gastrointestinal tract are actively involved in immune responses by producing and secreting cytokines. Alarmins or damage-associated molecular patterns including IL-33, IL-25, TSLP, and PGE2 are epithelial cell-derived cytokines that are central regulators of allergic responses.[1] Recently, the dual-allergen exposure hypothesis has been proposed, which suggests primary food allergen exposure through damaged and inflamed skin without prior gastrointestinal tract exposure can lead to Th2-type immune polarised responses and subsequent food allergen sensitisation. Consequently, food allergy development can be mechanistically linked to skin barrier dysfunction and potentially microbial colonization.[2] Both the gut and the skin are colonized by a dynamic ecosystem consisting of a diverse milieu of bacteria, fungi, viruses, bacteriophages and archaeal communities, that dictate the local environmental and nutrient conditions.[3] Components of each of these ecosystems are highly interactive and function together as a sophisticated holobiont. Skin commensal bacteria are involved in barrier maintenance, maturation of T cells, and activation of antimicrobial peptide production by keratinocytes.[4] Dysbiosis in the skin microbiome, such as increased *Staphylococcus aureus* abundance, is positively correlated with atopic dermatitis (AD) severity, and systemic sensitisation to food and alleroallergens.

Environmental exposures and immune development

Multiple studies have shown that human immune traits are significantly influenced by environmental factors. Some of the most important exposures in early life that determine functional programming of the infant immune system are associated with rural-specific and urban-specific factors. Rural or traditional farming lifestyles have been shown to modify innate (e.g., pattern recognition receptors – PRRs) and adaptive immune responses in children.[5] Differences in exposure to microbes, exposure to animals, dietary habits, use of cooking or heating methods that generate pollutants and socioeconomic status have all been shown to influence the development of the early life immune system.

We have previously shown that AD in South African children was associated with a distinct pattern of circulating cytokines (TARC, MCP-4 and IL-16) and elevated levels of specific IgE to food allergens and house dust mite.[6] However, the most significant effects on circulating serum cytokine levels were due to rural and urban exposures in this South African cohort. RNA-Seq analysis of PBMCs from this South African children's cohort revealed highly significant changes in immune cell gene expression profiles that were heavily dependent on the rural versus urban environment of the child.[7] AD was also associated with distinct changes in circulating immune cell gene expression, but far less than the changes induced by environmental exposures. Multiple pathways that are well described to regulate or suppress aberrant inflammatory immune responses were more highly expressed in rural children. IL-10 gene expression was highly upregulated in rural children's PBMCs, and IL-10 potently limits effector functions of antigen presenting cells and lymphocytes. Gene expression of additional IL-10 family members (IL-20R and IL-22) were similarly elevated. Inhibitory leukocyte immunoglobulin like receptors were increased in rural children (LILRB1 to LILRB4). These receptors are expressed on immune cells where they bind to MHC class I molecules on antigen-presenting cells and transduce a negative signal that inhibits stimulation of an immune response, thereby modulating cell activation thresholds and maintaining immune tolerance. G protein-coupled receptors (GPCRs) are involved in a wide array of physiological functions including important roles in regulating immune responses, attenuating inflammation, and promoting return to homeostasis. A surprisingly large number of GPCRs were differentially expressed in PBMCs from rural and urban children. The GPCRs with immune regulatory functions that were elevated in PBMCs from rural children include GPR132 (inhibits autoimmune responses), GPR183 (protective role in SLE and important for germinal centre reactions), GPR55 (negative regulator of γδ T cell migration), GPR17 (negative regulator of inflammatory cell recruitment and modulates TH2/TH17 cytokine expression), GPR84 (regulates TH2 effector cell function), GPR35 and GPR135 (activated by tryptophan metabolites), GPR31 (essential in the induction of oral tolerance by maintaining IL-10 producing intestinal RORγt+ Foxp3+ Treg cells), GPR171 (suppressor effects on T cell mediated effector responses), GPR15 (regulates preferential homing of Foxp3+ Treg cells to the large intestine), and GPR65 (regulates immune cell migration and maintains epithelial barrier homeostasis).

Environmental exposures and microbiota development

Epidemiological factors linking allergy risk with living environment, lifestyle and social interactions are also known to impact early-life gut microbiota assembly. During and following birth, infants acquire microbes from their mother (vertical transmission, e.g. bifidobacteria), and later, especially after the first 6 months, from the environment or from nearby non-parent hosts (horizontal transmission, e.g. clostridia). The complexity of the infant microbiome develops and matures during the first years of life and is supported by breastfeeding and the introduction of diverse complementary and solid foods.[8] In a study of Irish infants born during pandemic-enforced social restriction measures, multiple environmental factors were shown to influence microbiota development (CORAL study).[9] We examined correlations with a microbial exposure index generated by combining answers to questionnaire data relating to potential sources of microbial exposures (i.e. having a family member classified as an essential worker who was allowed to work outside the home, number of siblings, number of adults in the household, attending daycare outside of home, and having pets). The relative abundance of Clostridia was associated with the exposure index at 6 months of age after adjusting for birth mode and breastfeeding. At the genus level, all Clostridial genera showed a positive trend with the exposure index, which was statistically significant in 27/39 (69%) of the genera. In contrast, the relative abundance of *Bifidobacterium* was significantly negatively associated with the exposure index. At 6 months, *Bifidobacterium* was the only genus consistently and strongly negatively associated with health outcomes in terms of allergic phenotypes (food allergen sensitization and AD).

Following acquisition of potential colonizers, dietary habits strongly influence the growth and metabolism of microbial species in the gut. The cessation of breastfeeding leads to a rapid expansion in microbiota diversity and a shift to taxa that can utilize dietary fibers, accompanied by changes in production of immunomodulatory microbial metabolites. Plant based dietary sources (including legumes such as peanut and soya, tree nuts and sesame seeds) had a significant effect on microbiota composition at 12 months. Of note, the early introduction of potentially allergenic plants foods such as peanut and tree nuts protect against food allergy development. Our study suggests that these foods are utilized by the early life microbiota, which may play a role in their allergy protective effects complementing mechanisms associated with direct sampling by the immune system. Plant-based foods in general had similar effects as breastfeeding on microbiota composition, potentially due to microbiota-supporting fibers. The effect sizes of dietary components were greater in CORAL than that expected from previous studies, perhaps due to the reduced overall complexity of exposures contributing to the CORAL infant's microbiota, thereby allowing more accurate identification of dietary effects on specific taxa.

Microbial immunoregulatory metabolites

Metabolic and immune health are intimately connected via diet and microbiota. Nearly 90% of all immune cells in the body are associated with the gastrointestinal tract and these immune cells are continuously exposed to a wide range of microbes and microbial-derived compounds, with

important systemic ramifications.[10] Microbial-derived factors are integral components of the molecular circuitry that regulate immune and metabolic functions required for host physiology and survival. These host effects are partially induced by activation of host pattern recognition receptors to microbial-derived danger signals, but increasingly the role of secreted bacterial metabolites in shaping host immune function is being recognized. Immunoregulatory bacterial metabolites can trigger host GPCRs, aryl hydrocarbon receptor (AhR), nuclear hormone receptors such as the farnesoid X receptor (FXR) or can directly modulate gene expression through epigenetic mechanisms. Importantly, many immunoregulatory bacterial metabolites are derived from their metabolism of dietary ingredients (e.g. fiber, tryptophan), linking diet and lifestyle to protection from immune-mediated disorders via microbial mechanisms.[11] Microbial fermentation of dietary components in vivo potentially generates thousands of molecules, some of which regulate immune and metabolic functions. These in turn are thought to protect against aberrant inflammatory processes or hypersensitive responses, but also promote effector immune responses that efficiently eliminate pathogens, such as SARS-CoV-2.[12] One the of the microbial metabolites that are well known to exert immunoregulatory effects are short chain fatty acids (SCFAs). SCFAs are volatile fatty acids produced by the gut microbiota through fermentation of food components and refers to those with up to five to six carbons in straight or branched-chain formation. SCFAs have anti-inflammatory properties and influence the immune cells through various pathways via GPCRs and histone deacetylase inhibition.[13] We previously demonstrated that children with the highest levels of butyrate and propionate (≥95th percentile) in feces at the age of one year had significantly less atopic sensitization and were less likely to have asthma between 3 and 6 years. Children with the highest levels of butyrate were also less likely to have a reported diagnosis of food allergy or allergic rhinitis. Oral administration of SCFAs to mice significantly reduced the severity of allergic airway inflammation.[14] While individual microbes, individual dietary components and individual metabolites are certainly important, the overall community functional capacity and community metabolic outputs that underpin interactions with the host immune system are perhaps more relevant with regard to understanding disease risk. A low-risk microbe-diet configuration may generate sufficient levels of several regulatory metabolites that are associated with protection from aberrant inflammatory responses or sensitization to non-pathogenic epitopes such as those found in foods. In contrast, a high-risk microbe-diet configuration may consistently generate multiple pro-inflammatory metabolites that may contribute to a higher risk of inappropriate immune reactivity.

Conclusions

The interplay between the environment and barrier dysfunction drives Th2-type allergic responses. Nutritional, environmental, and lifestyle factors may underpin immune dysfunction and epithelial barrier disruption, potentially mediated via microbial metabolism. Targeting diet-microbial interactions may provide novel strategies to prevent or treat food allergen sensitization and food allergy.

Conflicts of Interest

L. O'Mahony reports consultancy with PrecisionBiotics, research grants from GlaxoSmithKline, Chiesi and Fonterra, and participation in speaker bureau for Nestle, Yakult, Reckitt and Abbott.

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Canine food allergy: pathogenesis and offending allergens

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Abstract

Food allergy is a relatively common skin disease counting for up to 20% of all allergic skin diseases in dogs. Clinically, it is associated with cutaneous and non-cutaneous signs with up to 40% of affected dogs demonstrating signs less than one year of age. The pathogenesis of canine food allergy is not completely understood. However, a complex systemic and gastroenteric immunological dysregulation is present in affected animals. This dysregulation is multifactorial, and it involves a decrease in local as well as systemic tolerance. The type of immune reaction involved (type I, III, and IV) is a significant contributor to this dysregulation. Structures and cross-reactivity among allergens are additional and relevant initiating factors to consider. For this reason, the physical structure, as defined by biochemical composition, is essential for determining the allergenic immunogenicity of a given protein(s). All such factors need to be kept in mind when selecting diets and re-exposing time in order to appropriately diagnose food allergy in dogs.

Glossary of abbreviations

GALT Gastrointestinal-associated lymphoid tissue
ILC3 Innate lymphoid cells type 4
SCFA Short-chain fatty acid
Th T helper

Introduction

In both dogs and humans, adverse food reactions encompass immunological and non-immunological disorders. The latter are more commonly referred to as food intolerances. Such types of intolerance may include food idiosyncrasy (e.g., anaphylactoid reaction to spoiled tuna), food poisoning/intoxication (e.g., bacteria and/or fungal toxins) and pharmacological and metabolic reactions to food[1] (e.g., reactions to chocolate or lactose). As to the former, food allergy (or food hypersensitivity) represents the main category of the immunological disorders associated with adverse food reactions.[1] In people food allergies are classified as IgE or non-IgE mediated, while in dogs this clear distinction is missing. In fact, although a type I hypersensitivity (IgE mediated) is most commonly seen in dogs, a delayed hypersensitivity reaction (not IgE mediated) is also observed. In addition, although less commonly reported, the involvement of a type III hypersensitivity reaction has been hypothesized in dogs.[2]

Food allergies are relatively common in people and, for some aspects, in dogs as well. In people, IgE mediated food protein allergy is estimated to affect up to 7.6% of children and 10.8% of adults in the USA alone.[3] Whereas IgE mediated allergic response to food-carbohydrate galactose-α-1,3-galactose (alpha-gal) in mammalian meat affects up to 96,000 to 450,000 individuals in the USA.[3] According to Food Allergy Research & Education (https://www.foodallergy.org/resources/facts-

and-statistics), over 33 million of Americans (1 in 10 adults and 1 in 13 children) have food allergies. More than half of adults (51%), and almost half (42%), of children with food allergies have experienced some form of severe reaction. In dogs, there are not many epidemiological studies focusing on the true incidence of food allergy. However, based on a critical appraisal review, the incidence of canine food allergy may be as high as 20% of allergic dogs.[4] A true breed predisposition has not been identified, although German Shepherd dogs, Retrievers and West Highland White terriers seem overrepresented.[5] The age of onset is variable, however up to 40% of the affected dogs start having clinical signs before the first year of age.[5]

Canine food allergy can manifest with a variety of clinical signs. These may include dermatological, gastrointestinal and respiratory symptoms. Of these, cutaneous are the most commonly reported[5,6] in dogs. These dermatological signs are indistinguishable from those of canine atopic dermatitis. This ambiguity in clinical manifestation(s) is an indication for food allergy to be one of the most important differentials for consideration when ruling out or ruling in atopic dermatitis in the canine species.[7]

Pathogenesis of canine food allergy

The pathogenesis of food allergy is extremely complex and not completely understood with most of the current knowledge coming from murine or human studies. Nevertheless, independently from the affected species, food allergy is characterized by an abnormal immune response associated with a reduced tolerance to innocuous allergens. The intestinal immune system plays a pivotal role in the maintenance of a properly functioning digestive system. This immune system is composed of several elements including epithelial and lymphocytic cells in constant interaction with a very diverse and abundant microbiota.[8] A disruption of this intestinal homeostasis has been associated with the etiology of intestinal disorders. In this context, dietary compounds significantly contribute to either the healthy maintenance or dysregulation of the intestinal immune system.[8] Food components such as vitamin A and D as well as AhR ligands have a direct modulating effect on the local immune response toward a more tolerant state. This effect is driven by the formation of retinoic acid and the stimulation of T regulatory cells and innate lymphoid cells type 3 (ILC3). The presence of fiber in the diet stimulates the production of short-chain fatty acids (SCFA) metabolites by the local microbiota.[8] In addition to producing SCFA metabolites, the local microbiota is essential in the direct activation of T and B regulatory cells as well as ILC3 and dendritic cells. This activation locally stimulates both innate and adaptive tolerance.[9] Oral tolerance is also directly related to the quantity of antigen directly interacting with the gastrointestinal-associated lymphoid tissue (GALT).[10] Indeed, a low dose of antigen is able to induce an active suppression through the activation of T helper (Th) type 2 and 3 secreting regulatory cells increasing the local concentration of suppressing cytokines like interleukin 10 and transforming growth factor β.[10] Similarly, a high dose of antigen may determine clonal deletion or anergy of the Th1 and Th2 cells.[10] Under these circumstances, it is clear how oral tolerance is the result of a cascade of events involving the host's local immune system (e.g., physical barrier and

GALT), environmental factors (e.g., breastfeeding and intestinal microbiota), and immune regulation (e.g., activation of Treg cells, dendritic cells and ILC3 cells).[11]

To the contrary, with food allergy such mechanisms of tolerance are failing. In particular, the epithelial intestinal barrier in allergic individuals is leakier that than of healthy.[12,13] This decrease in barrier integrity allows the penetration of a large number of allergens. While this increased uptake of allergens (generally proteins) is able to directly degranulate local mast cells through the post-sensitization activation of IgE on their surface, allergens can also stimulate the release of pro-inflammatory mediators through the stimulation of Th2 and ILC2 cells.[12-14] In addition to a defective barrier, the presence of an intestinal dysbiosis is also associated with food allergy due to an increased differentiation of Th2 cells promoting an IgE class-switching in activated B cells.[12]

Based on the information provided earlier, it is clear that local epithelial disruption, decreased tolerance, and gastrointestinal dysbiosis have a significant impact on the nature of immunological response mounted by the host. In humans, it is very clear how some food allergy manifestations may be predominantly characterized by a type I reaction (IgE-mediated reaction) or non-type I reactions (non-IgE-mediated or mixed IgE/non-IgE-mediated).[15] Clinically, such reactions are associated with anaphylaxis (IgE-mediated) or as eosinophilic disease (mixed IgE/non-IgE-mediated) or as celiac disease and food-dependent exercise-induced anaphylaxis (non-IgE-mediated).[15] Unfortunately, in dogs this distinction is not completely clear.[16] Clinically, most food allergic dogs are very similar to atopic dogs making these two diseases indistinguishable. Said that, some allergic dogs may present with a more classically defined type I reaction (e.g., urticaria and angioedema). Very recently two prospective studies[17,18] have aimed to identify the time needed for food allergic dogs to relapse post allergen re-exposure. The results of these studies suggested that a type I hypersensitivity reaction may be the most prominent immune reaction with the majority of patients reviewed showing symptoms within 1-3 days. They also highlighted the importance of allergenic load in triggering an allergic response post re-exposure (50% of previous diet).[18]

Allergens in canine food allergy

It is well-known that allergies occur when the host's immune system overreacts to certain substances (allergens) that are innocuous to most individuals. According to the Food and Drug Administration and the Food Allergy Research & Education, a total of nine major food allergens have been identified in people. These include milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, soybeans, and sesame. In children, peanut allergy has been reported to be the most frequent with a percentage equal to 25.2% of allergic children.[19] Peanut allergy was followed by milk (21.1%), shellfish (17.2%), and tree nuts (13.1%), with eggs, fish, wheat and soy affecting altogether up to 25.6% of allergic children.[19] On the other hand, based on two reviews, in dogs the most prevalent food allergens include beef (34-36%), dairy products (17-28%), wheat (13-15%), chicken (9.6-15%), and eggs 4-10%).[1,20]

Proteins are the most common source of allergens in both dogs and humans. To act as an allergen, a protein (or glycoprotein) requires specific characteristics. These include, a small size (molecular

weight between 10 and 70 kDa), thermoresistant, hydrophilic, resistant to acid degradation and resistant to proteases.[21] The association with lipids may also play a role in determining the allergenicity of a (glyco)protein. In fact, lipids can delay degradation, induce conformational changes, and increase sensitization to the allergens.[22] Finally, processing of food can also alter the allergenicity of a (glyco)protein. For example, the Maillard reaction can cause conformational changes resulting in blockage of existing epitopes, formation of new epitopes, and even enhancing the accessibility of epitopes.[23] In addition, the Maillard reaction produces specific products that are able to induce activation of pro-inflammatory and allergy-inducing Th2 and Th17 subsets.

The physicochemical characteristics of allergens play a role in cross-reactivity amongst them. Allergen cross-reactivity is one of the predominant pitfalls in the identification of a novel protein source in hypoallergenic (elimination) diets. This is such an impactful problem that in the past few years several studies have been published on possible cross-reactivity among otherwise unrelated allergens.[24-28] Such studies highlighted how different foods may contain similar proteins able to induce, at least *in vitro*, a strong cross-reactivity in dogs. In particular, they confirmed that a stronger cross-reactivity is present in phylogenetically related allergens alerting clinicians to the presence of panallergens (e.g., albumin). Panallergens are minor allergens defined as "homologous molecules that originate from a multitude of organisms and cause IgE cross-reactivity between evolutionary unrelated species."[29]

Conclusions

Although food allergies are relatively common in both humans and dogs, very little is currently known about the pathogenetic mechanisms associated with them. The clinical presentation can be highly variable, potentially because of the diverse immunological responses in individual patients. Alteration of both the innate and adaptive tolerance along with intestinal dysbiosis have been identified in food allergic patients. While relatively common in dogs, the lack of diagnostic tests available still leads clinicians to diagnose food allergies through the process of strict elimination diets. Due to this, it is essential to be able to identify the physiochemical profile of common allergens to be able to recognize possible cross-reactivity among different types of allergens. It is important to note that cross-reactivity may occur even if allergens are phylogenetically very distant from each other. Physiochemical similarities between allergens may bring to clinicians' attention the possible necessity of performing multiple strict elimination diet trials to reach a correct diagnosis of food allergy in dogs. This awareness will help clinicians weigh the pros and cons of using novel protein diets versus extensively hydrolyzed or amino acid-based diets before initiating the elimination diet.

Conflicts of Interest

The author declares no conflicts of interest.

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Gut barrier function and immune tolerance in healthy and allergic dogs

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Abbreviations

AHDS Acute hemorrhagic diarrhea syndrome
CPV Canine parvovirus
IBD Inflammatory bowel disease
PEG Polyethylene glycols
PI-IBS Post-infectious irritable bowel syndrome
SIC Serum iohexol concentration

Introduction

Food-responsive enteropathy represents the most frequent cause of chronic diarrhea in dogs.[1] Similar to findings in humans, there is also evidence from four studies in dogs that acute intestinal diseases associated with barrier dysfunction and dysbiosis can result in loss of oral tolerance and chronic intestinal signs.[2,3] This lecture aims to elucidate the mechanisms that initiate chronic disorders and enhance comprehension of the defensive mechanisms inherent in the gut barrier.

Mucosal Immunology and Inflammation

At mucosal sites, the “outside world” is typically separated from the inner world by a single layer of epithelium.[4,5] The mucosal immune system is present in various locations, such as the intestinal tract, respiratory tract (particularly the upper respiratory tract) and urogenital tract.[3] Each of these sites has developed its own specific set of cell populations, whereas the mucosa of the gastrointestinal tract serves as an immune organ housing the largest concentration of lymphoid and myeloid cells within the body. This high number of immune cells in the mucosa is unique for the gastrointestinal tract and referred to as "**physiologic inflammation.**" The reason for this, and the difference from other mucosal sites, is that the intestine harbors a vast population of microorganisms, primarily bacteria, ranging from billions to trillions.[6] These microorganisms, along with their byproducts and ingested food, constitute a **substantial antigenic burden** that must be managed by the mucosal immune system. The unique conditions of this environment and the corresponding challenges have led to the formation of a distinctive immune system, comprising inductive lymphoid follicles known as gut-associated lymphoid tissue (GALT), as well as effector cells dispersed within the epithelium (intra-epithelial lymphocytes, IELs) and in the lamina propria (LP) as mononuclear cells (LPMCs).[3] In preserving mucosal homeostasis within the intestinal mucosa, a crucial role of the immune system is the discrimination between potentially harmful antigens, such as pathogenic bacteria and beneficial molecules derived from food or commensal bacteria. Unlike the systemic immune system, which reacts rapidly within seconds to foreign antigens, the mucosal immune system is poised to respond but predominantly maintains a state of tolerance. The immune system plays a crucial role in controlling the body's reaction to a diverse

range of antigens introduced through the oral route. A reduction in intestinal barrier function leads to an increased allergen availability within the systemic circulation, which has the potential to trigger detrimental immune responses. Thus, **disruption of oral tolerance** can lead to food allergies and chronic inflammatory enteropathies.[1,3,4]

Intestinal Barrier Function

The intestinal barrier serves as the boundary between the external environment and the internal surroundings. An effective intestinal barrier facilitates the uptake of nutrients and fluids while concurrently obstructing the passage of detrimental elements such as toxins and bacteria from traversing the intestinal epithelium into the underlying tissue.[4,5]

The intestinal barrier is mainly composed of a **physical barrier** (mucus layer, epithelial layer), and the underlying **lamina propria** with its immune cells. The **commensal bacteria** present in the lumen of the intestinal tract already play a crucial role in barrier function by inhibiting the colonization of pathogens through competitive inhibition.[6] Certain specific bacterial species additionally possess the capability to ferment complex carbohydrates in the colon, resulting in the production of short-chain fatty acids. Especially the short-chain fatty acid butyrate has the potential to enhance the production of mucus and exhibits other potential functions in improving the intestinal barrier.

The intestinal tract is lined by a **mucus coat**, which is produced by goblet cells.[5] This mucus coat is composed of a mixture of glycoproteins known as mucins. Mucus serves to protect the intestinal epithelium through several mechanisms such as its stickiness/adhesive properties, its ability to bind bacteria/reducing the penetration of microorganisms and its flow actively moving luminal contents away from the surface of epithelial cells, preventing direct contact with various antigens.

The mucus lining in the intestinal tract comprises two distinct layers:

Loose Outer Layer: This outer layer is characterized by its loose structure and contains bacteria. It provides a habitat for commensal microorganisms and helps maintain a symbiotic relationship between the host and beneficial bacteria.

Dense Inner Layer: In contrast, the inner layer is denser and is devoid of bacteria. This layer serves as a more protective barrier, shielding the underlying epithelial cells from direct contact with microorganisms and luminal contents, thus contributing to the defense of the intestinal epithelium.

The mucous layer comprises IgA, a highly prevalent antibody in mucosal secretions. Through various mechanisms, IgA works to neutralize pathogenic bacteria, promoting the preservation of the commensal flora.[3]

The next element of the intestinal barrier is the epithelium, consisting of a single layer of cells. This layer encompasses various cell types including enterocytes, Paneth cells, and goblet cells, each contributing distinct functions that collectively establish a tight barrier against the intestinal lumen. Aside from their role as a physical barrier, intestinal epithelial cells (IECs) communicate with immune cells and thus actively participate in both innate and adaptive immune responses within the gut lining, crucially contributing to the maintenance of intestinal equilibrium. Tight junctions

(e. g. claudins, zonal occludens) effectively seal the intercellular spaces between epithelial cells and dynamically regulate solute transport.[4,5]

Beneath the intestinal epithelium lies the lamina propria, housing a variety of innate and adaptive immune cells including neutrophils, T-regulatory cells, macrophages, and mast cells. These cells swiftly respond to foreign invaders, serving a crucial dual purpose: defending the host and maintaining mucosal balance by regulating inflammation.

Assessment of the Intestinal Barrier Function

There exists a range of methodologies employed to evaluate intestinal barrier function in vivo (e. g., multi sugar absorption test, intestinal biomarkers), ex vivo (e. g., Ussing Chamber) and in vitro (e. g., epithelial cell culture, organoids), each offering unique benefits and limitations.[5]

The primary techniques utilized for evaluating the integrity of the intestinal barrier function involved solutes ingested orally and subsequently detected in urine samples. The ratio between large pore markers (e. g., ^{51}Cr-EDTA; disaccharides such as lactulose; polyethylene glycols (PEG) with a molecular weight of approximately 1000 Da) and small pore markers (e. g. PEG 400 Da; monosaccharides (mannitol and rhamnose)) are used to assess permeability of the intestine.[4,5]

In addition, several blood biomarkers serve as indicators of compromised barrier function. Examples include lipopolysaccharide (LPS), zonulin and intestinal fatty acid-binding protein (I-FABP).[4,5] However, these biomarkers have not been critically assessed in dogs with intestinal disorders and caution should be taken when using commercially available assays (e. g., zonulin), which are not validated for dogs. Improved assessments of intestinal permeability have been achieved through the utilization of iohexol, a radiographic contrast medium, administered orally and subsequently measured in the serum. An optimal oral iohexol dosage for an intestinal permeability serum test in dogs has been defined and reference values established. Measurement of serum iohexol concentration (SIC) has been recently utilized to assess intestinal permeability in dogs with acute hemorrhagic diarrhea syndrome (AHDS), which is characterized by necrotizing enteritis associated with loss of epithelial integrity on histopathology. Results indicated significantly higher SIC in dogs with AHDS compared to healthy controls. There was a significant correlation between the clinical activity AHDS index and SIC and between SIC and serum albumin concentrations. Especially dogs with severe AHDS demonstrated significantly higher SIC than those with mild to moderate disease. Interestingly, another study evaluating long-term consequences of AHDS showed a higher prevalence of signs of chronic GI disease in the dogs with a previous episode of AHDS compared to control dogs.[2] From these results it was concluded that severe intestinal mucosal damage and associated barrier dysfunction might trigger chronic GI disease later in life. Further studies are needed to make a link between severe barrier dysfunction and sensitization of the immune system in dogs with acute enteritis.

Risk Factors for the Development of Chronic Enteropathies/Food Allergies

In people, the prevalence of food allergies as well as other allergic and inflammatory disorders, such as asthma and inflammatory bowel disease (IBD), is rising, especially in Western societies.[3,5] Adverse food reactions (AFR) are also frequently reported in dogs with cutaneous signs (estimated

prevalence 8-25%).[1] Food-responsive enteropathy represents the most frequent cause of chronic diarrhea in dogs - approximately 50-60% of dogs with chronic diarrhea respond to dietary changes.[1] There is evidence that in acute disease, barrier dysfunction as well as dysbiosis can lead to loss of oral tolerance and sensitize the immune system to food components and the intestinal microbiota.[7] Due to destruction of the epithelial mucosal barrier an increased number of food antigens and bacteria can pass the intestinal barrier and influence the immune system. Acute enteritis is a well-recognized trigger of chronic diseases in humans.[2,5,6] Long-term consequences of acute intestinal problems can manifest as intestinal and extra-intestinal disorders. Most important long-term sequelae affecting the intestinal tract include irritable bowel syndrome (IBS) and IBD. Main extra-intestinal consequences include reactive arthritis, Guillain-Barré Syndrome, and hemolytic uremic syndrome. Data about long-term consequences of acute gastrointestinal disorders in dogs are sparse. One recently published study in puppies with canine parvovirus (CPV) infection showed that significantly more CPV-infected dogs (30/71; 42%) compared to control dogs (8/67; 12%) had developed chronic gastrointestinal signs later in their lives.[7] Chronic intermittent diarrhea usually had started during the first year of life (25/30; 83%) and responded in many dogs to dietary changes (19/30; 63%). Since most dogs relapsed whenever dietary management was discontinued, it was concluded that dogs with former CPV infection are predisposed to develop gastrointestinal food sensitivities. A second study published in 2022 by Sato et al. confirmed that a parvovirus enteritis is a significant risk factor for chronic GI signs in dogs. The prevalence of persistent GI signs was significantly higher in post-parvo dogs compared to control dogs (57% vs 25%, $p < 0.001$).[8] It is hypothesized that negative events on the gut microbiota and permeability especially **early in life**, whilst it is in a dynamic and vulnerable state, have a fundamental impact on microbial consortia's resilience and development of the immune system. However, also adult dogs with severe destruction of the intestinal mucosa and thus intestinal barrier dysfunction like in AHDS have an increased risk to develop chronic problems. For example, the prevalence of chronic GI disease in dogs with a previous episode of AHDS was significant higher compared to control dogs (AHDS 28%; controls 13%; p=0.03) over a similar observation time (median 4 years; range, 1-12 years).[2] The risk for post-infectious irritable bowel syndrome (PI-IBS) is specifically high in humans with enteritis caused by protozoa or parasites. For example, 1,252 people with verified giardiasis during an outbreak in 2004 in Bergen, Norway, were evaluated after 3 years, and the development of long-term consequences were documented by a questionnaire. The prevalence of PI-IBS reported in this study was 47.9% (339/707) in the exposed and only 14.3% (149/1042) in the control group.[9] An unpublished study in dogs showed similar findings. Dogs afflicted with giardiasis, characterized by acute diarrhea during their first months of life, exhibited a higher likelihood of developing chronic GI disease later in life compared to control dogs (*Giardia* 29%, 14/49; controls 10%, 5/50, p=0.02). Interestingly, there was also a higher prevalence in chronic pruritus in dogs with a previous *Giardia* infection (16/49; 33%) compared to dogs without a history of acute diarrhea due to giardiasis (4/50; 8%) (p=0.002). The concurrent occurrence of persistent GI symptoms and chronic pruritus may suggest the presence of food allergies impacting both the digestive and integumentary systems.[1]

Conclusions

There is strong evidence in human medicine and some evidence in dogs that acute intestinal disorders associated with intestinal barrier dysfunction can lead to different long-term consequences. Currently the main focus should lie on restoring intestinal microbial homeostasis and intestinal integrity especially in young dogs with acute enteritis. Further studies are needed to identify specific risk factors leading to food allergies. The future challenge lies in identifying nutritional or pharmaceutical compounds that positively impact barrier function not only in the acute but also in the chronic phase of barrier dysfunction offering potential for prolonged remission in affected individuals.

Conflicts of Interest

The author declares no conflicts of interest.

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Impact of nutrition on allergy and tolerance in dogs

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Abstract

The pathogenesis of loss of tolerance and allergic disease is multifactorial; however, epithelial barrier breakdown plays a major, if not the central, role. Similarly, restoration of functional immunotolerance and mitigation of the clinical consequences of atopic disorders, including adverse cutaneous and gastrointestinal reactions, requires a multimodal approach with modification of nutrients and other dietary factors at the forefront of therapy. This seminar will build on the previous sessions discussing food allergy pathogenesis in the skin and gut and will offer established and explorative nutritional solutions to restore barrier function and reduce immune responses in dogs.

Glossary of abbreviations

AD Atopic dermatitis
CIE Chronic inflammatory enteropathy
DHA Docosahexaenoic
EPA Eicosapentaenoic acid
FMT Fecal microbial transplantation
IBD Inflammatory bowel disease
LA Linoleic acid
PUFAs Polyunsaturated fatty acids
SCFAs Short-chain fatty acids
UC Ulcerative colitis

Introduction

The pathogeneses of atopic dermatitis (AD) and chronic inflammatory enteropathy (CIE) in the dog have similar features. Epithelial barrier breakdown is thought to be a major contributor to disease for both conditions. Barrier dysfunction increases bacterial and allergen translocation and triggers pro-inflammatory immune responses that can have both local and systemic effects. Research from our laboratory demonstrates that patients can have abnormal gut barrier function without overt signs of disease. These patients are at-risk, many from genetic factors, and often develop disease following exposure to environmental pressures such as antibiotics. Nutritional control of AD and CIE is highly dependent on recognition and intervention before disease becomes established. The answer to how we intervene in these patients is complicated by the fact many factors contribute to barrier dysfunction (e.g., immune dysfunction, microbial dysbiosis, decreased intestinal mucus layering, malnutrition, or co-morbidities such as diabetes mellitus), the result of which is a heterogeneity in treatment response.[1] This explains why some dogs with AD show improvement with a modified fatty acid diet and why some dogs with CIE respond to probiotic therapy or fecal microbial transplantation, whereas others show no benefit. How do we improve our ability to treat these patients with nutritional approaches? First, we develop diagnostics to identify dogs before or

at earlier stages of disease when nutritional solutions are more likely to be successful. Second, we develop tools to identify and target the specific causes of abnormal barrier function in our patients rather than treating their symptoms. Third, we use a multimodal trial and error approach.

Nutritional approach to loss of tolerance

For simplicity of these proceedings, nutritional approaches are outlined individually; however, modification of a single nutritional factor is unlikely to result in significant patient improvement. Nutritional interventions for immune-mediated disorders must be multimodal. Advances in the field of "omic" disciplines (e.g., transcriptomics, metabolomics) will make it possible to create a personalized nutrition plan for the prevention or treatment of allergic and immune-mediated disorders. Until then, a trial-and-error approach is used to promote a healthy microbiota, confer barrier protection, and attempt to restore immunotolerance. As AD and CIE share features of epithelial barrier dysfunction, oxidant injury, and inflammation, these are attractive targets for nutritional approaches. Most studies would suggest that bioactive compounds are the safest and most effective when used early in the course of disease and supplied within their natural food matrix. The reader should take note that some of the studies listed below use experimentally induced models of disease in species other than dogs or naturally occurring models of inflammatory bowel disease (IBD) in humans, which does not mimic CIE in dogs. More randomized, blinded, placebo-controlled studies are needed to provide efficacy data for specific nutrition-based interventions in dogs with AD or CIE.

PUFAs

Lipids including cholesterol and polyunsaturated fatty acids (PUFAs) play important roles for epithelial and epidermal barrier functions. In the skin the ω -6 PUFA, linoleic acid (LA), maintains the trans-epidermal water layer, prevents penetration of the stratum corneum by allergens, and provides fluidity and stability to the epithelium.[2] Provision of the ω-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), also helps to reduce production of inflammatory eicosanoids. Diets enriched with PUFAs decrease pruritus and have a steroid sparing effect in dogs with AD.[1,3] Manipulation of diet including the LA concentration as well as the dietary ω-3 to ω-6 ratio in order to positively impact epithelial health and help reduce inflammation has been explored in both CIE[4,5] and AD[6,7] As with most nutritional interventions, earlier supplementation is more likely to demonstrate a benefit and there is a heterogeneity in treatment response.

Prebiotics

Resident microbiota, including bacteria, fungi, protozoa, viruses, and archaea, play an important role in cutaneous and intestinal gut health. Microbiota compete with pathogens for nutrients, generate postbiotics and antimicrobial substances, and participate in crosstalk with the immune system.[8] Bacterial communities in the healthy canine large intestine are characterized by a predominance of Firmicutes, Fusobacterium, and Bacteroidetes with lesser amounts of facultative anaerobes such as Proteobacteria and Actinobacteria. The causal link of dysbiosis in the pathogenesis of AD is suspected but has not been proven;.[9,10] however, intestinal dysbiosis, especially during development, is a recognized risk factor for CIE in the human and dog.[11,12,13] Strategies to promote a healthy microbiota include the use of fecal microbial transplantation

(FMT), probiotics, prebiotics, and postbiotics. The reader is referred to other summit proceedings that will go into more detail about the use of FMT and probiotics. Prebiotics such as inulin, fructooligosaccharides, glucans, and resistant starches are non-starch polysaccharides that are largely found in plant, fungal, and algal sources. Prebiotics are resistant to host degradation and undergo bacterial fermentation in the host lower intestine. Most prebiotic fibers enrich microbial communities, such as Firmicutes, that ferment the prebiotic fibers into beneficial postbiotics.[8] These benefits can also be observed in extra-intestinal locations such as the skin or urinary tract. Prebiotics can also enhance antioxidant activity, reduce inflammation, improve gut barrier function, and prevent degradation of the host mucus layer via provision of an alternative nutrient source.[14] Several studies have demonstrated a beneficial effect of dietary prebiotic fibers on stool quality and inflammatory scores in dogs with CE[15,5,16] Dietary prebiotics might have a preventative effect in some humans with a high risk of AD.[17] Although the preventative effect of prebiotics in dogs with AD is unknown, the combination of prebiotics and probiotics has been demonstrated to reduce the severity of dermatitis in dogs.[18,19] The effects of prebiotics are dependent on their source and quality, dosage, duration, and timing of administration.

Postbiotics

Postbiotics, including exopolysaccharides (e.g., β-glucan), antioxidant enzymes, bacterial lysates, polyamines, tryptophan-derived metabolites, vitamins, and short chain fatty acids (SCFAs), are largely the end products of microbial metabolism. Postbiotics are postulated to provide immunomodulation with wide reaching effects much like probiotics. Most postbiotics have a more stable shelf-life and the potential for a higher safety margin. In a double blind, placebo-controlled study, children with AD receiving an orally administered bacterial lysate (OM-85) and standard treatment daily had decreased incidence of new flare events compared to those receiving standard therapy alone.[20] Orally or rectally administered butyrate improves histologic inflammatory scores in patients with UC.[21] There are studies reporting a potential benefit of postbiotic administration in healthy dogs and dogs subjected to acute stress;[22] however, to date, no published studies report on their use in dogs with AD or CIE.

Vitamins and minerals

Patients with inflammatory disorders, especially those with intestinal inflammation, are at risk for vitamin and mineral deficiencies including vitamins A, B, and D; zinc; calcium; and magnesium. Correction of deficiencies is associated with improved disease severity. Much attention has been paid to hypovitaminosis B, particularly with hypocobalaminemia and CIE and is covered in detail elsewhere.[23]

Provitamin A (a.k.a carotenoids including β-carotene, lycopene, lutein, and zeaxanthin) and vitamin A (retinoids) promote cutaneous and intestinal health through regulation of epithelial cell proliferation and differentiation and enhancement of barrier function. Additionally, retinoic acid promotes immunoglobulin A (IgA) class switching of B cells in mucosal tissues and helps mediate development of T regulatory cells over T-helper 17 differentiation.[24] Reduced plasma carotenoid and retinoid concentrations can be observed in humans with AD[25] and UC[26] and correction of deficiency is associated with improvement in clinical signs. Vitamin A deficiency is known to

cause several dermatoses in dogs, but further study is needed to determine if low carotenoid or retinoid concentrations contribute to AD or CIE in dogs.

Vitamin D is best recognized for its role in serum and calcium homeostasis. However, vitamin D also aids in the body's immune response and in epithelial cell differentiation, proliferation, and maintenance of the epithelial barrier. Because dogs do not synthesize vitamin D following ultraviolet light exposure and are reliant on gastrointestinal absorption, they are particularly at risk for vitamin D deficiency with malabsorptive gastrointestinal diseases and inflammatory disorders. Hypovitaminosis D has been identified as a negative prognostic indicator for dogs with protein-losing enteropathy.[27] Correction of hypovitaminosis D in dogs with CIE is assumed to increase survival although there are no published studies to confirm this hypothesis. Dogs with AD and lower pretreatment calcifediol concentrations are more likely to have a suboptimal response to steroid therapy.[28] And, cholecalciferol treatment can reduce pruritus and skin lesion scores.[29] Therefore, monitoring of blood vitamin D concentrations and treatment of hypovitaminosis D should be considered in patients with refractory CIE or AD.

Vitamin E is a powerful antioxidant that protects against lipid peroxidation and suppresses expression of IL-4, which is essential for IgE isotype switching. Lower plasma vitamin E concentrations have been documented in dogs with AD compared to healthy dogs,[30] and vitamin E supplementation did lessen clinical signs of AD compared to those receiving placebo.[31] In an unpublished study evaluating fat-soluble vitamin concentrations in dogs with CE compared to healthy controls, 5 dogs with CE had much lower alpha-tocopherol concentrations compared to healthy dogs, although the difference between groups was not significantly different.[32] Measurement of vitamin E, as is the case with vitamin A, is challenged by a lack of commercially available laboratories to measure blood vitamin E in dogs.

Zinc is critical for the formation of the stratum corneum and in essential fatty acid conversion. Zinc cannot be created by dogs and, therefore, must be consumed in the diet. Dogs with chronic gastrointestinal diseases might be particularly at risk for zinc deficiency due to malabsorption and luminal loss.[33] No beneficial effect has been observed in dogs with acute diarrhea[34,35] but, to the author's knowledge, there are no studies evaluating the benefit of zinc supplementation in dogs with CE. Zinc promotes LA metabolism and deficiency can cause similar clinical signs as that observed with LA deficiency. Feeding a diet enriched with zinc and LA in excess of minimum requirements to healthy Labrador retrievers for 12 weeks resulted in less transepidermal water loss as compared to those that did not receive the same amount of supplementation.[2]

Glutamine

Glutamine is a conditionally essential amino acid and is the preferred energy source for rapidly dividing cells such as enterocytes. Glutamine can have anti-fibrotic, anti-inflammatory, and antioxidant activity and stimulates tight junction protein expression.[36] In certain disease states such as protein-losing enteropathy, increased utilization and loss of glutamine could render the body glutamine deficient, further exacerbating intestinal hyperpermeability and bacterial translocation. Although glutamine supplementation does improve disease severity in

experimental models, data on the use of glutamine for humans with IBD are less convincing.[37] Like other nutrients, glutamine supplementation might have more of a benefit when used before or early on in the course of disease. For example, glutamine administration to low birth weight infants decreased the incidence of AD and gastrointestinal infections later in life.[38] Oral glutamine is rapidly absorbed by the small intestine and is less likely to have a beneficial effect on the colon. Currently, there are no published studies evaluating the effects of orally or parenterally administered glutamine in dogs with CIE. These are needed before any recommendations regarding additional glutamine supplementation can be made.

Bioactive compounds

Phytochemicals and herbals such as polyphenols, sulforaphane, ginger, and cannabinoids have been explored as natural treatments for a variety of conditions due to their antioxidant and anti-inflammatory activities. Polyphenols are organic compounds that are particularly abundant in plants. Examples of polyphenols include flavonoids (e.g quercetin and genistein), curcuminoids, epigallocatechin gallate (a green tea flavonoid), catechin, epicatechin, berberine, proanthocyanidins, anthocyanins, and resveratrol. Intake of natural polyphenolic compounds can promote the growth of beneficial bacteria such as *Bifidobacteria*, enhance tight junction integrity, increase mucus secretion, and help scavenge reactive oxygen species.[39] Polyphenols have variable bioavailability and are susceptible to degradation during food processing; however, several studies suggest that polyphenols might concentrate in the intestinal epithelium in the presence of inflammation. Some polyphenol extracts at levels higher than those found naturally in dietary sources and high doses of synthetic phenolic antioxidants (e.g., butylated hydroxyanisole [BHA], butylated hydroxytoluene [BHT]) can be harmful, and therefore phenolic compounds from natural sources are preferred.[40]

Both ginger and licorice extract have polyphenolic compounds that exhibit anti-inflammatory and antioxidant activity in the gut and skin. In a clinical trial of humans with ulcerative colitis (UC), patients ingesting 2000 mg/day of dried ginger powder for 12 weeks had significantly decreased disease severity and increased quality of life scores compared to those receiving placebo.[41] A Japanese formula containing ginger root along with Astragalus root, licorice, jujube, ginseng, white Atractylodes rhizome, and Chinese angelica root has also been explored in uncontrolled studies of AD in humans. A complete diet containing turmeric and licorice extracts and a modified fatty acid composition improved pruritus scores and reduced the need for additional therapies in dogs with AD.[42] More randomized, controlled studies in dogs with AD or CIE are needed to understand the specific benefit, if any, associated with the use of ginger or licorice extract.

Herbal supplements containing extracts from plants and algae such as *Boswellia serrata*, *Andrographis paniculate, Glycyrrhiza uralensis*, *Spirulina* have also shown promise in reducing disease severity in patients with UC[43] and dogs with AD.[44]

Additional considerations

Environmental factors - Dietary factors including food processing are known to contribute to the development of and serve as a treatment for the treatment of immune-mediated disorders

including AD and CIE. Extensive hydrolyzation of peptides or feeding amino acid-based diets can decrease immunoglobulin E (IgE) crosslinking and mast cell degranulation and result in improvement in some dogs with AD or CIE. What is unclear is the impact of avoidance or minimization of exposure to environmental factors such as dietary advanced glycation end product or emulsifier content, preservatives, and microplastics, pollution, detergents, and household cleaners on reducing risk of AD or CIE in dogs. More studies are needed to understand the role of exposure to these environmental factors in the development of barrier dysfunction.

Diet variation - In at-risk breeds, dietary variation could be a method to reduce allergic reactions. Diet diversity during pregnancy or during early life can reduce the odds of food sensitization later in life in humans.[45] Longitudinal studies of dogs are needed to better understand the benefit of this practice.

The role of stress - The link between physical and psychosocial stressors and IBD is well recognized in humans. Humans with IBD are twice as likely to suffer from anxiety and depression.[46] Stressors also have a demonstratable role on gut function in dogs.[47,48] The effects of these stressors can be seen at sites distant from the gut including the skin. This relationship is defined as the "gut-brain-skin axis". In one study of dogs with AD, investigators determined that pruritus severity was associated with increased frequency of undesirable behaviors including excitability, reduced trainability, and hyperactivity.[49] In a retrospective study of owners of 271 dogs, dogs with psychological disorders including non-social fear and anxiety had an increased severity and frequency of skin disorders.[50] It can be difficult to determine which came first, disease or psychological condition; however, as shown by these studies, stressors can impact the severity of disease and therefore represents an important therapeutic target. Acquisition of the dog's history should include questions that identify stressors in the dog's life. Clients should be educated about ways to minimize stress such as environmental modifications, daily exercise, and nutritional interventions.[51] Weaning and maternal separation are some of the major stressors in a dog's life and both come at a time when the gut microbial environment is highly dynamic.[52] Interventions to reduce stress and to stimulate the immune system and bolster the epithelial barrier through nutritional interventions such as dietary colostrum or antioxidants (e.g., alpha lipoic acid) might influence immediate mortality risk as well as decrease risk factors for disease later in life.[53]

Delivery systems

Innovative nutrient and drug delivery systems have the potential to deliver functional foods and drugs to the specific intestinal segment of interest whilst reducing the potential for adverse effects caused by systemic absorption. Nanoparticle delivery systems involves the use of nontoxic, edible biopolymers such as nondigestible proteins or polysaccharides to deliver nutrients and/or drugs to the site of interest. For example, lutein, a carotenoid with numerous benefits as listed above, is poorly soluble and poorly bioavailable. It is also easily broken down by pH changes and high moisture content but could be delivered effectively to the intestinal tract via nanotechnology.[54] Nutrient delivery of polyphenols has been explored experimentally,[55] but there are no published studies evaluating the potential for nanocarriers to deliver targeted nutrients in dogs.

Conclusions

The epithelial barrier plays the central role in the prevention of allergic and other immune-mediated disorders. Modification of nutritional and environmental factors can be used to decrease local and systemic inflammation, restore normobiosis, and enhance epithelial barrier function. Nutritional management of disease is largely dependent on early recognition of barrier breakdown and prompt intervention with a multi-modal, patient-centric approach.

Conflicts of Interest

The author declares no conflicts of interest.

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Overview and clinical implications of immunity and inflammation in the GI tract

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Abstract

The gastrointestinal (GI) tract is home to a large percentage of immune cells of the body and when the gut barrier, microbiome, and immunity are functioning normally, many infectious agents and potential allergens are excluded and the animal remains normal. Transient GI inflammation associated with innate and acquired immune responses is generally beneficial in limiting the effect of bacterial, fungal, parasitic, and viral pathogens and resolves with time. However, when normal GI physiological responses are compromised or hyperresponsive, clinical signs of disease like vomiting, diarrhea, inappetence, and weight loss occur and can persist. An excellent review of immunonutrition as a science applied to small companion animals was presented in the first section of this Summit (Dr. Satyaraj). While still in a phase of relative infancy in pets, this field is moving forward rapidly now that progress has been made in new and more affordable diagnostic methods to assess the microbiome and performance of a variety of "omics" including proteomics, transcriptomics, genomics, metabolomics, lipidomics, and epigenomics. Results from multiple newer techniques will be presented in this section of the Summit. In this specific lecture, results of several studies will be used to emphasize how immunonutrition can actively modulate the immune system of dogs or cats leading to systemic effects.

Glossary of abbreviations

CDV Canine distemper virus
Center Center for Companion Animal Studies
Con A Concanavalin A
GI Gastrointestinal
SF68 *Enterococcus faecium* strain SF68

Introduction

In this 4th section of the 2024 Companion Animal Nutrition Summit focusing on Gastrointestinal Immunity and Inflammation, the speakers will be providing updates on:

1. Clinical implications of GI immunity (Lappin),
2. New approaches to immunomodulation and diagnostics (Manchester),
3. The relationship between the intestinal microbiome, dysbiosis, and inflammation in dogs and cats (Suchodolski), and
4. The comparative aspects of nutrient effects on the intestinal microbiome in humans and other animals (Hoffman)

Each of the speakers are active in the field and provide different perspectives on gastrointestinal immunity and inflammation. Emphasis in each lecture will be placed on what is currently known as it relates to dogs, cats, or people as well as providing directions for the future. The speakers will also provide information on how to use nutrition in the management of cases with gastrointestinal inflammation or systemic infectious diseases.

The science of immunonutrition has been advancing in human and companion animal medicine.[1-3] In the first section of the 2024 Companion Animal Nutrition Summit, the lecture entitled "Immunonutrition – Companion Animal Perspective" was presented by Dr. Ebenezer Satyaraj. Dr. Satyaraj sorted the key components of immunonutrition into 4 stages:

Stage I: Complete Nutrition
Stage II: Optimizing Macro & Micronutrients
Stage III: Active Modulation of the Immune System
Stage IV: 'Personalized' Nutrition

Our research group at the Center for Companion Animal Studies has focused in part on the use of dietary supplements in the management of health and disease in dogs and cats and actively collaborates with many of the speakers at the 2024 Companion Animal Nutrition Summit, including Drs. Manchester, Satyaraj, and Suchodolski. One of the Center's areas of interest is to assess the use of nutritional supplements to actively modulate the systemic immune system which will be the focus of this lecture.

In most countries, there are minimal regulations concerning supplements and how to choose a supplement was recently reviewed.[4] With the exception of osteoarthritis in dogs, there is generally not enough data concerning supplements or enhanced therapeutic diets to assess multiple studies by metaanalysis.[5] However, there have been increasing numbers of peer reviewed publications providing a more evidence-based assessment for the use of supplements in managing dermatological and gastrointestinal syndromes in pets.[6-13] There is some evidence for use of certain veterinary supplements like probiotics to modulate immune responses to lessen inflammation associated with inflammatory bowel disease.[12,13] However, the number of studies documenting supplements inducing systemic immune stimulation are small. In humans, interest in use of probiotics that can induce anti-viral responses increased during the SARS-CoV-2 pandemic.[14] Dogs and cats also develop GI and systemic coronavirus infections as well as multiple other GI and systemic bacterial, fungal, parasitic infections that could potentially benefit from systemic immune stimulation.[15] Some strains of probiotic bacteria are known to induce systemic immune responses, but the number of studies documenting those effects in dogs or cats is small.[13,14,16-20]

Evidence for systemic immunomodulation using dietary supplements in dogs

In one of the first companion animal studies of a probiotic that is commercially available in some countries, *E. faecium* strain SF68 (SF68) was fed to a group of puppies vaccinated for canine

distemper virus (CDV) and compared over time with a control group that was administered the same vaccination protocol but was not fed the probiotic.[17] A number of findings suggested an immune modulating effect of the probiotic. The puppies supplemented with SF68 had increased serum and fecal total IgA concentrations, maintained higher CDV-specific IgG and IgA serum concentrations of time, and had an increased percentage of circulating B lymphocytes when compared with control puppies. The effect on canine distemper virus-specific IgG and IgA antibodies in serum was seen after the puppies had been supplemented for 31 and 44 weeks. It was believed that SF68 prevented the decline in antibody titers observed in the controls by maintaining high levels of antibodies in the supplemented puppies due to a probable immune stimulating effect on B lymphocytes. Another commercially available probiotic mixture available in some countries also showed some evidence of B lymphocyte stimulation in supplemented healthy dogs that developed increased plasma IgG levels.[13]

The previous study of SF68 was performed using puppies which presumably were still developing mature immune responses.[17] A follow up study was performed in the Center to determine whether the immune modulating effects of SF68 could be apparent as soon as 4 weeks after starting supplementation (*Manuscript in review*). Age-matched, clinically healthy, young adult beagles (n = 7) were chosen for study. Blood and serum samples were collected from each dog prior to supplementation and then monthly for 12 weeks. Flow cytometry was used to measure percentage of B cells expressing surface-bound IgG or major histocompatibility complex class II (MHC II,) the percentage of CD4+ T cells expressing MHC II, and the percentage of CD8+ T cells expressing MHC II or CD11a. T lymphocyte proliferative responses of all dogs to a non-specific mitogen (Concanavalin A) were assessed by flow cytometry. For multiple B and T lymphocyte parameters, statistically significant differences compared with baseline were detected as early as 4 weeks after starting SF68 supplementation (***Figures 1 and 2***).

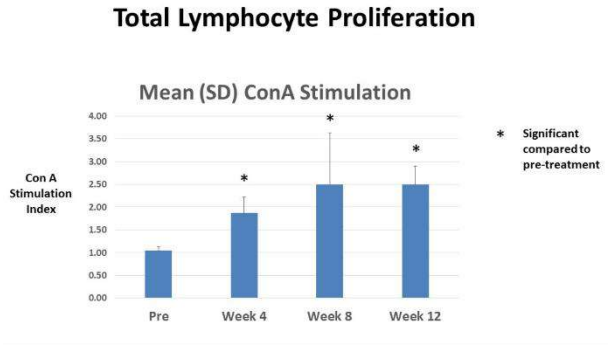

Figure 1

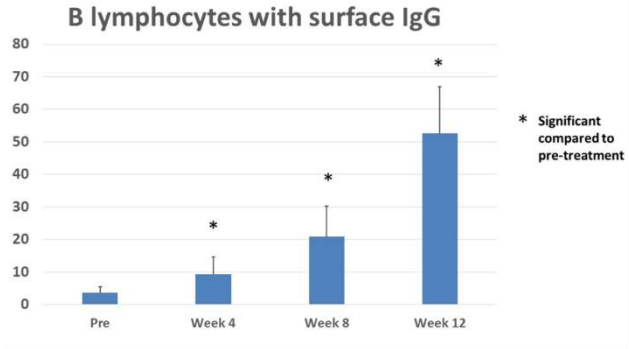

Figure 2

Generalized demodecosis is common in dogs and has been associated with either immune exhaustion or pre-existing immune deficiency.[15] The Center completed a pilot study that rescued

pit bull terrier dogs with generalized demodicosis from local shelters to provide free treatment. All the dogs were administered a drug known to have anti-*Demodex* effects and were randomized into an SF68 supplementation group or a placebo group. Over time, SF68 supplemented dogs had improved clinical scores and mite kill, suggesting a systemic immune-stimulating effect (*Manuscript in review, 2024*).

Evidence for systemic immune modulation induced by a dietary supplement in cats

A similar experiment with SF68 to evaluate the safety and immunological effects when fed to healthy research kittens was performed.[18] That study investigated whether feeding SF68 to kittens would enhance non-specific immune responses or specific immune responses to FHV-1, feline calicivirus, and feline panleukopenia virus vaccination. Starting at 7 weeks of age, one group of 10 kittens was fed SF68 daily and the other group was fed a placebo. All kittens were monitored to 27 weeks of age. A number of the measured humoral immune responses were numerically greater in the kittens that were fed SF68 when compared to the placebo group but the differences did not reach statistical significance. For example, the mean FHV-1-specific serum IgG concentrations were greater in the treatment group when compared with the placebo group at 15, 21, and 27 weeks of age. However, at 27 weeks of age, the treatment group had a significantly higher percentage of gated lymphocytes positive for CD4+ (mean 13.87%) than the placebo group (mean 10.61%, $p = 0.022$). It was concluded that *E. faecium* strain SF68 was safe and some evidence for immune modulation occurred.[18]

To determine whether the immune-modulating effects of SF68 noted in the healthy kitten trial could be of clinical benefit, healthy cats that were normal carriers of FHV-1 were studied. In this pilot study, it was hypothesized that feeding strain SF68 would decrease recurrence of clinical disease, frequency of episodes of FHV-1 shedding, and the total number of FHV-1 DNA copies shed over time in cats with chronic FHV-1 infection.[19] Twelve normal cats carrying FHV-1 were fed either SF68 or a palatability enhancer as a placebo. Clinical signs of disease, FHV-1 shedding, FHV-1-specific humoral and cell-mediated immune responses, and changes in the fecal microbiome were monitored and evaluated. After an equilibration period, the housing of the cats was changed from individual to group housing multiple times over a five-month period. SF68 was well tolerated by all cats. Fecal microbial diversity was maintained throughout the study in cats supplemented with *E. faecium* strain SF68, but diversity was decreased in cats fed the placebo, indicating a more stable microbiome in cats fed SF68. The cats fed *E. faecium* strain SF68 had significantly fewer episodes of conjunctivitis than the placebo group during the supplementation period, suggesting that probiotic administration lessened morbidity associated with the FHV-1 carrier state. However, experiments to assess the cause of these clinical effects were not performed.

Due to increased interest in supplements with anti-viral properties stimulated by the SARS-CoV-2 pandemic and to further evaluate possible antiviral mechanisms associated with SF68, an experiment was recently completed.[14,20] Two groups of four young adult cats were fed a

commercial product containing SF68 and psyllium or a placebo and fed the same diet. On Day 28, total RNA was extracted from peripheral blood mononuclear cells and bulk RNA sequencing performed using an Illumina-based platform. Sequence files were aligned with the reference cat genome and assembled using Partek flow software. Gene set expression analysis (GSEA) was performed using open-source software, with significance was set at FDR of < 0.05 and $p < 0.1$. Leukocyte transcriptomic data from Day 28 revealed that cats fed SF68 had 45 significantly upregulated genes and 33 significantly downregulated genes. The TNF-a and TGF-b signaling pathways were significantly upregulated while interferon-alpha and interferon-gamma pathways were significantly downregulated. Increases in IL-12 were noted in the supplemented cats using the serum assay. Serum from supplemented cats showed evidence for suppressing feline infectious peritonitis virus in a feline macrophage cell line. Continued studies on inhibition of enteric and FIP inducing strains of coronavirus are ongoing.

Conclusions

Continued study of immunonutrition for modulating not only GI immunity but systemic immune responses as well is indicated.

Conflicts of Interest

I direct the Center for Companion Animal Studies at Colorado State University which a non-profit organization that currently has donations or sponsored research with 15 companies including Nestlé-Purina Pet Care. I also serve on the Purina Advisory Board. However, the lecture presented herein will only include material that has been published as a peer reviewed abstract or full manuscript and/or is on approved product labels.

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New approaches to immunomodulation and diagnostics

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Abstract

Chronic enteropathy in dogs is a very common clinical problem with a poorly understood pathogenesis. This session will include a summary of results from recent immune assay and transcriptomic investigations of canine chronic enteropathy. Attendees will gain appreciation for the potential role of bile acids in modulation of canine macrophage molecular programs, as well as the many unanswered regarding these complex metabolites. Results of a single cell RNA-sequencing based investigation of the duodenal mucosa in health and in CE will be explored, implicating important roles for epithelial and myeloid cells in disease. Using these modern approaches to study CE will facilitate specific, evidence-based classification of patients, toward the ultimate goal of providing individualized care and improved outcomes.

Glossary of abbreviations

BA	Bile acids
CA	Cholic acid
CE	Chronic enteropathy
LCA	Lithocholic acid
scRNA-seq	Single cell RNA-sequencing (scRNA-seq)

Introduction

Incomplete understanding of the pathogenesis underlying CE in dogs has impaired individualized management of this disease. This leaves clinicians to pursue a trial-and-error approach, monitoring for improvement in clinical signs. While most dogs improve clinically with diet and/or medications, a subset of patients fail to respond to all interventions.[1] New approaches to CE understanding are necessary to categorize patients, target treatments, and improve outcomes.

Bile acids as important metabolites in canine CE

Data from untargeted metabolomics studies have implicated bile acids (BA) as potentially important molecules in the pathogenesis of CE in dogs.[2] These molecules have been long recognized for their role in digestion and assimilation of fats across species. Specifically in GI diseases, BA are implicated in a variety of conditions ranging from development of colorectal cancer[3] to susceptibility to *Clostridioides difficile* colitis[4]) to flares of irritable bowel syndrome (IBS).[5] Bile acid sequestrants are an effective therapy for alleviating diarrhea in a subset of human IBS patients.[6]

In dogs, evidence suggests that intestinal bile acid pools are altered in dogs with CE. To date, the main themes involve loss of the typical predominance of secondary over primary BA in the feces in CE dogs compared to healthy.[7,8] However, consideration of these results must recognize that intestinal BA homeostasis may be dramatically disrupted by antimicrobial exposures,[9,10] and this

could be a confounding factor in comparing BA composition in feces from healthy compared to CE dogs.

Connecting the quantitative shifts in fecal BA pool to disease manifestations has been largely unstudied in dogs. We therefore investigated potential immunomodulatory properties of different unconjugated bile acids through *in vitro* experiments with canine macrophages.[11] Both the primary bile acid cholic acid (CA) and the secondary bile acid lithocholic acid (LCA) influenced LPS-induced cytokine production via canine monocyte-derived macrophages similarly, with suppression of TNF-α secretion and enhancement of IL-10 secretion. Neither BA altered the expression of the BA receptor TGR5. Transcriptomic analysis of a canine macrophage cell line revealed that CA activated inflammatory signaling pathways in macrophages involving type II interferon signaling and the aryl hydrocarbon receptor, whereas LCA activated pathways related to nitric oxide signaling and cell cycle regulation. Thus, we concluded that both CA and LCA are active modulators of macrophage responses in dogs, with differential and shared effects evident with sequencing analysis. This initial study provides a putative connection between disrupted BA homeostasis and manifestations of CE in dogs, realizing the limitations of the experimental model. Further work is needed to explore the multifaceted roles of these metabolites in health and disease.

Chronic enteropathy pathogenesis – zooming out

To date, much investigation of the cellular drivers of CE in dogs has focused on immune cells. This is understandable as canine diseases are approached from the context of better understood conditions in humans such as ulcerative colitis. However, direct parallels between human and canine diseases have been difficult if not impossible to find. Many CE dogs improve with dietary modification, yet gluten does not appear to be a trigger as is the case in human celiac disease. A subset of CE dogs improves with immunomodulation, but these dogs do not share the histological features or systemic consequences of a patient with Crohn's disease. It is possible that CE dogs are more akin to humans with irritable bowel syndrome (IBS), also known as disorders of the gut-brain axis. Given the broad heterogeneity among CE dogs, and the failure of canine disease to recapitulate well described human conditions, a more global approach is needed. This would consider contributions to pathogenesis from beyond immune cells, and even beyond the gastrointestinal tract in isolation.

Our group elected to apply single cell RNA-sequencing (scRNA-seq) technology to the study of CE in dogs. This technology allows for characterization of transcriptomic activities in heterogeneous samples. Since its creation in 2009, scRNA-seq has added to the body of knowledge on a diverse range of GI topics in mice and in humans. Inflamed and uninflamed ileal biopsy samples from 12 Crohn's patients analyzed with scRNA-seq highlighted the importance of CCR2 ligand producing stromal cells.[12] Patients with an enrichment of these cell types proved refractory to anti-TNF agent therapy. Subsequently, the investigators identified this significant cell type within 4 independent bulk RNA-seq datasets, thus confirming their theory that the cell signature correlated with refractoriness to TNF blockade. This approach has also advanced understanding of development of the intestinal immune system and regional differences along the length of the gut, of rare cell

subtypes like tuft cells, and contributions of non-immune cells to GI homeostasis.[13] Understanding of celiac disease was advanced by Atlasy and colleagues who showed that celiac patients in remission were distinct from healthy controls, and that celiac individuals had expanded intraepithelial cytotoxic T lymphocytes with concomitant reduction in natural intraepithelial lymphocytes (IELs).[14] Thus, scRNA-seq is a powerful tool to investigate intestinal tissues in terms of cellular composition and molecular programs.

Drawbacks of scRNA-seq exist. As with any transcriptome-based analysis, it must be recognized that gene expression is not an exact surrogate for protein abundance or function. Translation rates, protein synthesis alterations, changes in protein half-life, and other factors may result in a disconnect between transcript and protein concentration.[15] Assignment of cell identities based on transcriptomic data can be challenging and relies upon the quality of the reference genome and iterative analyses of cell subtype gene expression. Automated tools can be massively helpful with this but have not been established for all tissues in all species. Single cell RNA-sequencing fails to identify splice variants and identify targets of antigen receptors. Processing of tissues can bias for certain cell types[16] and therefore impede accurate characterization the cellular make up of heterogeneous tissues. In this case, complementary techniques such as flow cytometry and immunohistochemistry are needed. As compared to bulk RNA-seq, scRNA-seq is much more labor intensive and expensive. However, once tissue-specific cell atlases are generated, deconvolution applications can help estimate the cellular composition of tissues analyzed with bulk RNA-seq.[17,18]

Single cell RNA-sequencing analysis of the duodenal mucosa in dogs with CE

We recently cataloged and compared the diversity of cells present in duodenal mucosal endoscopic biopsies from 3 healthy beagle dogs and 4 dogs with CE using scRNA-seq.[19] Through characterization of 35,000 cells, we identified 30 transcriptomically distinct cell populations, including T cells, epithelial cells, myeloid cells, and plasma cells. Both healthy and CE samples contributed to each cell population. T cell subtype proportions exhibited no quantitative differences comparing CE to healthy and were dominated by $CD8^+$ T cells. Among the myeloid cells, neutrophils from CIE samples exhibited inflammatory gene expression signatures (SOD2 and IL1b). Numerous differentially expressed genes were identified in epithelial cells, with the most significantly differentially expressed gene encoding for a 2-pore potassium channel. Pathway analysis of one epithelial cell subtype indicated reduced oxidative phosphorylation gene programs in CE cells. Overall, this work revealed a profound cellular heterogeneity in canine duodenal mucosa and provided new insights into molecular mechanisms which may contribute to GI clinical signs in CE. The cell type gene signatures developed through this study add to the foundation of understanding of canine intestinal physiology, with further work particularly needed to explore novel cell subtypes identified.

Further exploration of these initial findings is required before new CE categorization schemes and treatment strategies can be generated. However, with these initial signals, unique strategies are implicated. For example, targeting overactive neutrophils could be considered. Moreover, if further studies reinforced a role for epithelial metabolism and barrier function, specific therapies targeting those pathways would be indicated.

Conclusions

Chronic enteropathy is a common syndrome in dogs, with much to be discovered regarding its pathogenesis. Future investigations should explore the features that distinguish unique clinical presentations. This will require careful clinical evaluation of patients along with multiomic analysis of disease relevant samples (i.e., intestinal biopsies, feces). In the future, this will ideally allow for stratification of patients based on their unique disease characteristics and individualized medicine, as well as improved translational studies involving dogs and their human companions.

Conflicts of Interest

The author declares no conflicts of interest.

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The relationship between intestinal microbiome, dysbiosis, and inflammation in dogs and cats

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Abstract

Chronic enteropathies (CE) are a common clinical syndrome in dogs and cats causing chronic intestinal signs. They are difficult to diagnose and manage due to the lack of specific markers for response to therapy. The pathophysiology of CE is complex and includes changes in the immune system, intestinal function, and the intestinal microbiome. The current classification solely based on response to treatment (ie, food- vs. steroid-responsive) is currently being questioned, as new research findings indicate that CE is a syndrome with many overlapping unspecific features, often due to chronic irreversible changes in intestinal function and persistent dysbiosis. This highlights the need for a staging system of intestinal damage and dysfunction. Future work should focus on how a multimodal therapy approach encompassing immunomodulation and microbiome restoration can help in the long-term management of CE to manage current clinical signs and potentially help improve mucosal healing.

Glossary of abbreviations

BA	Bile acids
CE	Chronic enteropathy
DI	Dysbiosis index
GIT	Gastrointestinal tract
SCFA(s)	Short-chain fatty acid(s)

Introduction/Background

The gastrointestinal tract is a complex system that encompasses various functions by the host and the resident microbiota. Within the gastrointestinal tract (GIT), dietary substrates are broken down into smaller micronutrients, which subsequently are absorbed by active or passive transport systems in the intestinal brush border. Balanced host and microbiota functions are needed for optimal physiological functions.

The microbiome encompasses various microorganisms such as bacteria, viruses, fungi, and protozoa within the gastrointestinal tract (GIT). Bacteria make up the majority of these with >95% of sequenced microorganisms.[1,2] The importance of viruses and fungi remains poorly understood. The microbiome acts as an important immune and metabolic organ.[3] Intestinal bacteria interact with the immune system of the host through various mechanisms. These include communications through microbial surface molecules on bacterial cells that interact with receptors on host cells (e.g., dendritic cells, Toll-like receptors). The communication between bacteria and host is also mediated through microbiota-derived metabolites. In brief, bacteria break down and metabolize dietary compounds (e.g., fiber, protein, fat) or host molecules (e.g., primary bile acids) into

metabolites. These function as signaling molecules that affect function within the GIT but also other organ systems. These metabolites can provide energy, can be immune-modulatory, can regulate intestinal motility, and/or they can act on the gut barrier. For example, some bacteria, such as *Faecalibacterium* and *Turicibacter*, ferment dietary carbohydrates to SCFAs. Indole compounds are metabolized from dietary tryptophan. Selected *Bifidobacterium* spp are involved in this pathway. Intestinal bile acid metabolism and conversion by bacteria is particularly important in maintaining host health and normal microbiota.[4] Briefly, primary BAs are released into the small intestine after a meal to facilitate fat digestion, and a small percentage of BA will be converted by intestinal microbes to secondary BA in the large intestine. The latter BAs have important signaling effects on the host. In physiological amounts, they act as signaling molecules, have glucose-lowering effects, and are anti-inflammatory.[4] They also regulate and suppress potential pathobionts such as *C. difficile*, *C. perfringens*, and *E. coli*.[5] A lower abundance of *C. hiranonis* and decreased conversion of primary to secondary BAs is associated with intestinal dysbiosis due to antibiotics or chronic enteropathy in a subset of dogs and cats.[6] However, if present in excessive amounts, secondary bile acids can also lead to diarrhea or even intestinal inflammation. This indicates the need for the proper balance of microbiota and their metabolites.

Some intestinal bacteria also produce immunomodulatory molecules. For example, *Faecalibacterium* spp., an important core bacterium that is often decreased in chronic intestinal disease in humans, dogs, and cats,[7] secrete anti-inflammatory peptides.[8] Organs outside the GIT also have receptors for bacterial metabolites produced in the intestine. For example, short-chain fatty acid (SCFA) receptors are found in the kidney and lung, while bile acid receptors are expressed in the lung and pancreas.[9] Therefore, changes in the concentrations of these microbial-derived metabolites may impact other organ systems. An altered intestinal bile acid pool (i.e., an abnormal ratio of primary to secondary pile acids) is associated with diabetes, as bile acids can stimulate insulin secretion in the pancreas. SCFAs have been recognized as microbial molecules involved in gut-brain axis signaling.[10]

The enteric environment in response to diet or antibiotics

Through various direct and indirect mechanisms, the intestinal microbiota is in contact with the intestinal epithelium, mucus layer, the immune system, and the luminal environment (***Figure 1***). Depending on the extent of changes within the intestinal environment, these can affect the microbiota composition, and severe dysbiosis is a biomarker of an abnormal gut environment in disease. In addition, the altered microbiota can contribute to clinical signs in a subset of patients.

There are some differences in the microbial changes depending on the underlying disease process or inciting cause.[6] The fecal microbiota is in part influenced by diet, but the extent of changes in total microbiome composition is typically limited in healthy animals. A recent meta-analysis demonstrated that the microbiota responds to different levels of macronutrients (i.e., protein and fat) in some individual taxa, but overall these shifts were relatively small with a small statistical effect size, as there was almost complete overlap in microbiome composition between macronutrient levels.[11] In contrast, broad-spectrum antibiotics like metronidazole, tylosin, or

clindamycin have major effects on the microbiome, inducing major shifts and changes in function, which persist for at least several weeks in some animals.[12] Various microbial metabolites that are associated with intestinal health (e.g., SCFA, fecal bile acids) are negatively impacted by these antimicrobials and correlate with the changes in the microbiota composition. Acid suppressant therapy with the proton pump inhibitors leads to increases in lactic acid bacteria in the stomach and duodenum[13] and to a mildly increased fecal dysbiosis index (mainly due to an increase in *Streptococcus* spp, but normal *C. hiranonis*). These changes are transient and revert within 14 days after the end of therapy.

Figure 1 – The intestine in health and disease. A healthy intestine (left) is characterized by a balanced microbiome, an established mucus layer (green) separating luminal bacteria from the epithelial cells, a normal epithelial cell barrier, and a regulated immune system.

In chronic inflammatory enteropathy (right), various changes may occur, with all of them potentially contributing to clinical signs. Loss of mucus allows luminal bacteria to attach to epithelial cells, stimulating pro-inflammatory cytokines. A broken barrier leads to translocation of food and bacterial antigen, which also activates the immune system. Loss of transporters in the brush border leads to malabsorption of dietary compounds, which can lead to bacterial overgrowth. The inflammation (changes in pH and oxygen on mucosal surface) and the low-grade malabsorption of nutrients (provides substrate for bacterial overgrowth) both can contribute to intestinal dysbiosis.

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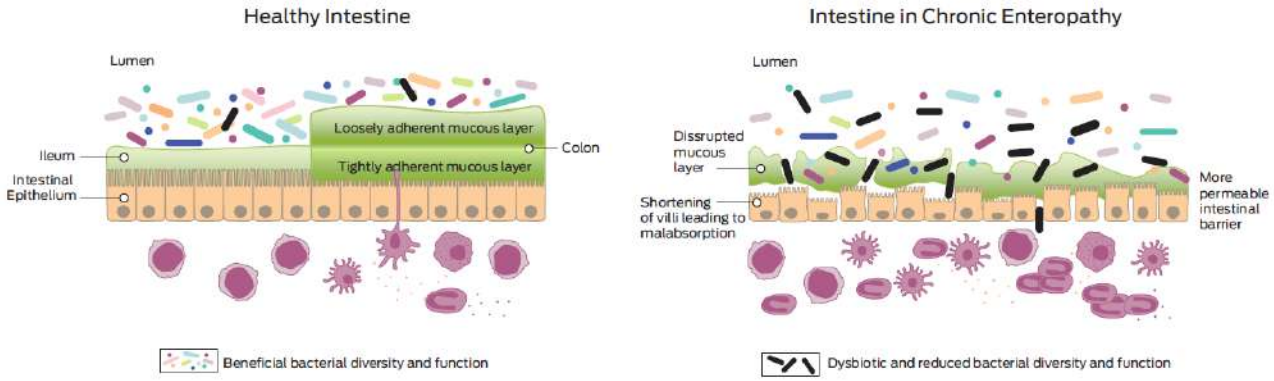

The enteric environment in intestinal disease

In intestinal disease, intestinal inflammation and structural changes leading to changes in intestinal function (e.g., absorption) will also influence microbiota composition. The extent and pattern of these changes differ between acute and chronic diseases. Several studies have shown that in acute uncomplicated as well as acute hemorrhagic diarrhea (AHD), changes in microbiome composition typically show increased abundance of enterotoxigenic and net-F encoding *C. perfringens* that is self-limiting, as independent of treatment (antibiotics vs probiotics), the abundance decreases within a few days. *C. hiranonis*, an important core bacterium in dogs and

cats remains typically with the reference interval in acute diarrhea, and the dysbiosis index is either not or only mildly increased and also normalizes within a few days, even in cases of severe AHD.[14,15] This has been also confirmed by metabolic analysis indicating that in AHD, various metabolites that are markers of intestinal damage (fecal long-chain fatty acids, fecal cholesterol, nervonic acid)[16] are increased at the time of presentation, but these markers normalize very quickly within a few days, and their normalization correlates with normalization of the microbiome and clinical recovery with a few days (unpublished data). Furthermore, intestinal protein loss and intestinal inflammation also normalized within 3 days in dogs with AHDS.[17]

This is in contrast to microbial and metabolic changes in animals with chronic enteropathies (CE). These are often accompanied by systemic and localized inflammatory changes. Non-invasive markers like calprotectin or C-reactive protein often decrease with successful therapy and correlate with clinical remission.[18] However, mucosal infiltrates remain typically abnormal for a prolonged time. Furthermore, structural changes in the architecture of the intestine indicate chronic mucosal remodeling (eg, shortened and blunted villi) which is associated with damage of the mucus layer and changes in function (ie, changes in the expression of transporters).[19] This results in malabsorption due and altered oxygen levels at the mucosal surface and often leads to increases in aerobic bacteria (e.g., *E. coli*) and decreases in anaerobic bacteria, which correlate with intestinal cytokine expression.[20] As an example, *C. hiranonis* is decreased in 50-70% of dogs with CE, reflecting a dysbiosis pattern associated with chronic intestinal disease. Of note is that these microbiota and metabolomic changes typically persist for at least several months (if not years) and only moderately correlate with clinical remission, as has been shown in various studies.[5,16] This is likely due to persistent changes in intestinal function due to chronic mucosal remodeling and fibrosis.[16,21] Another important new finding is that only a subset of animals with chronic enteropathy has detectable intestinal dysbiosis and/or these metabolic changes. This has been confirmed by next-generation sequencing, quantitative PCR (Dysbiosis Index) and metabolic profiling.[5,6,16,22] This highly suggests that the extent of chronic intestinal inflammation and mucosal remodeling varies between individual animals. Therefore, there is a need to develop a staging system of intestinal damage and dysfunction, to better characterize the extent of changes in individual animals. This will allow for a more accurate prognosis and recognition of which animals need long-term management of the underlying intestinal disease. This will also allow better individualized multi-modal treatment approaches.

Conclusions

Chronic enteropathies encompass various abnormalities in the function of the GIT, such as chronic mucosal remodeling with subsequent loss of function and malabsorption that is associated with inflammation and persistent dysbiosis. The extent of these changes varies between individual patients, and this is likely the reason why the response to therapy varies between animals. Of importance is that many changes on the functional level and microbiome dysbiosis persist for months to years, even if patients are in clinical remission. Therefore, future research should focus on the staging of the intestinal disease to better define the different

underlying pathologies, and define clinical remission vs deep remission (i.e., improvement in mucosal healing). This will allow us to better tailor multi-modal approaches to the subsets of disease, which in turn may allow for better long-term management of intestinal disease. Finally, earlier detection of intestinal changes before clinical signs manifest also may improve the long-term outcome of patients.

Conflicts of Interest

The author is an employee of the Gastrointestinal Laboratory which offers gastrointestinal function testing on a fee-for-service basis, serves on the Nestle Purina advisory board, and is the Purina PetCare Endowed Chair for Microbiome Research.

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Comparative aspects of nutrient effects on the intestinal microbiome in humans and other animals

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Abstract

Over the past 15 years, our comprehension of the gut microbiome has undergone a paradigm shift, evolving from a mere collection of gastrointestinal microbes to a pivotal determinant of human and animal health. This transformation is underscored by fundamental concepts such as a mutual relationship involving evolution, development and metabolism, elucidating the intricate microbial ecosystem within the gut. Initially, correlations between gut microbiome composition and health biomarkers laid the groundwork for diagnostic tools, but recent mechanistic studies have unveiled genetic and molecular underpinnings, offering numerous therapeutic opportunities. The symbiotic relationship between humans, animals, and their gut microbiomes is deeply rooted in a co-evolutionary process, shaping microbial diversity and functional capabilities with implications for health and disease. Diet emerges as a crucial determinant of gut microbiome composition and function, exerting profound effects on metabolic pathways and immune responses. Urbanization and changes in dietary patterns have rapidly altered our microbiome, leading to decreased diversity and potential health implications. Carbohydrates, as primary energy sources for microbial fermentation, elicit distinct responses from microbial communities, influencing their composition and metabolic activities. Understanding these dynamics offers novel strategies for promoting microbial diversity and metabolic health.

Introduction

Over the past 15 years, our understanding of the gut microbiome has undergone significant expansion. Initially considered a mere collection of microbes residing in the gastrointestinal tract, we now recognize its pivotal role in human and animal health. Co-evolution, co-development, co-metabolism, and co-regulation are fundamental concepts shaping our understanding of this intricate microbial ecosystem.[1] Initially, studies focused on correlations between the gut microbiome composition and health biomarker,[2] laying the foundation for diagnostic tools. However, recent studies have propelled us into the realm of mechanistic studies, unraveling the genetic and molecular relationships underlining the gut microbiome function.[3] These developments have unveiled myriad opportunities for therapeutic interventions, ranging from dietary modulation to the use of live biotherapeutics and targeted interventions aimed at specific microbial enzymes and metabolites.[4]

1. Microbiome, Humans, and Animals: An Ancient Relationship

The relationship between human and animal host and their gut microbiomes is the result of a co-evolutionary process.[5] Throughout history, humans and animals have cohabited environments rich in microbial diversity, fostering intricate symbiotic relationships. This coexistence has

influenced not only the composition of the gut microbiome but also its functional capabilities,[6] with simultaneous co-selection of characteristics that are advantageous to both parts in the system. Understanding the evolution of this relationship provides valuable insights into the dynamic interplay between host and microbiota and its implications for health and disease.

2. Diet and the Gut Microbiome

Diet exerts a profound influence on the composition and function of the gut microbiome, serving as a key determinant of microbial diversity and activity. The intricate interplay between dietary components and microbial communities shapes metabolic pathways, modulates immune responses, and contributes to overall host health. In humans, we know that long term dietary patterns are associate with the current microbiome composition, with the balance between the protein and carbohydrate intake having strong effects on major gut microbiome taxa.[7,8] As populations shift their dietary patterns, we also see a shift in the gut microbiome composition,[9] associate with the onset and progression of many chronic diseases. Investigating the impact of diet on the gut microbiome unveils novel strategies for promoting health and preventing disease through dietary interventions and personalized nutrition approaches.

3. Our Changing Microbiome

In evolutionary terms, changes have occurred very rapidly in our lifestyle, resulting in a swift decline in global microbial diversity, including gut-associated microbial diversity. Diet, as the main factor modulating the gut microbial community, has shifted significantly, both in quantity of macronutrients as well as in way that food is accessed and consumed, with profound consequences for the gut microbiome. Additionally, urbanization is associated with decreased gut microbial diversity, such as the loss of members of the *Segatella copri* complex, both in number of different species colonizing the gut, as well as the overall abundance of its members. Lower gut microbial diversity is also associated with the onset and development of chronic diseases. *Segatella* is one of the taxa particularly modulated by carbohydrate intake.[10,11] Understanding the drivers of these changes, including urbanization, industrialization of food production, and antibiotic usage, provides crucial insights into the factors shaping our evolving microbiome and its implications for human health.

4. Effects of Carbohydrates on the Gut Microbiome

Carbohydrates play a fundamental role in shaping the gut microbiome, serving as a primary energy source for microbial fermentation. Different types of carbohydrates, including fiber, sugars, and complex carbohydrates, elicit distinct responses from microbial communities, influencing their composition and metabolic activities.[12] Some unavailable carbohydrates induce a response only certain gut microbiome background, such as the effect observed by resistant starch on a *Segatella* rich gut microbiome context. Exploring the effects of carbohydrates on the gut microbiome provides valuable insights into dietary strategies for promoting microbial diversity and metabolic health.

Conclusions

The gut microbiome, once viewed as a passive bystander, has emerged as a central player in human and animal health. Our understanding of the host-microbiome relationship has evolved significantly over the past decade, revealing a complex interplay between diet, host genetics, microbial communities and consequent health.[13,14] As we continue to unravel the mechanisms governing these interactions, novel therapeutic avenues emerge, offering promise for personalized interventions to promote health and prevent disease.

Conflicts of Interest

The author declares no conflicts of interest.

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