veterinary focus
#28.2
The worldwide journal for the companion animal veterinarian
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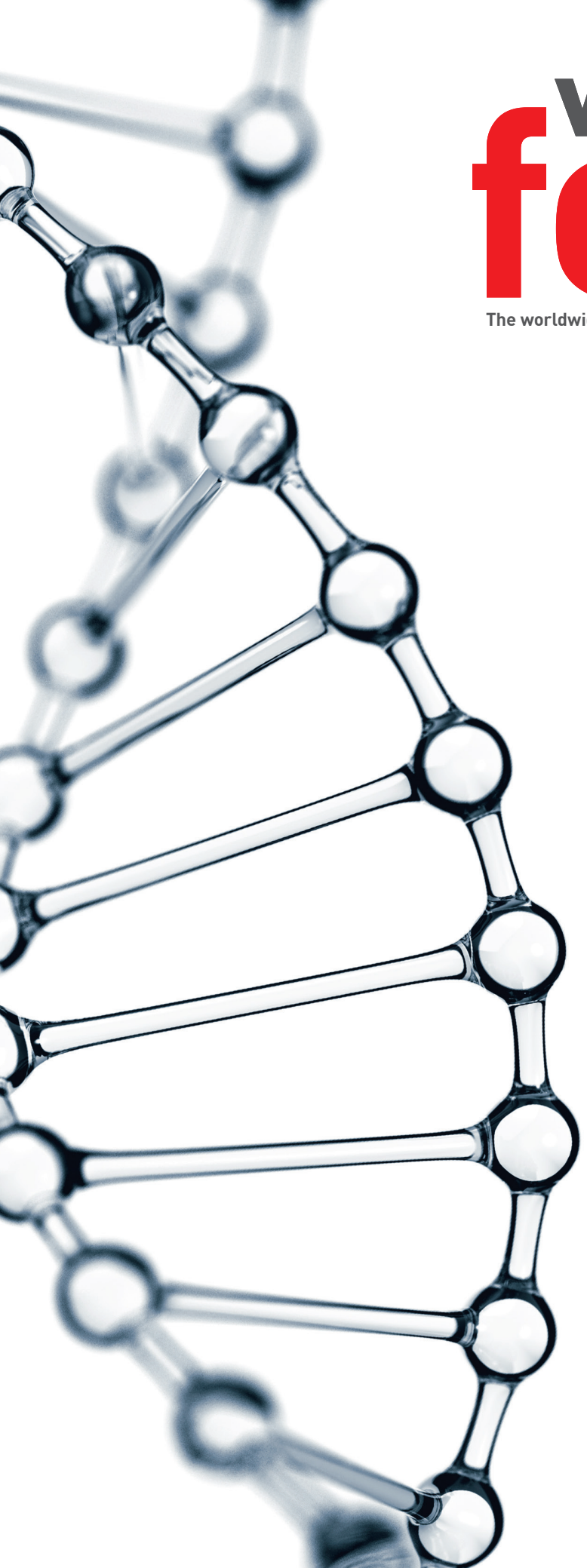

GENETICS AND HEALTH

veterinary focus #28.3

The worldwide journal for the companion animal veterinarian


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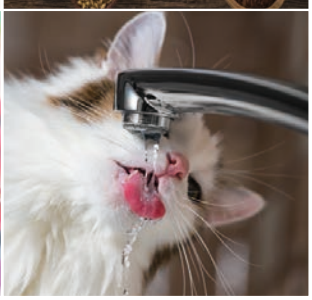
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COMING UP...

In our next issue of *Veterinary Focus*, we will look at different aspects of nutrition:

- Feline feeding behavior
  Jon Bowen, UK
- Dog breeds and their influence on food-related diseases
  Giacomo Biagi, Italy
- The Lewisburg Pet Health and Nutrition Center (PHNC)
  Sally Perea, USA
- The role of Vitamin D in canine disease
  Valerie Parker, USA
- Dietary considerations for dogs with chronic enteropathies
  Adam Rudinsky, USA
- Drinking behavior in cats
  Stefanie Handl, Austria
- Grain-free diets – good or bad?
  Maryanne Murphy, USA
- The benefits of feeding wet diets
  Megan Shepherd and Jessica Benson, USA

ROYAL CANIN®

VARIETY IS THE SPICE OF LIFE

"When a man is tired of London, he is tired of life"

So said Samuel Johnson, the renowned 18th century essayist, moralist and critic, a man who appreciated diversity and believed that the city offered almost unlimited opportunities for novel entertainment, stimulating dialogue and motivating challenges. He was essentially expressing the idea that everyone likes variety, and he was surely right. We would all concur with the sentiment that variety is the spice of life, and things would be very boring if we were all the same.

And so it is with our pets – some people like the long ears of a Bassett, others are drawn to the prick ears of a Corgi; some admire the sleek coat of a Siamese, whilst others prefer the long hair of a Norwegian or the distinctive markings of an Egyptian Mau. And as every veterinarian knows, when breeding for certain features – whether that is ear shape, coat color or facial contours – it is possible to inadvertently introduce unwanted characteristics at the same time.

But keeping up with the diversity of breed problems can be a challenge in itself. Dr. Johnson also said "Knowledge is of two kinds. We know a subject ourselves, or we know where we can find information upon it" and *Veterinary Focus* offers clinicians the latter, addressing as it does some of the breed-related issues that are to be found in our patients today.

Ewan McNEILL
Editor-in-chief

• Focus on *Veterinary Focus*

A **mutation in the canine *ABCB1* gene** results in affected animals being unusually sensitive to many different drugs commonly used within veterinary practice, and clinical signs of toxicity can occur when normally therapeutic doses are administered to dogs with one or two copies of the mutation.

p14

Perianal fistula disease has a suggested multifactorial etiology, including immune dysfunction, food allergy and a genetic predisposition for German Shepherd dogs.

p21

p32

Several **hereditary erythrocyte defects** have been reported in dogs and cats, but such disorders are often only considered after speculative treatments for immune and infectious causes of anemia have failed.

veterinary focus #28.2

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ROYAL CANIN

MAINTAINING GENETIC DIVERSITY: WHY IT MATTERS

It may seem dramatic to claim that many dog and cat breeds, including some very popular ones, could be classified as endangered or critically endangered, but Casey Knox and Katie Lytle present a thoughtful discussion on genetic diversity – or the lack of it – in our pet population, and why it matters.

KEY POINTS

1. Only genetic variants that are passed to the next generation can affect future diversity.
2. Genetically associated disease is not limited to purebred dogs and pedigree cats; such diseases can and do spill over into the mixed breed population.
3. Genetic testing can be performed to assess an animal's overall level of diversity and identify the presence of known disease-causing genetic mutations, thereby informing individualized care.
4. It has been estimated that some dog breeds lost more than 90% of the founders' genetic variants by the sixth generation due to breeding practices.

What is genetic diversity?

The astonishing variety seen within the estimated 400 domestic dog breeds worldwide is a product of, and testament to, their intimate relationship with human development over the last 14,000 years and the selective breeding that has taken place in that time. Consider that the 2 lb (0.9 kg) teacup Chihuahua and the 200 lb (91 kg) Great Dane exhibit a 100-fold size difference, the largest range observed within a mammalian species, and we can begin to understand the degree of impact that humans have had on the domestic dog. To date, 19 million unique genetic variations have been found across the canine genome (1). The pedigree domestic cat, on the other hand, shows less variability and a shorter history of intentional human breeding; there are approximately 70 recognized cat breeds, and the majority of these were only developed in the past 80 years. In both dogs and cats, however, relatively few gene variants, or alleles, are responsible for the variety of physical traits humans have carefully propagated through breeding, compared to the overall genetic diversity found within each species.

We know that in many things "variety is the spice of life" and research has shown that genetic diversity within a species is no different. When we think about the genetic diversity of a population, we consider the variety of genes present within a population *in its totality*. This includes alleles influencing physical appearance as well as biologic processes (see **Figure 1**). In contrast, within an individual we describe genetic diversity as internal diversity, hybrid vigor, or *heterosis*. Diversity can have direct and profound impacts on population health and long-term survival. Zoo conservationists are well aware of these risks, and thus have developed advisory groups and species survival plans for many of the animals in their care, with the goal of cooperatively working together to maximize genetic diversity, appropriately managing the demographic distribution and long-term sustainability of a species or subspecies (2). When we consider our companion animals from this viewpoint, we come to realize that our dog and cat breeds can be thought of in much the same way, as they represent isolated populations with a limited number of individuals, reared and bred primarily in captivity.

Genetic diversity is the resource or "tool library" on which a population relies when faced with a new challenge, whether that is a maladaptive DNA mutation, exposure to a novel virus, or an environmental challenge. The most obvious benefit of a more diverse gene pool is a reduction in the likelihood of recessive, maladaptive mutations pairing up in each generation, manifesting as

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Katherine M. Lytle,

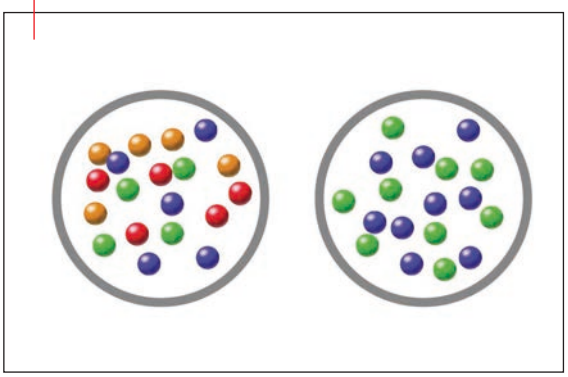
DVM, MPH, MS, Wisdom Health™, Vancouver, WA, USA

Katie Lytle has a passion for science, pets, and people. A graduate of the University of Florida, she has worked in private practice in both mixed animal and small animal settings. She now works for Wisdom Health™ where she is a Genetics Research Project Manager, working to bring genetic disease test arrays to pet owners, breeders, and veterinarians.

disease. We know from the *1,000 Genomes Project* that maladaptive mutations are present in every human; this is termed "genetic load". Researchers have found that the average human has 50-100 disease-causing mutations, as well as 250-300 loss-of-function mutations (3). It is reasonable to assume that dogs and cats would also, on average, carry maladaptive mutations, and recent research has supported this idea. In a study of nearly 7,000 purebred dogs, representing 230 breeds, each animal was tested for 93 risk-associated variants. The researchers found that 17.8% (N = 1,208) of the dogs carried at least one of the tested variants, while 2.5% (N = 170) were genetically affected for a tested disease (4), a result that challenges the idea that genetic disease-associated variants in our canine population are rare. The genetic load of disease-associated mutations is not limited to purebred dogs however. Following the evaluation of nearly 35,000 mixed breed dogs for 13 disease mutations, a separate study found that two of the mutations were detected at a high enough frequency that the "assumption that mixed-breed dogs do not suffer from single-gene genetic disorders is shown [...] to be false" (5).

The cat, on the other hand, has not been subjected to a similarly broad assessment of the genetic health of the fancy cat breeds. However, a comprehensive health survey of over 8,000 cats did find evidence that supported breed specificity among several of the conditions queried (6). Furthermore, evaluation of insurance claims in both Japan and Sweden have also highlighted that certain breeds are more likely to report a particular diagnosis (7,8). In Japan, for example, a cardiovascular diagnosis was more likely from a Scottish Fold, American Shorthair, Persian, Maine Coon, Norwegian Forest Cat, Ragdoll or Bengal than a crossbred cat (7). Although the exact mechanism of action of inbreeding depression on hybrid vigor is not known, many experts believe it to be related to the pairing of disease and loss-of-function mutations. As our understanding of the genetic underpinnings of diseases afflicting our small animal companion breeds expands, we expect to see a paradigm change in our understanding of the impact of genetic disease in our patient population: genetic disease is not rare in our companion animal species.

Figure 1. Two hypothetical representations of the alleles, or gene variants, present in a population. The population on the left has more variants and is therefore more diverse.



How common is low genetic diversity?

In conservation biology, a species is regarded as "endangered" when there are fewer than 500 fertile breeding individuals, because efforts to avoid inbreeding become difficult or impossible at this stage, and the species may not be able to survive indefinitely. A "critically endangered" species is defined as a population with less than 50 genetically unique, fertile breeding individuals, also called the "effective population size" (N_e) (9). In populations this size, genetic theory indicates that inbreeding depression will likely impact the health of the population in the immediate to intermediate future. Given the overall size of dog and cat populations, it may be surprising to the average practitioner to learn that many of our dog and cat breeds would be classified as endangered or critically endangered.

A study estimating the effective population sizes of several common dog breeds, using eight generation or more pedigrees obtained from the UK Kennel Club, found that eight of the ten breeds investigated – Akita Inu, Boxer, English Bulldog, Chow Chow, Rough Collie, Golden Retriever, German Shepherd Dog, and English Springer Spaniel – had effective population sizes of between 33 and 76 dogs (10). A more recent US study using "whole pedigrees" going back to the earliest documented relatives found that effective population sizes were less than 100 animals in nine of the eleven breeds studied, a finding reflected in diversity levels measured by both

pedigree calculations and tested genetic measures. Worryingly, Golden Retrievers in the US were traceable to an effective population size of 6.5 dogs (11). These genetic bottlenecks often occurred within the first decades of a breed's formation; it was estimated that seven of the studied breeds lost over 90% of the founders' genetic variants by the sixth generation, highlighting the severe effects of the breeding practices commonly used. The Golden Retriever, in particular, showed a severe bottleneck, with 10% of the sires used each producing more than 100 registered offspring, followed by the Labrador Retriever with 5% (10). These studies suggest that many of our most popular and common breeds are effectively endangered when evaluated by the parameters applied in conservation biology.

In cats, a global assessment of the overall genetic diversity of representative populations has found that fancy cat breeds have less overall genetic diversity than randomly bred cats. The cat breeds tended to have 10% lower heterozygosity on average when compared to the random bred populations, and several breeds – principally the Burmese and Singapura – were noted to be at risk of suffering from the effects of low diversity (12). Interestingly, one study found that levels of genetic diversity or inbreeding in a cat breed could not entirely be predicted based on a breed's popularity or how long the breed has existed (13). As in dogs, evidence suggests that decisions made by the breeders themselves may be the most significant influence on a particular breed's genetic health and diversity.

How does low diversity come about?

Only genetic variants that are passed to the next generation can affect future diversity. The most influential force in purebred dog and pedigree cat diversity is breeder behavior. Genetic diversity is easily lost within a closed population, but takes a very long time to be gained naturally, assuming the population can survive long enough to re-acquire diversity. Irrespective of the cause, following a genetic bottleneck **(Figure 2)** the resulting decreased genetic diversity may preclude the population from overcoming a new challenge such as an emerging infectious disease. Due to the global nature of most companion animal species, it is unlikely for a single challenge to completely eliminate either the canine or feline species, but unhealthy breeding trends occurring over many generations pose a greater threat to extinction of individual breeds.

Mechanisms that can lower population genetic diversity include:

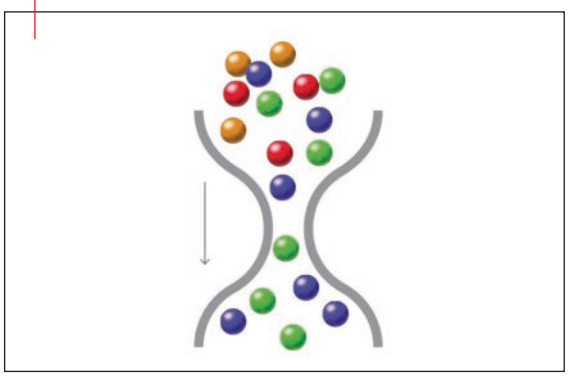
- "Popular sire syndrome" (sometimes called "Matador breeding")
- Inbreeding
- Overuse of males vs. females
- Consolidation of breeding populations by removal of geographic, climatic, or political boundaries
- Infectious disease
- Loss of the lifestyle a certain breed was developed for (*e.g.*, some sled-dog breeds, puffin hunting dogs, duck decoy dog)
- Significant human social events, *e.g.*, warfare

Although dogs were initially bred to perform a particular task (*i.e.*, function-bred), physical appearance is a common goal of modern breeders, while modern cat breeding has focused almost exclusively on physical attributes. The efforts of human-directed matings in dogs and cats have sought to achieve these repeatable results, whether that is a physical "type" or a behavior. Even though our ancestors lacked an understanding of genetics, the concept of heritability has long been accepted. By heavily breeding a particular male that "breeds true" (suggesting high heritability of the trait for his lines), breeders increase the likelihood of producing the sought-after attribute with the least time and investment. Breeding closely related animals is also well accepted to "set type" or create more consistency in the offspring by taking advantage of heritability. Prior to the creation of large, closed-breed dog registries in the late 1800's, and the more modern introductions of parentage testing and artificial insemination, inbreeding effects were tempered by deliberate outcrossing or accidental introductions from other breeds or lines, as well as limitations to

"Genetic diversity is easily lost in a closed population, but it takes a long time to be gained naturally... assuming the population can survive long enough to do so."

Casey A. Knox

Figure 2. A genetic bottleneck, regardless of cause, reduces diversity in the population.



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overbreeding males. Breeds were defined based on phenotype, or physical appearance, or behavior, and less so by pedigree. Today, the major dog registries such as the American Kennel Club are closed registries, meaning no new blood lines can be added to the existing registry; a dog must be related to dogs already in the registry in order to be recorded. On the other hand, both major cat registries in the USA have provisions in place to allow outcrosses in certain cases. Not surprisingly, cat breeders are more apt to outcross than their canine counterparts.

Breeders today often follow similar guidelines as those first established long ago. However, the environment in which these decisions are made is often influenced by the broader breeder network in which they participate. This homogeneity in breeding behavior patterns is potentiated by the internet, which allows greater communication and less geographic isolation of individuals, thereby impacting a larger percentage of the breeding population and potentially risking loss of alleles (14). As breeders are encouraged to breed "the best to the best" it is natural for certain lines to be overemphasized in pedigrees, sometimes on a countrywide basis. In breeding a "popular" sire or dam, or relying heavily on particular lines, other potential mates are excluded. The genes they carry may be beneficial, albeit less common, and are likely to be lost entirely from the population's gene pool if the popular sire or matador breeding is extreme. If the males are bred more often than the females, as is the case in many domesticated species, this will also reduce the size of the genetic population. All of these practices serve to create more consistency in the offspring and the total breed population for the target behavior or physical type, but do so by simultaneously decreasing genetic diversity. This in turn increases the chances of witnessing the negative impact of reduced genetic diversity in the subsequent generation. In general, both dog and cat breeders regularly employ pedigree analysis, with dog breeders also routinely making use of artificial insemination. Only in the latter situation is veterinary advice or assistance sought – the majority of breeding decisions occur devoid of veterinary involvement, even though the outcome can directly affect our patient population.

How does low diversity manifest?

Signs of low diversity are remarkably similar across plant and animal species. A recent evaluation of more than one million humans from over 100 cultural backgrounds found that 10% of the world's population are the offspring of second cousins or closer relatives. When inbred or low-diversity individuals were compared to their peers, researchers found evidence of infertility. Those with moderate inbreeding were 1.6 times more likely, and children of incest (first-degree relatives) 4 times more likely, to be childless than their outbred peers. Researchers also found reduced height and decreased educational performance were associated with inbreeding (15). Research in dogs has found similar negative effects, which are in general proportional to the level of inbreeding **(Box 1)**. There is increased risk of genetic disease, of both complex and simple Mendelian genetic disease, with decreased diversity (16,17). Decreasing diversity is also associated with increased risk of autoimmune disease (18). It is worth noting, too, that the mixed breed and random-bred animals are not protected from genetic disease simply by their lack of pedigree; they owe their genetics in many cases to the purebred or pedigreed population and are thus also impacted (5). Signs of infertility, such as decreased litter size and increased puppy mortality, have been associated with decreasing diversity (19-21). In Bernese Mountain dogs, researchers found that lifespan decreased by 21 days for every 1% increase in the coefficient of inbreeding (COI) (22). Little research is available on the impact of genetic diversity on behavior and performance, but preliminary data suggests hunting ability also decreases (23).

Box 1. Signs of low diversity in dogs include:

Decreased lifespan
Decreased litter sizes
Decreased size
Decreased fertility (ability to conceive)
Increased puppy mortality pre- and post-weaning
Increased risk of genetic disease
Increased susceptibility to autoimmune disease and/or infection

Studies evaluating the impact of reduced diversity in cat breeds are underrepresented, but the effect is anticipated to follow a similar pattern to those found in dogs and humans.

How is genetic diversity measured?

When it comes to assessing an individual's genetic diversity, the most common approach has been to calculate the coefficient of inbreeding (COI) (24). A COI seeks to determine the statistical likelihood that two random alleles at a certain locus in an individual are identical because of common ancestry, meaning the two gene variants came from the same ancestor that was present in both the sire and dam's ancestry. As more familial relationships exist among the individuals in a family tree, the higher the resulting COI percentage. For example, a breeding between full siblings or a parent and offspring would produce a litter with a COI of 0.25 or 25%, while half-sibling matings result in 0.125, and first cousin crosses yield 0.0625. In order to perform the analysis however, a few assumptions are made: the most distant generation used in the analysis is composed of completely unrelated individuals, all offspring of the parents are identical, and any individuals missing from the family tree are unrelated. Thus, COI calculations are heavily dependent on the completeness and accuracy of the pedigree and are approximations only. But as the COI increases, so does the rate of homozygosity and the risk of encountering genetic disease.

"The decreased genetic diversity that follows a genetic "bottleneck" may preclude a population from overcoming a new challenge, such as a novel infectious disease."

Katherine M. Lytle

Initial research suggests direct genetic measures of diversity are more sensitive and useful than COI as a breeding tool (25). Because COI calculations using standard software becomes quite slow or impossible beyond about 10 generations, and many breeds do not have pedigrees back to foundation individuals, it is rare for COI to be calculated beyond 5-8 generations. Furthermore, when calculating a COI, there is no way to account for the impact of inaccuracies in the pedigree; one study estimates the error rate for dogs to be between 1-9% (26) which could substantially impact the COI. Hence the use of direct measures of diversity through genetic testing is a more substantive assessment of an individual's genetic diversity, as it is not bound by the assumptions applied to the COI calculation and is therefore a reflection of the individual's genetic state **(Figure 3)**. When using whole genome sequencing (WGS) or single nucleotide polymorphism (SNP) chip-based diversity measures, it was found that even whole pedigree calculations significantly underestimated COI compared to direct measurements, likely due to the limitations in this method as described earlier. Most breeds studied fell in the range of half to full sibling average relation (COI = 0.125-0.25) (11).

What does the practitioner need to know?

Veterinary medicine, like human medicine, is entering an era of individualized care, and for good reason. Standard practice in human medical care involves obtaining a medical history from new patients that includes questions pertinent to their family's medical history, as well as their individual medical records. Having access to information of this kind is rare for the veterinarian, who is often presented with a single individual, disconnected from familial history, and sometimes without even basic medical records for the patient. Low genetic diversity to a degree that impacts health is relatively uncommon in humans, and legislation exists to prevent it in most countries, whereas high rates of inbreeding or low diversity in mixed-breed or purebred dogs and pedigreed cats is common (12), and genetic disease in dogs (4) and cats (6) is also commonplace. In addition, many of these dogs and cats are of mixed, unknown ancestry, most with inaccurate breed labels applied, giving the false impression of breed-specific risks that may not be present, while predilections that should be considered may be overlooked (27). In counseling cat and dog breeders, education around proper

Figure 3. The diversity curve observed in US field lines of Labrador Retrievers is to the left of the US and UK show lines of the breed, reflecting that field lines are more diverse on average than the show lines.

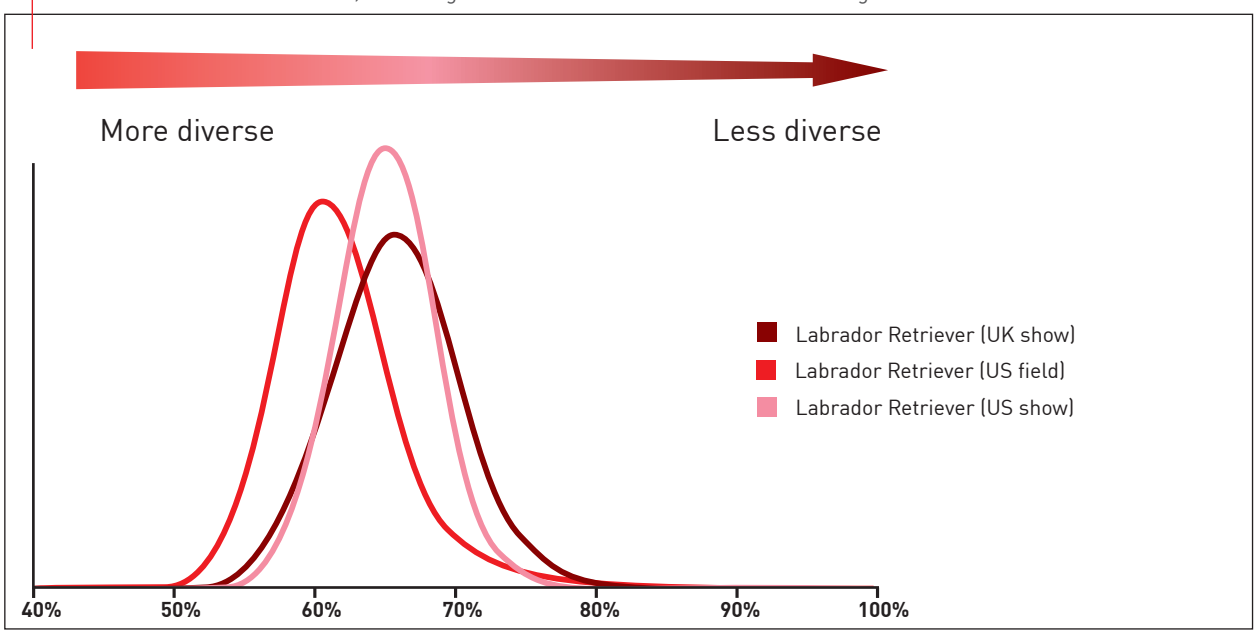

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Figure 4. The Singapura is one of the cat breeds with a small genetic base.

genetic "pre-screening" of breeding individuals for health and diversity – not only of those individuals, but the larger breed population – should be discussed. Encouraging breeders to consider aspects of population genetics, and how the strategy of their own kennel or cattery fits within it, is essential to the future genetic fitness of the breed in question **(Figure 4)**.

In humans, pharmacogenetic panels are commonly requested prior to certain treatments such as anti-depressives, and veterinarians should be aware that similar tests now exist prior to instituting treatment for their patients, most notably *ABCB1* (previously referred to as *MDR1*) testing prior to anesthesia, chemotherapy, and dermatologic treatments (28) **(see page 14).** Commercial genetic testing for known disorders, as well as breed ancestry and diversity testing, are available for dogs, and increasingly for cats as well (29-31).

CONCLUSION

Moving forward, practitioners should work from the presumption that genetic disease in both pedigree cats and purebred dogs (as well as mixed-breed) is quite common. Breed and genetic health screening information, as well as diversity data, can be invaluable when objectively counseling owners and treating pets in everyday practice. Genomic testing methods have proved to be important tools in the analysis of genetic diversity and disease mutations, and should be leveraged to reveal relevant information about our patients; this will enable us to both deliver better pet healthcare and help preserve and improve the diversity and health profile of subsequent generations.

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CLINICAL APPLICATIONS OF GENETIC TESTING

Our understanding of genetics has advanced at a remarkable pace in the last few years, and as a result the options for genetic testing are increasing dramatically. Benefits are already starting to become apparent for the veterinarian, as Jamie Freyer and Angela Hughes describe in their overview of the current situation.

KEY POINTS

1. Genetics is a rapidly changing field, and it will be essential to incorporate genetic testing into the veterinary practice of the future.
2. Ancestry testing can be a helpful tool in the individualization of healthcare plans for mixed-breed dogs, since visual identification of a breed is unreliable.
3. The types of genetic testing that can be useful in clinical practice include parentage testing, direct mutation testing, and phenotype testing.
4. Genetic disease testing is important in both purebred and mixed-breed pets, and judicious use of such tests is the key to decreasing the risk of genetic disease in the pet population.

Introduction

The field of genetics has progressed far beyond most people's recollections of Darwin's Galapagos finches and Mendel's wrinkled peas. In fact, there has been exponential growth in this area within the last few years alone. Most major animal species have had their genomes sequenced, including the human, dog, cat, horse, pig, cow, and mouse. With the new tools available, scientists have learned a tremendous amount about how traits and diseases occur and are inherited.

This knowledge is changing the way that we, as veterinarians, approach our patients. For example, it was only a few decades ago that a serious side effect of ivermectin was identified in some Collies (1) **(see page 14)**. It is now known that the molecular cause is a small deletion in the multi-drug resistance gene which can eliminate the function of a vital drug-transport pump in the blood-brain barrier (2), and it is now evident that this mutation is not only present in many pure- and mixed-breed dogs other than the Collie, but that it involves many drugs beyond ivermectin (3). Such knowledge makes us better clinicians and leads to better care of our patients.

Understanding and utilizing these genetic advances enables clinicians to provide comprehensive care for patients and increase client awareness of the value of that care. We can provide individual care plans for our patients by tailoring our approach not only to our patient's life stage, but also to their breed. Identifying breed-specific risks and customizing a patient's care based on their breed background improves the client's relationship with the veterinary practice and allows for more rapid diagnosis and earlier medical intervention.

Most veterinarians probably already take some breed differences into account when making medical decisions or speaking with owners, but it is important to develop a consistent message that the entire team can relay for each breed. This should be discussed with clients at an early point in any treatment so that they can be prepared, and the best possible wellness plan can be created for their pet. Well-informed owners are more likely to visit the clinic for routine care more often, perform the recommended preventative care, and recognize when pets are ill sooner.

Obviously, this type of breed-specific healthcare is easily applied to the portion of patients that are purebred dogs or pedigree cats, but now this benefit can be extended to mixed-breed canine, and increasingly feline, patients as well. DNA testing to evaluate the purebred ancestors within the recent lineage of a mixed-breed dog has been available for a decade now and the technology is constantly improving. Based on this information, veterinarians

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can help clients better understand their dog and create an individualized wellness plan for them. In this day and age it is a medical imperative to understand each patient's breed history and treat them appropriately.

Phenotypes and genotypes

Though many clients and veterinarians may feel relatively confident in estimating a dog's breed based on visual cues such as coat length, coat color, or other characteristics, the association of breed with phenotypical (appearance-based) features is not as intuitive as it might seem. While some traits show a simple dominant/recessive mode of inheritance, others are polygenic, or caused by a combination of multiple genes, and therefore the donor breed(s) may be difficult to pinpoint. Additionally, dominant traits can come from many generations back in a dog's ancestry, as their method of inheritance makes them comparatively "easy" to pass along.

Common misconceptions regarding appearance abound. For example, it is often thought that the offspring of a dog with short hair and a dog with a long coat should have a medium length coat; in actuality, short hair is dominant in nearly all cases, so such offspring are usually short coated. Another example is the belief that a particular color pattern – such as merle or tan points – is unique to a certain breed; in fact, there are approximately twenty breeds that carry merle coloring, and even more that can carry tan points. The reality is that mixed-breed dogs can have ancestries that belie their appearances, and breed combinations that may be considered unusual are actually fairly commonplace.

A few illustrations can exemplify how difficult it can be to guess a dog's ancestry based on looks alone, and how different the appearances of dogs with similar ancestries can be **(Figures 1-3)**. Essentially, visual identification of breeds is much more complicated than many realize, and studies have shown it to be largely inaccurate (4), so guessing at breed identification should not be relied upon when making important medical decisions. To fully utilize genetic data and breed-specific healthcare in both pure- and mixed-breed patients, veterinarians and their staff should have a basic knowledge of genetics and the types of genetic and phenotype testing available.

Figure 1. The dog on the left was adopted as a "Labrador/Shepherd" mix. The breed makeup of the dog on the right could also be thought to include Shepherd, or possibly Akita. In fact, both dogs are American Staffordshire Terrier/Chow Chow crosses. Both dogs also exhibit some dominant traits, such as short coat, black coloration, or a dark facial mask.

Figure 2. Although quite different in appearance, these dogs are both a mix of Cocker Spaniel and Chihuahua.

Figure 3. These American Staffordshire Terrier/Yorkshire Terrier mixes both illustrate a trait known as "furnishings", the beard and eyebrows commonly associated with Terrier breeds. This is a dominant trait that is often inherited from many generations back in a dog's ancestry, a factor which may make visual identification of the breed difficult. The dog on the right also shows a trait known as chondrodysplasia, a shortening of the limbs commonly seen in the Yorkshire Terrier.

A genetics overview

The basic blueprint for life, or genetic material, is deoxyribonucleic acid (DNA). DNA is composed of two polymer strands of nucleotide bases: adenine, thymine, guanine, and cytosine. These strands can be replicated in preparation for cellular division or transcribed into ribonucleic acid (RNA) and translated into functional proteins. DNA is contained in the nucleus of most cells in the body and is arranged into chromosomes. In dogs, these chromosomes consist of 38 autosomes and a pair of sex chromosomes, X and Y, while cats have similar genetic information packaged into 18 autosomes and a pair of sex chromosomes. Each offspring inherits one set of autosomes and a single sex chromosome (X or Y) from each parent. Within each of these chromosomes, sequences of bases combine to make genes which are essentially instructions for cells to create different proteins.

"Understanding and utilizing recent genetic advances enables clinicians to provide comprehensive care for patients, and increase client awareness of the value of that care."

Jamie L. Freyer

Dogs are one of the most diverse species on the planet. How can a single species range from a tiny Chihuahua to a huge Great Dane, with essentially the same DNA instructions in each? The answer is in the alleles. Alleles are small sequence alterations at the level of the DNA that are often passed onto future generations. Some alleles are translated into differences in the proteins, potentially resulting in a structural or health-related difference between individuals. The dogs within a breed tend to share a lot of the same alleles, therefore they tend to look and act similarly.

As opposed to dogs, where selective breeding for many centuries has created hundreds of distinct breeds, most cat breeds have been created only within the past century and are often based on single gene traits such as coat color or type. For example, the Exotic is a shorthaired Persian and the Selkirk Rex is a curly-coated Persian. Thus, cat breeds, and consequently their alleles, tend to be much more homogenous than dog breeds, resulting in fewer breed-specific genetic diseases and morphologic differences. While the genetic offerings available for cats have traditionally lagged behind their canine counterparts, they are continuing to progress, with over 60 disease and trait mutations now available for testing.

The benefits of genetic testing

Perhaps the most obvious benefit of genetic testing is through disease prevention, by allowing breeders to selectively choose animals that do not produce affected offspring. Remember that disease carriers can continue to be used in breeding programs as long as they are responsibly bred to appropriate clear mates; it is also advisable that potential carrier offspring are tested before breeding.

In addition to utilizing genetic testing for pre-breeding screens, we can also use the information that they provide to care for the general population. As in human medicine, veterinary medicine is moving toward individualized care based on assessments of each animal's risk for disease, with widespread genetic testing becoming the norm for both pure- and mixed-breed patients to assist with early disease detection.

Genetic testing

There are several types of genetic tests available to veterinarians. One such test is used to verify the parentage of a litter, whereby the DNA can be used to examine genetic "markers", or places in the DNA where mutations have created several alleles, to identify whether an offspring genetically matches to the proposed sire and dam. These same markers can be used to confirm an individual's identity in forensics cases or in situations involving a lost or stolen pet.

When the specific mutation that causes a disease or trait is known, that genetic location can be examined in an animal's DNA to determine if the individual carries one or two copies of the specific mutation. This is called a *direct mutation test.* Other genetic tests are based on the concept of genetic linkage, using markers that flank the area of interest in order to predict the genotype at that location.

Technology continues to evolve to make testing a large number of markers feasible. Because of this, more advanced and complex genetic tests are available for research and commercial applications, including genetic disease screening in both dogs and cats, and ancestry testing for dogs.

Phenotype testing

While our knowledge of genetic traits and diseases is growing rapidly, there are still some inherited disorders that are likely multifactorial, or whose genetic cause remains unknown. Because of this, we are unable to directly probe the DNA to determine whether a dog or cat will suffer from these conditions or potentially produce offspring that will. Instead, the patient must be examined for clinical features that can indicate the allele(s) that they likely carry.

As previously mentioned, phenotype is defined as the outward product of the genes. Therefore, a dog with alleles to produce a brown coat has a brown "phenotype". Examples of commonly used phenotype tests include radiography for hip dysplasia and elbow dysplasia, ultrasonography for cardiac disease, and laboratory testing or physical examinations for ocular, thyroid, skin, and auditory diseases.

Case studies

Each breed of dog has its own genetic diseases of concern. For example, it is common knowledge among veterinarians that von Willebrand's type 1 is frequently found in the Doberman Pinscher, and that Dalmatians are predisposed to urolithiasis due to hyperuricosuria. Genetic research continues to identify the presence of clinically relevant mutations in breeds for which the disease has previously not been characterized. One study established the presence of a number of mutations in new breeds, including hyperuricosuria in the Lagotto Romagnolo, a relatively rare breed (5). Mixed breed dogs pose their own unique challenges with regard to genetic disease testing; it is important to understand how the identified genetic risk variants will clinically manifest in dogs of mixed breed ancestry in order to provide proper counseling to clinicians and owners.

One example is the case of a 1.5-year-old female mixed-breed dog (25% each Labrador Retriever and Rat Terrier, 12.5% each Siberian Husky, Golden Retriever, and Australian Shepherd) named Bear **(Figure 4)** who enjoyed running and playing at her local park. On two separate occasions while she was at exercise she collapsed and was unable to

Figure 4. This dog, with a breed makeup that includes 25% Labrador Retriever, experienced episodes of collapse before it was discovered that she carries two copies of the gene for exercise-induced collapse. This knowledge may allow her owner to prevent future episodes, and will reduce the stress experienced by the owner should any episodes occur.

"Visual identification of breeds is much more complicated than many realize, and is largely inaccurate; guessing at breed identification should not be relied upon."

Angela Hughes

stand without assistance, prompting the owner to take her to the emergency clinic for evaluation. While a medical cause could not be identified, Bear successfully recovered from each episode, but not without causing her owner significant concern and financial investment. Genetic panel testing later revealed that Bear was positive for two copies of a mutation in the dynamin 1 gene that is responsible for exercise-induced collapse, as described in several Retriever breeds (6) – explaining the episodes of collapse. Based on this information, the owner was provided with proper counseling with the aim of avoiding future collapsing episodes, and advice on how best to treat them if they did occur.

Numerous accounts of mixed-breed dogs with one or two copies of the multidrug sensitivity (*ABCB1* – previously referred to as *MDR1*) mutation have also been noted. These accounts generally relate to an appreciably delayed recovery from anesthetic procedures that include the use of acepromazine and butorphanol as part of the anesthetic protocol. Processing and elimination of both medications are known to be affected by the *ABCB1* mutation. Owners and clinicians have reported that these dogs require up to four days to return to normal levels of activity and mental acuity compared to dogs without the *ABCB1* mutation who receive the same anesthetic protocol; these animals typically return to normal activities by the following day. Because of this, it is recommended that alternatives to these medications be selected (or dosages reduced) for dogs known to be carrying one or two copies of the *ABCB1* mutation.

Note that a certain "appearance"of mixed-breed dogs does not always indicate the need for *ABCB1* testing. For example, many veterinarians use the "white feet, don't treat" method of identifying patients that may benefit from testing for the *ABCB1* mutation. This colloquialism is actually a misnomer; white spotting is seen (most commonly on the feet) in many dogs who have no herding ancestry, and many herding breed mixes do not actually have

Table 1. Useful resources for genetic disease information.

Resource name	Notable features	URL
Canine Health Information Center	OFA sponsored centralized canine health database. Breed-specific testing protocols.	www.caninehealthinfo.org
Canine Inherited Disorders Database	Searchable genetic disease database with a more clinical focus.	cidd.discoveryspace.ca
Companion Animal Eye Registry	Ophthalmic genotype and phenotype database	www.ofa.org/diseases/eye-certification
Genesis Program	Personalized health recommendations based on breed and size information	www.genesis4pets.com
Inherited Diseases in Dogs	Searchable database of genetic disease information and resources	www.vet.cam.ac.uk/idid
International Partnership for Dogs	Numerous breed- and health-related resources including blogs and online presentations/classes	www.dogwellnet.com
Langford Vets	List of applicable genetic diseases by cat breed	www.langfordvets.co.uk/diagnostic-laboratories/diagnostic-laboratories/general-info-breeders/genetic-diseases-and-cat
My Breed Data	Disease prevalence information searchable by breed and disorder	www.mybreeddata.com
Online Mendelian Inheritance in Animals	Searchable database of genetic disease information and resources	omia.org/home/
Orthopedic Foundation for Animals (OFA)	DNA repository, stores genotype/phenotype information for research	www.offa.org
PennHip	Hip dysplasia phenotype testing information	www.pennhip.org
WSAVA Hereditary Disease Committee	News and resources related to genetic disease	www.wsava.org/Education-1/Hereditary-Disease-Committee-Resources
	Database of available dog and cat tests and laboratories	research.vet.upenn.edu/penngen/AvailableTests/TestsAvailableatLabsWorldwide/tabid/7620/Default.aspx

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Figure 5. This dog's appearance and breed makeup (Beagle, Chihuahua, and Pomeranian) would not necessarily indicate a need for *ABCB1* testing. However, she is heterozygous for the mutation, information which should be used to inform future medical choices.

white feet. Additionally, because there are so many generations of interbreeding with mixed-breed dogs, it is no longer only dogs with recent herding ancestry that carry the mutation **(Figure 5)**.

Genetic counseling in practice

When a patient is diagnosed with a potentially inherited condition, if a DNA test is available it can be used to confirm the diagnosis and possibly provide the client with treatment and prognostic information as described in the examples above. Wherever possible, the owner should be encouraged to inform the breeder of the findings in a constructive way; ethical breeders care deeply about their animals and are trying to produce healthy animals to improve their breed. Additionally, many congenital disorders, whether inherited or environmental, may occur sporadically, and a breeder cannot improve their breeding program if they are not provided with information – good or bad – about the offspring they are producing.

The future

While it is inherently difficult to predict the future of veterinary medicine, there are many exciting research programs underway that hold significant promise. A number of those studies are attempting to understand the genetic influences on more complex diseases including hip dysplasia, atopy, and many cancers. Researchers are also trying to better understand breed structure and diversity, and have developed tools to allow breeders to make better decisions in order to produce healthier puppies and kittens.

It can, however, be daunting for a busy clinician to find accurate disease prevalence information for particular breeds, especially for less common breeds. Good sources of information include the national breed club websites, certain resources available online, and recently published textbooks (to ensure the information is current). Some particularly useful websites are included in **Table 1**.

CONCLUSION

Overall, genetics is an exciting and quickly evolving field of veterinary medicine. Incorporating genetics into practice and focusing on breed-specific concerns will improve the clinician's medical and diagnostic skills, help owners understand the cause and course of a disease (which in some cases will allow for early treatment and delayed disease onset), and enable breeders to produce healthy offspring, all of which will increase client satisfaction. By raising awareness of genetic diseases and the availability of testing, veterinarians can work to alleviate – and perhaps someday eliminate – genetic diseases in the companion animal population.

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THE *ABCB1* GENE IN DOGS

All clinicians will be aware of ivermectin sensitivity in Collies, but what is generally not recognized is that the gene responsible is much more widespread than first thought; moreover the mutation can cause adverse reactions to many other drugs apart from ivermectin, as Cindy Cole explains.

KEY POINTS

- Many breeds of dog carry the *ABCB1* gene mutation and can be susceptible to various drugs used routinely in veterinary medicine.
- Wherever possible such drugs should be avoided in dogs carrying the mutation, but if necessary the clinician should adjust the dosage after careful consideration of the factors involved.

Previously referred to as *MDR1* (Multi-Drug Resistance), the *ABCB1* gene codes for P-glycoprotein (P-gp), an ATPase transporter that moves small molecules (substrates) across the cell membrane. The importance of P-gp was underscored in 2001 when a mutation in the *ABCB1* gene was identified as the cause of ivermectin sensitivity in Collies (1,2). Initially, the clinical significance of the mutation was thought to be limited to macrocyclic lactones, a drug class which includes ivermectin, milbemycin oxime, selamectin, and a number of other molecules commonly used as parasiticides. However, it is now recognized that there is a long list of P-gp substrates, many of which are commonly used in veterinary medicine **(Table 1)**. The mutated *ABCB1* gene results in production of a truncated protein, leading to altered handling of these medications, and clinical signs of toxicity occur when normally therapeutic doses are administered to dogs with one or two copies of the mutation. Various reports have implicated the mutation with toxicity to many different drugs, including loperamide **(Figure 1)** (3), acepromazine (4) and the chemotherapeutic agents vincristine, vinblastine, and doxorubicin (5).

Table 1. Drugs currently known to be substrates for P-glycoprotein.

Chemotherapeutic agents	Cardiac agents	Other
Doxorubicin Mitoxantrone Paclitaxel Vinblastine Vincristine	Digoxin Diltiazem Losartan Quinidine Verapamil	Amitriptyline Phenytoin
Antibiotics	**Steroids**	**Opioids**
Doxycycline Erythromycin Itraconazole Rifampin Tetracycline	Aldosterone Cortisol Dexamethasone Estradiol Hydrocortisone Methylprednisolone	Butorphanol Morphine Loperamide
Immunosuppressants	**Antiemetics**	**Macrocyclic lactones**
Cyclosporine A Tacrolimus	Domperidone	Ivermectin Milbemycin oxime Selamectin Moxidectin
H2-antihistamines	**H1-antihistamines**	
Cimetidine Ranitidine	Fexofenadine Terfenadine	

What breeds are affected?

The mutation is thought to have originated in herding dogs in Britain in the 1800's (6). Although today many of the herding breeds are commonly affected, the breed distribution of the mutation is not straightforward. Collies have the highest frequency of the mutation, with up to 75% of some populations carrying at least one copy (6). Other commonly affected breeds include a number of herding breeds, such as Australian Shepherds and McNabs. Genetic analysis reveals that most likely a single mutation occurred in a shared ancestor of the herding breeds.

Unexpectedly, two breeds in the sighthound group, Long-haired Whippets and Silken Windhounds, also carry the *ABCB1* mutation, and this appears to be a fairly recent phenomenon (6). It has been suggested that the gene mutation was introduced as these breeds were being developed some decades ago, and the implications are clear; if breeders want to create new breeds or designer dogs, undesirable conditions, such as the *ABCB1* mutation, may be unknowingly introduced. The frequency of the mutation in some of the more common breeds is shown in **Table 2**.

What's the risk?

Many veterinarians are familiar with the mutation in herding breed dogs, but may not realize that mixed breed dogs are also at risk. Because the mutation is inherited in a dominant manner, even dogs possessing one copy of the gene are at risk for responding adversely to certain drugs, and ideally all dogs should therefore be tested to determine their *ABCB1* status.

The dosage of some (but not all) medications that are P-gp substrates need to be decreased when administered to affected dogs. The degree to which the dose should to be reduced depends both on the drug and whether the dog carries one or two copies of the mutation. For example, although digoxin, cyclosporine, doxycycline, morphine and most other opioid

Table 2. Breeds affected by the *ABCB1* mutation (frequency %).

Collie	70%
Australian Shepherd, Mini	50%
Australian Shepherd	50%
Long-haired Whippet	50%
McNab Collies	30%
Silken Windhound	30%
Chinook	25%
English Shepherd	15%
Shetland Sheepdog	15%
German Shepherd	10%
Old English Sheepdog	5%
Border Collie	< 5%

analgesics are P-gp substrates, no increased sensitivity to these agents has been observed, and no change in dose is currently recommended (7).

Drugs that require dose adjustments when used in dogs with *ABCB1* mutations are shown in **Table 3** (7). It is difficult to recommend a general dose adjustment for any of the drugs transported by the ABCB1 protein, so it is safest to avoid administering these agents to dogs with the mutation, or if necessary consult a clinical pharmacologist. A clinical pharmacologist should also be consulted prior to administering agents with narrow therapeutic windows, such as chemotherapeutic agents, as they need to be administered with extreme care to dogs. One extremely important point, however, is that all medications approved by government regulatory authorities (*e.g.*, FDA, EMEA) to prevent heartworm disease in dogs are safe and effective regardless of the dog's *ABCB1* status. Higher doses of these macrocyclic lactones, which are commonly used to treat conditions like demodectic mange, can be toxic to dogs carrying the *ABCB1* mutation, even causing death in puppies or infirm animals.

CONCLUSION

The *ABCB1* mutation is present in many more dog breeds than just Collies or even herding breeds. Mixed breed dogs may be at particular risk because veterinarians may not suspect they have the mutation and therefore their *ABCB1* status is rarely determined. While toxicity associated with ivermectin and the other macrocyclic lactones is most closely associated with the *ABCB1* mutation, various other commonly used medications are also P-gp substrates. Veterinarians should recommend testing all dogs to determine their *ABCB1* status in order to provide the safest and most effective therapy for their patients.

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Figure 1. Roxy developed neurological signs following administration of a "therapeutic" dose of loperamide. Subsequent genotyping revealed her to have two copies of the *ABCB1* mutation.

© Courtesy of Dr. Katrina Mealey

Table 3. P-gp substrates commonly used in veterinary medicine that require dose adjustments in dog with *ABCB1* mutations (7).

Acepromazine
Butorphanol
Apomorphine
Loperamide
Ivermectin*
Milbemycin*
Moxidectin*
Selamectin*
Chemotherapeutic agents (Vinblastine, Vincristine, Doxorubicin, Paclitaxel)

*FDA-approved formulations for the prevention of heartworm disease are safe in all dogs regardless of *ABCB1* status.

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COPPER-ASSOCIATED HEPATITIS IN DOGS

Copper-associated hepatitis resulting from copper overload has been well documented in Bedlington Terriers for many years now - and the faulty gene has now been almost eliminated - but other breeds can still be at risk, as Hille Fieten discusses in her paper.

KEY POINTS

1. Copper-associated hepatitis is a hereditary disease present in a number of dog breeds; the dietary levels of copper and the copper:zinc ratio influence copper levels in the liver.
2. To determine copper status in a dog, a histological liver biopsy with quantitative copper measurement is needed.
3. Treatment consists of feeding a low copper/high zinc diet and using D-penicillamine to chelate the copper; long-term dietary management may be an effective supportive measure to delay copper re-accumulation in the liver.
4. Lifelong, continuous chelation therapy is, except in the Bedlington Terrier, not recommended due to the risk of copper and zinc deficiencies developing.

Introduction

Copper is an essential trace element which plays a vital role in a wide variety of biological processes. However, in excessive amounts copper is extremely toxic, because free copper ions can generate reactive oxygen species that damage proteins, lipids and DNA. In order to prevent toxic effects, copper homeostasis in the body is tightly regulated by many different copper-binding proteins (1).

Copper contained in food or drinking water is absorbed via the gastrointestinal (GI) tract. At the basolateral side of the enterocytes, the copper transporter ATP7A is responsible for the movement of copper across the basolateral membrane into the portal circulation **(Figure 1)**. Through the portal system copper reaches the liver, which has a central role in the metabolism, storage and excretion of the element. Within the hepatocytes, copper is chaperoned to distinct cellular organelles and incorporated into proteins to exert its different functions. Hepatocytes also have a storage function for copper and regulate redistribution of copper to other organs in the body. Excess copper is transported over the canalicular apical membrane of the hepatocytes and excreted in the bile. The copper transporter ATP7B, which is structurally related to ATP7A, plays an important role in this excretion process **(Figure 1)**. The protein COMMD1 is believed to be important for proper functioning of ATP7B in the process of biliary excretion of excess copper.

The importance of the transporters ATP7A and ATP7B in copper homeostasis is illustrated by the disruptive effects of hereditary defects in each of these proteins in human patients. Babies and children with mutations in *ATP7A* develop Menkes disease, a lethal disease that is characterized by a severe copper-deficiency phenotype, with neurological defects (2). Mutations in *ATP7B* cause Wilson disease in humans, whereby copper overload in the liver and neuronal tissues results in liver failure and/or neurological or mental disease (3).

Etiology

Copper-associated hepatitis resulting from copper overload in the liver in dogs shows similarities with Wilson disease in humans, except for the fact that neurological phenotypes are not recognized in dogs. The prime example of hereditary copper-associated hepatitis is seen in the Bedlington Terrier **(Figure 2)**. In this breed, a deletion of the second exon of the *COMMD1* gene results in complete absence of the COMMD1 protein in the liver, resulting in decreased copper excretion into the bile **(Figure 1)** (4). In this breed, extreme hepatic copper values have been measured, up to 10,000 mg/kg dry weight liver, and the accumulated hepatic copper inevitably leads to liver cirrhosis. Fortunately, with the development of a DNA test this disease has almost been eradicated from the Bedlington Terrier population.

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After graduating from Utrecht University in 2006, Dr. Fieten obtained her Masters degree in genetic epidemiology at Erasmus University in 2011 before studying for her PhD, which focused on copper-associated hepatitis in Labrador Retrievers. She currently works as an internal medicine specialist at Utrecht University with a focus on hepatology, and she is the president for the Society of Comparative Hepatology. She was recently appointed director of the Expertise Center Genetics for Companion Animals, which aims to decrease the incidence of hereditary disease in dogs and cats.

In a number of other breeds, copper-associated hepatitis is being recognized with increasing frequency. Pedigree studies performed in the Labrador Retriever, Dobermann, West Highland White Terrier, Skye Terrier and Dalmatian have a confirmed hereditary background. A study in the Labrador Retriever detected mutations in the copper transporters ATP7A and ATP7B, with respective decreased and increased hepatic copper levels (5); a female predisposition was also noted. Further, there was a significant relationship between copper intake in the diet and hepatic copper levels, which therefore poses a risk factor for the development of copper-associated hepatitis.

In other dog breeds a similar complex etiology in the development of copper-associated hepatitis is suspected, whereby gene mutations predispose for disease, but the eventual development of clinical

Figure 1. The diagram shows how copper uptake and excretion is regulated by ATP7A and ATP7B transporters. ATP7A located at the basolateral membrane of enterocytes facilitates uptake of copper from the enterocytes into the portal circulation. Subsequently, copper is taken up by hepatocytes and excess copper is excreted via the bile, a process facilitated by ATP7B. In Bedlington Terriers a deletion in the *COMMD1* gene results in complete absence of the COMMD1 protein in the liver, with subsequent decreased copper excretion into the bile. In the Labrador Retriever a combination of mutations in the ATP7A and ATP7B proteins may influence copper homeostasis (5). Copper accumulation in the liver will occur due to decreased function of the ATP7B protein caused by the ATPB^{R1453Q} amino acid substitution. This effect will be attenuated if ATP7A function is concurrently hampered by substitution of the ATP7A^{T327I} amino acid, which results in copper accumulating in the enterocytes and subsequent increased shedding of copper in the feces. The presence of the *ATP7A* mutation alone could theoretically predispose to copper deficiency.

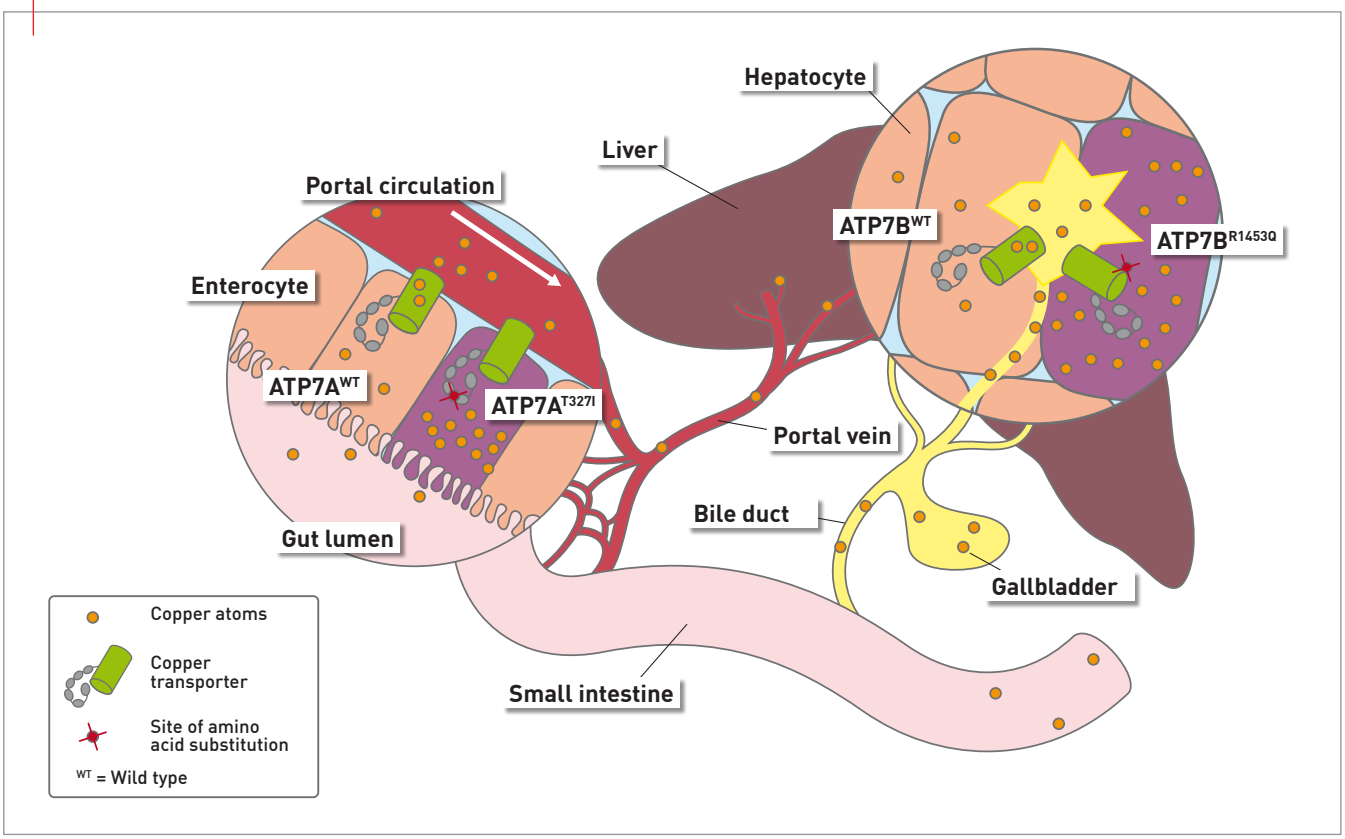

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Figure 2. The prime example of hereditary copper-associated hepatitis is seen in the Bedlington Terrier. However, with the development of a DNA test the disease has almost been eradicated from the breed.

disease depends on environmental influences, in which copper intake plays an important role (6).

Clinical signs

In dogs with early stages of hepatic copper accumulation without overt liver damage, clinical signs are usually absent. When copper continues to accumulate, causing hepatocyte damage, a rise in transaminases, especially ALT, is one of the first laboratory abnormalities, but this can still be very subtle. The process is usually chronic, in which multiple cycles of copper accumulation, hepatocyte damage, phagocytosis of copper-loaded, damaged hepatocytes by macrophages, initiation of inflammation and fibrosis formation occur subsequently. When hepatitis becomes severe or when liver cirrhosis is present, clinical signs appear. The age at first clinical presentation is variable and can range from 2-11 years of age, although the majority of dogs present in middle age (6-7 years).

"Histological evaluation of liver tissue is the only way to diagnose copper-associated hepatitis in breeds other than the Bedlington Terrier."

Hille Fieten

Initially, clinical signs can be subtle and non-specific, including a decrease in activity, decreased appetite, and vomiting. Later, the clinical picture will develop into that of end-stage liver disease, with signs including weight loss, icterus, ascites and hepato-encephalopathy. Clinical signs can be accompanied by increases in liver enzymes, bilirubin and bile acids, a drop in albumin levels and a decrease in coagulation factors (especially fibrinogen) and, with the development of portal hypertension and collateral blood vessel formation, increased blood ammonia levels.

Clinical and laboratory abnormalities cannot distinguish liver disease induced by hepatic copper overload from any other cause of chronic hepatitis. In the Bedlington Terrier, hemolytic crises have been described as the result of massive release of copper from hepatocytes into the circulation, but this has not been reported in other breeds.

In some dogs, including Labrador Retrievers, Fanconi syndrome has been described, resulting from concurrent copper accumulation in the proximal renal tubuli. This is reversible with chelation therapy (7).

Diagnosis

Histological evaluation of liver tissue is the only way to diagnose copper-associated hepatitis in breeds other than the Bedlington Terrier. In this breed the disease is monogenetic and the presence of two copies of the *COMMD1* gene mutation inevitably leads to copper-associated hepatitis if the element is present in the diet or drinking water.

In the Labrador Retrievers, a risk prediction based on *ATP7A* and *ATP7B* genotype may be an option if the dog has a genotype within the extreme categories. However, the actual copper status is dependent on dietary intake of copper during the dog's lifetime, which is often difficult to estimate and which complicates the reliability of the risk prediction for an individual dog based solely on genotype.

Liver biopsies should be evaluated by routine hematoxylin and eosin staining, using the Gordon and Sweet method to detect reticulin, and rubeanic acid or rhodanine for copper. The localization of copper in the centrolobular region of the liver lobule is characteristic for primary copper toxicosis. An inflammatory mononuclear or mixed infiltrate accompanies the copper-loaded hepatocytes. In the more advanced stages of the disease apoptosis and necrosis of affected hepatocytes and copper-loaded macrophages (Kupffer cells) can be recognized in association with the centrizonal hepatocytic copper accumulation **(Figure 3)**. In advanced disease, apoptosis, necrosis, regeneration and typical centro-central bridging fibrosis is seen, eventually leading to end-stage micro- or macro-nodular liver cirrhosis.

To quantify the amount of copper in the liver, a histological grading system, ranging from 0 (no copper), to 5 (diffuse panlobular presence of hepatocytes with many copper-positive granules,

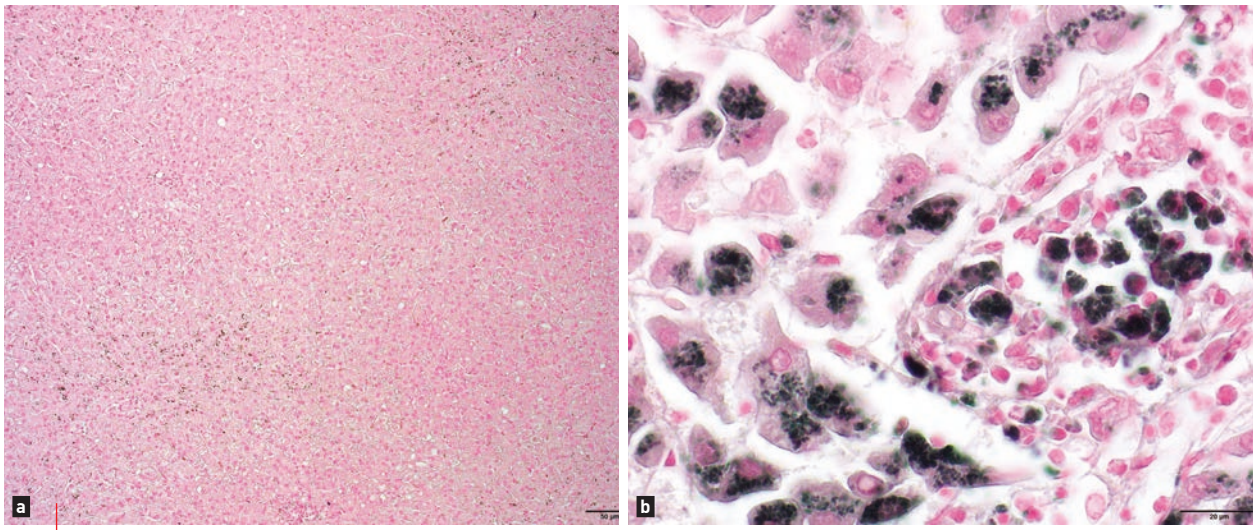

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Figure 3. Histological images of a liver from a dog with copper toxicosis (rubeanic acid stain). This dog was attributed a copper score of 3+. The zonal distribution of copper with accumulation in the centro-lobular areas is clearly demonstrated **(a)**. Magnification shows copper accumulation within the hepatocytes, indicating previous hepatocytic damage with subsequent phagocytosis of cellular debris and copper **(b)**.

usually associated with copper-containing macrophages) is employed. A score of 2 or higher is considered abnormal. When copper is present on histology, measurement of copper in an additional liver sample is warranted. Copper concentration in liver tissue can be assessed quantitatively by irradiation of biopsies and measurement of the induced copper radioactivity or by spectrophotometric methods. Liver biopsy specimens must first be freeze-dried and a dry weight liver (dwl) sample should then be processed. A copper concentration between 150-400 mg/kg dwl is considered normal for the dog. Bedlington Terriers can have hepatic copper concentrations exceeding 10,000 mg/kg dwl, whereas in other dog breeds, levels of up to 4,000 mg/kg dwl have been reported.

Treatment options

The therapeutic approach for copper-associated hepatitis is to create a negative copper balance. This can be achieved by restricting copper uptake, or preventing copper uptake by extra zinc supplementation, and by promoting copper excretion with copper-chelators.

Dietary management

Restriction of copper uptake can be achieved by feeding a diet with a low copper concentration and by avoiding copper-containing mineral supplements. Balanced, low copper diets are commercially available for dogs in the form of "hepatic support diets". An additional benefit of these diets is that they are suitable to feed to dogs with signs of hepato-encephalopathy; they are also usually very palatable, which can be beneficial for animals with a decreased appetite.

Dietary adaptation may prevent further copper accumulation in dogs in early stages of the disease (8), however, response to diet differs between individuals and lifelong monitoring of hepatic copper concentration remains necessary. In Bedlington Terriers with extreme hepatic copper accumulation, dietary intervention is not effective as a sole therapy.

After successful chelation therapy, a low copper/high zinc diet can be beneficial for long-term management of patients, as it delays re-accumulation of hepatic copper (9). Zinc can decrease copper absorption from the gastro-intestinal tract by induction of metallothioneins in the enterocytes that bind copper. In this way copper is lost in the feces during turnover of enterocytes. In some dogs, a single course of D-penicillamine and subsequent dietary adaptation may be enough to prevent progression of the disease.

Chelation therapy

D-penicillamine binds copper at its SH-group and promotes urinary copper excretion (10). It forms relatively stable chelates with all biologically active trace metals, including iron and zinc, and also promotes urinary excretion of these metals. The recommended dose is 10-15 mg/kg twice daily, and

Table 1. Medication for copper-associated hepatitis.

Drug	Dose	Adverse effects	Remarks
D-penicillamine	10-15 mg/kg q12H, separate from meals	Anorexia, vomiting	Most commonly used prediction model for treatment duration available for Labrador Retrievers (11)
Zinc salts; - zinc acetate - zinc gluconate - zinc sulphate	5-10 mg/kg elemental zinc q12H	Generally well-tolerated, but GI side effects may occur	Should not be the sole therapy in clinical cases Slow onset of action Monitoring of plasma zinc concentrations necessary

the drug should be administered separately from meals by 1-2 hours to increase absorption **(Table 1)**. D-penicillamine is absorbed in the stomach and upper intestine. Adverse effects that are often encountered in dogs are anorexia and vomiting, but these may be avoided by gradually increasing the dose. Additionally, the tablet formulation (compounded capsules vs. enteric coated tablets) may have an effect on the occurrence of side effects. Treatment should be continued until normalization of hepatic copper is achieved, which requires regular liver biopsies for assessment of the copper concentration **(Figure 4)**. Bedlington Terriers usually require lifelong, continuous D-penicillamine treatment, whereas in other breeds continuous treatment may lead to a deficiency of copper and possibly zinc. In these dogs an intermittent treatment regime with annual histopathological evaluation of liver biopsies is recommended. The required treatment duration can be calculated based on the copper concentration in the pre-treatment liver biopsy (11).

Zinc

Oral zinc blocks copper uptake in the enterocytes. The recommended dose is 5-10 mg/kg of elemental zinc twice daily **(Table 1)**, with the high end of the dosage range used initially and then reduced for maintenance (12). Again separating administration from meals by 1-2 hours is recommended, as food can decrease the efficacy of the drug.

Excess zinc can be harmful; plasma zinc levels above 1000 µg/dL can cause hemolysis, so to ensure safety, plasma zinc concentrations should be monitored during treatment. Note that it can take three months before dosing with oral zinc effectively blocks copper uptake from the intestines, so this therapy is therefore not recommended as the sole treatment for cases of clinical copper-associated hepatitis.

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Figure 4. Regular liver biopsies (for example, by an ultrasound-guided needle method) are required to monitor the copper concentration in order to assess the efficacy of therapy.

CONCLUSION

Copper-associated hepatitis is a hereditary disease in which dietary copper intake is an important risk factor. The condition poses both a diagnostic and a therapeutic challenge, as clinical signs often occur when liver damage is already present, and the clinical and clinico-pathological abnormalities are not specific for copper-associated liver disease. However, with a relatively early diagnosis and strict monitoring and management, affected dogs may have a normal life expectancy.

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HOW I APPROACH... PERIANAL FISTULA DISEASE IN DOGS

Perianal fistula disease is a common and difficult problem that is seen all too often in clinical practice. Its chronic, progressive nature can make it a real challenge for the veterinarian, but Lindsay McKay offers some pointers to optimize therapy and control the risk of recurrence.

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KEY POINTS

1. Perianal fistula disease is a condition where draining tracts form in the skin and deeper tissues of the areas surrounding the anal region of dogs.
2. Over 80% of affected animals are German Shepherd dogs, and although the etiology is multifactorial, a genetic predisposition seems indisputable.
3. Recognition of an immune-mediated etiology has led to more effective treatment and a better understanding of the need for chronic maintenance therapies to keep the disease in remission.
4. Cyclosporine (with or without ketoconazole) is the most successful therapy, along with tacrolimus for mild forms of the disease or as maintenance therapy. Surgery, including anal gland sacculectomy, may be needed for some dogs.

Introduction

Perianal fistula disease (PFD), also known as canine anal furunculosis, is a condition where draining tracts form in the skin and deeper tissues of the areas surrounding the anal region of dogs. For most affected animals it is a painful and debilitating condition, with clinical signs that vary from licking of the affected area to hemopurulent discharge and odor, to difficulty defecating or even obstipation. Although a wide variety of breeds can be affected by this condition, German Shepherd dogs are over-represented, suggesting a genetic predisposition. Early recognition and treatment is important so that affected animals maintain a good quality of life, and thorough client communication is key, as most dogs will require extended maintenance therapy to keep the disease in remission.

Etiology

Our knowledge of PFD has changed dramatically from when this disease was first described in the 1960's. Originally it was thought to be the result of anatomic factors such as **(i)** a broad-based tail **(ii)** low tail carriage and **(iii)** increased density of apocrine sweat glands in the region surrounding the anal canal (1). For decades the disease was treated with surgical intervention, ranging from tail amputation, debridement and debulking of the fistulous tracts, to anal sac removal. While surgical

intervention may be necessary in some cases, most veterinarians now usually employ medical management to treat PFD. This new approach comes from our more recent understanding that this disease is – at least in part – due to an immune dysfunction. Canine PFD shares many characteristics of certain variants of Crohn's disease in humans, including clinical signs, histopathology, and response to cyclosporine therapy (2-6). Crohn's disease is thought to develop from an autoimmune attack against cells of the gastrointestinal tract or associated microbial antigens (7). A specific causative antigen has not been identified for canine PFD, but it has been proposed that the inflammation is due to inappropriate immune responses to normal flora in the feces or skin of the perineal area (5). Additionally, as with people, a genetic predisposition to the development of the disease has been identified (8,9); over 80% of dogs diagnosed with PFD are German Shepherd dogs (GSD) (10). Research revealed that GSDs with a specific MHC class II allele and haplotype are five times more likely to develop perianal fistulae (9). Lastly, it is also thought that there is a strong correlation between PFD, colitis, and food allergy in dogs (11). Thus, PFD has a complex multi-factorial pathogenesis that likely varies between dogs, especially breeds other than GSD.

Signalment

Whilst PFD is most often seen in GSDs, with studies indicating up to 84% of affected dogs belonging to this breed, reports note other affected breeds include Labrador Retrievers, English Bulldogs, Beagles, Spaniels, Collies, Border Collies and Old English Sheepdogs, as well as mixed-breed dogs (10). This disease is more common in middle-aged dogs, with a mean age of onset of four to seven years, but no definitive sex predilection has been identified (10).

Clinical signs and diagnosis

The diagnosis of PFD is made based on correlation of the pet's signalment, history, clinical signs, and physical examination findings. When a dog presents with PFD, the owner will most often report signs including painful defecation, straining while defecating, blood in stools, constipation or obstipation, diarrhea or ribbon-like stools, increased frequency of defecation, purulent perianal discharge and/or bleeding, perianal licking or scooting, offensive odor and/or weight loss (10). On examination of the perianal area it is common to see multiple fistulous tracts that can involve the whole circumference of the anus in severe cases, as well as moist dermatitis and hemo-purulent discharge **(Figures 1-3)**. Physical examination should include rectal examination; given the pain associated with this condition, sedation or general anesthesia may be required to obtain an accurate clinical assessment. It is very important to correctly diagnose PFD and separate it from other differentials such as chronic anal sac abscessation with secondary fistulae, colitis, perianal tumors (including anal sac adenocarcinoma), caustic injury, and/or untreated dog bite wounds (1). A study showed that 50% of patients with PFD also had a histopathological diagnosis of colitis (12). As both colitis and perianal fistulae can have very similar clinical signs, if both PFD and colitis are present, then colonoscopy and biopsy may be necessary to fully delineate the extent of the patient's disease. Perianal fistulae may also extend to involve the anal sac, and this can affect the dog's overall prognosis as it often makes treatment of the perianal fistulae

Figure 1. A mild case of perianal fistula disease, with a small number of small fistulous tracts.

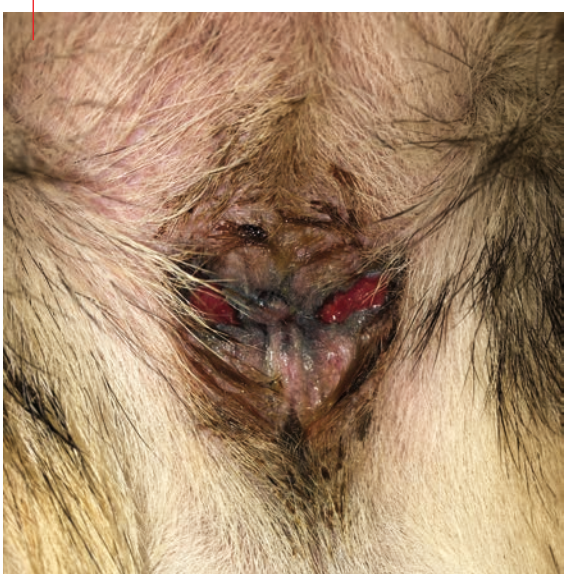

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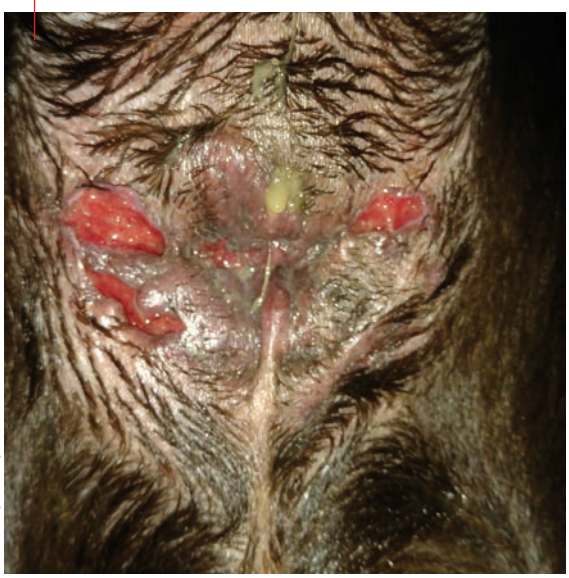
Figure 2. A moderate case of perianal fistula disease with several large fistulous tracts.



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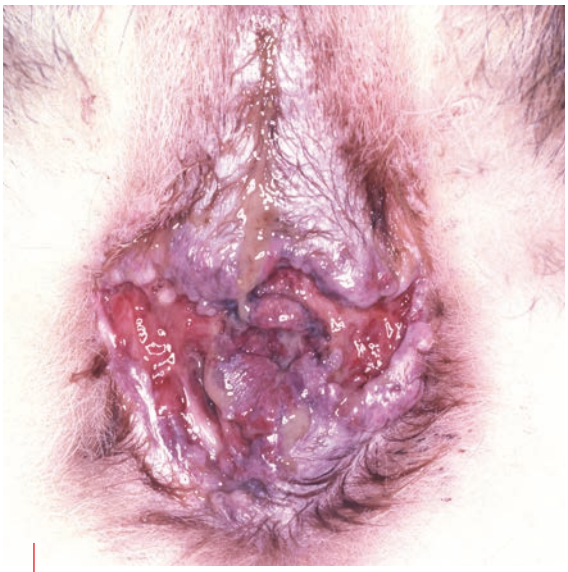

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Figure 3. A severe case of perianal fistula disease approaching 365-degree involvement of the perineal area.

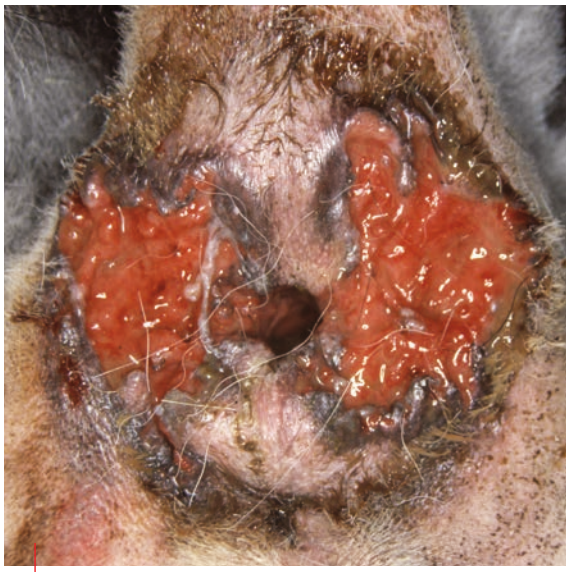

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Figure 4. A severe case of perianal fistula disease with large fistulous tracts that involve the perianal tissues, anus and extend into the rectum.

more difficult, with a higher rate of reoccurrence. I also feel that cytology of the perineal area is important to identify secondary infections, such as *Staphylococcus* bacteria. If intracellular bacteria are present within inflammatory cells, especially diplococci, this likely signals a secondary bacterial skin infection that warrants antibiotic therapy.

Treatment

PFD is a chronic progressive inflammatory condition that tends to increase in severity over time and often has periodic flares. Spontaneous healing is extremely uncommon and lifelong therapy is generally required to keep the disease in remission (10). It is managed with a combination of medical management, dietary therapy and (in some cases) surgery.

Surgical therapy

While PFD was initially described as an anatomic problem with the need for surgical correction, medical management is now the mainstay of therapy. Surgical treatment was usually performed to remove necrotic tissue and destroy the epithelial lining to prevent recurrence, but the reported success rate varied from 48-97% based on method, with recurrence rates approaching 70% (10). Surgical complications were commonly reported, with anal stenosis in up to 15% of cases and fecal incontinence in up to 27% of cases (10). However, when medical management, dietary therapy and surgery were combined, one study noted complete or near complete resolution of fistulae in 88% of cases; almost 80% of dogs had no clinical signs and the remaining 20% had only mild or intermittent clinical signs on a one-year follow-up (13). In this study, 33 affected dogs were initially treated with cephalexin, metronidazole and sulfasalazine in conjunction with a white fish and potato diet for up to 6 months and then had *en bloc* surgical excision of fistulous tracts and bilateral anal sacculectomy. The novel protein diet was continued after surgery. Fecal incontinence was not reported in any of the dogs, and this combination of medical and surgical therapy was associated with overall less complications than previous reports of surgical case management. Medical management with immune suppressive or immune modulatory therapies is very much my first choice treatment for PFD given the current understanding of its etiology and the high rate of recurrence and potentially serious complications seen in some cases managed with surgery. However, for cases of perianal fistulae with concurrent anal gland sacculitis, or if there is communication of a fistulous tract with the anal sac **(Figures 4 and 5)**, surgery may be needed to remove the affected anal sac if medical therapy alone is not effective. I see this scenario uncommonly, but it is an important cause of recurrent PFD and does generally require anal sacculectomy.

Medical therapy

The suspected immunologic etiology of PFD and the similarity to its human counterpart, Crohn's disease, has led to the use of immune-suppressive or immune-modulatory therapies as the primary means of treatment. As PFD is a chronic lifelong condition, the first phase of treatment can be considered the induction phase, whereby the clinician seeks to treat until the disease is in complete or near-complete remission with control of clinical signs. There is then a second phase when maintenance medications are utilized to keep the disease controlled long term. The most common medications used during the induction phase include cyclosporine (with or without ketoconazole), glucocorticoids, azathioprine, and

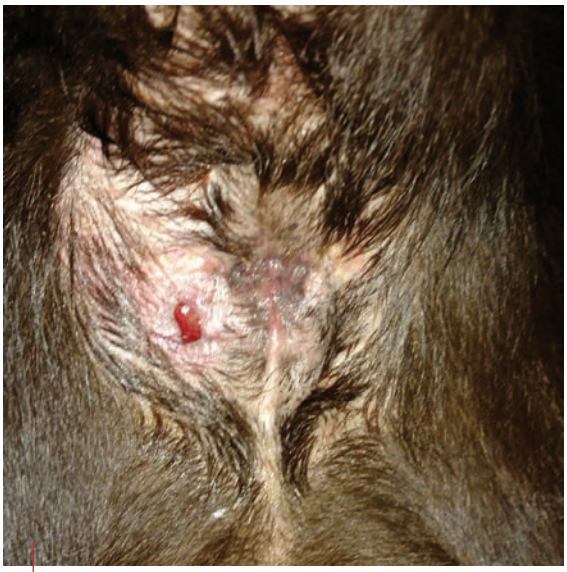

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Figure 5. Post-medical management; a draining tract remains located over the left anal sac. This is the same dog as in **Figure 2** and eventually required anal gland sacculetomy.

topical tacrolimus. Prednisone, at immune suppressive doses (starting at 2 mg/kg q24H), is not my first choice as sole therapy due to the low efficacy reported in the literature; prednisone completely resolved 33% of fistulae, with partial resolution in only another 33% of patients (10). Another immune suppressive therapy, azathioprine, has also been used with moderate success in the treatment of PFD. Given the lag period of several weeks to reach optimal blood levels of azathioprine, concurrent use of prednisone is recommended during the induction phase. The induction dose of azathioprine is 2 mg/kg q24H until fistula remission is achieved, then dropping to 2 mg/kg q48H and lowering to maintenance doses of 1 mg/kg q48H as able. Complete or partial remission was reported in 64% of the 14 dogs treated with azathioprine and prednisone (14). Laboratory monitoring, including complete blood counts and serum chemistries, is needed to monitor for myelosuppression and liver toxicity if using azathioprine. A recent study looked at the use of mycophenolate mofetil in a single dog for treatment of PFD. Mycophenolate mofetil is a lymphocytic immunosuppressive agent used to treat a wide variety of immune mediated diseases, however it was not helpful in resolving PFD after 4 weeks of treatment with the single case reported (15).

"While perianal fistula disease was initially described as an anatomic problem that required surgical correction, medical management is now the mainstay of therapy."

Lindsay W. McKay

The most successful medical therapy for PFD, and my treatment of choice, is cyclosporine. This is a calcineurin inhibitor that inhibits IL-2 transcription preventing the activation and proliferation of T lymphocytes. This immunomodulatory action is thought to treat the suspected immune dysfunction in PFD (16). Looking at several studies that report the resolution of clinical signs and complete clinical remission of PFD treated with cyclosporine, all clinical signs were reported to have resolved in 69-100% of dogs, with full remission seen in 69-93% of dogs (17-20). However, in some of these studies, the authors reported recurrence rates of approximately 50% when cyclosporine therapy was discontinued (17,20). The underlying immune-mediated etiology and high recurrence rate supports a continued maintenance therapy for management of PFD. When using cyclosporine as sole therapy, initial doses range from 4-8 mg/kg q24H until lesions are in remission (11,21). Marked clinical improvement can be seen in as little as two weeks of initiating therapy (17). Once all lesions are in remission, then cyclosporine can be tapered to maintenance dosing. I prefer to maintain the same total daily dose and lower the number of days per week that the medication is given. The ultimate goal is to discontinue the cyclosporine over three to six months, with concurrent use of tacrolimus as maintenance therapy **(Box 1)**. Although some patients will still require cyclosporine, most will tolerate some reduction in the dosing regimen. There has not been a correlation between cyclosporine trough concentrations and efficacy of PFD treatment, thus routine monitoring of cyclosporine levels is not currently recommended (16). The most frequent side effects of cyclosporine are gastrointestinal (GI) upsets (anorexia, vomiting, soft stools or diarrhea) with chronic side effects including gingival hyperplasia and hirsutism. Rarely, papillomatosis, atypical bacterial or fungal infections and psoriaform-like dermatitis have been reported.

As cyclosporine is a costly medication and most affected dogs are large breeds needing higher doses, ketoconazole can be added to decrease the dose of cyclosporine needed. Ketoconazole competitively inhibits the cytochrome P450 3A enzyme, leading to a prolonged serum half-life of cyclosporine and higher blood levels of the drug (22). Recommended dosing protocols for combination therapy with cyclosporine and ketoconazole include doses ranging from 0.5 mg/kg q12H to 5 mg/kg q24H for cyclosporine and doses ranging from 5-7.5 mg/kg q12-24H for ketoconazole (11,22). My current induction therapy of choice is cyclosporine starting at 2.5 mg/kg q24H combined with ketoconazole at 7.5 mg/kg q24H. These combination protocols are estimated to reduce the cost of therapy by up to 70% with no change in efficacy when compared to cyclosporine alone (21).

Ketoconazole can also cause GI upset and (more rarely) hepatotoxicity, thrombocytopenia, or skin reactions including pruritus and alopecia.

Tacrolimus is a topical calcineurin inhibitor with similar immunomodulatory actions as cyclosporine. It can be used as the sole treatment for mild cases of PFD **(Figure 1)**. One study looking at tacrolimus as a sole therapy reported clinical remission in 50% of treated dogs, with significant improvement in clinical signs in 90% of those treated. However, as is common with PFD, recurrence following cessation of therapy was seen in around 50% of cases (23). Another study examined the use of tacrolimus in conjunction with prednisone, a novel-protein diet, and a short course of metronidazole. The authors reported complete resolution in 87% of dogs without relapse over a 2-year period (24). Maintenance therapies used in this study included tacrolimus applied every one-to-seven days, with 73% also continuing on a novel-protein diet and 33% receiving prednisone intermittently to q48H (24). I feel that tacrolimus can be used for treatment of mild PFD (initially as twice-daily treatment) but it is also well suited as a long-term maintenance therapy, applied q24-72H, to prevent flare of clinical signs and fistula recurrence. As a maintenance therapy, treatment can be initiated once oral immunomodulatory therapies like cyclosporine and ketoconazole have begun to control the clinical signs and the perineal area can be treated topically by the owner.

There are new therapies on the horizon for the treatment of PFD. Several recent human clinical trials have used mesenchymal stem cells for management of fistulizing Crohn's disease with positive results. A small pilot study of six dogs with refractory PFD were treated with one injection of human embryonic stem cell-derived mesenchymal stem cells and all were lesion-free three months after injection, but two dogs did relapse six months following the treatment (25). This therapy is currently still in the research phase and not clinically available.

Box 1. How I treat with cyclosporine/ketoconazole/tacrolimus; from induction to maintenance therapy.

- **Initial visit;** induction therapy started. Cyclosporine (2.5 mg/kg q24H) combined with ketoconazole (7.5 mg/kg q24H). Add oral antibiotics if secondary infection is present, *e.g.,* cephalexin (22-30 mg/kg q12H). Plan recheck in 30 days.
- **Recheck 1;** add tacrolimus applied q12H to the affected areas. As long as PFD is in remission, start taper of cyclosporine and ketoconazole. Dose maintained, but frequency reduced to 5 days per week (*i.e.,* skipping Wednesday and Sunday). If PFD is not in remission, maintain current dosing regimen with added tacrolimus. Plan recheck in 30 days.
- **Recheck 2;** if PFD remains in remission, continue the taper of oral medications by reducing dosing frequency to q48H and maintain q12H application of tacrolimus. If PFD is now fully controlled, start taper as instructed above. If PFD is still not in remission, consider 25% dose increase of oral medications. Plan recheck in 30 days.
- **Recheck 3;** if PFD remains in remission, continue the taper of oral medications from q48H to twice weekly and maintain tacrolimus q12H applications. Plan recheck in 30 days.
- **Recheck 4;** if PFD remains in remission, discontinue oral medications and maintain tacrolimus q12H applications. Plan recheck in 30 days.
- **Recheck 5;** if PFD remains in remission, lower application of tacrolimus to q24H. Plan update in 30 days. If pet is still doing well, tacrolimus application may be decreased further until the lowest frequency of application that keeps the fistulas in remission is determined – this is often q24H to weekly.

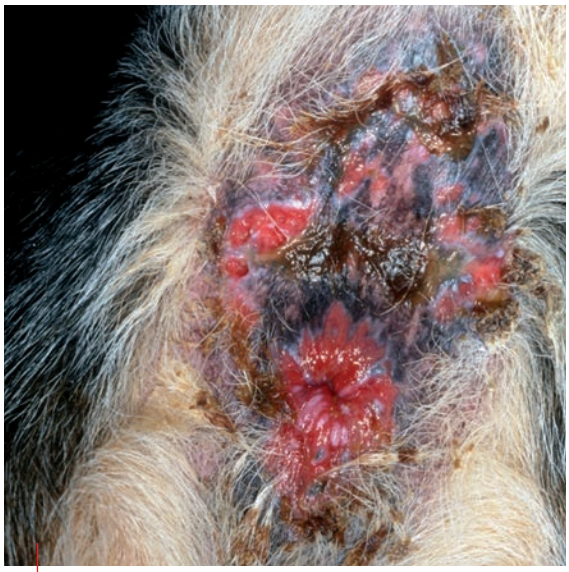

Figure 6. A moderate to severe case of perianal fistula disease with concurrent mucocutaneous pyoderma.

Dietary therapy

Due to the suspected link between PFD, colitis, and food allergy in some dogs, an elimination diet with either a novel-protein or hydrolyzed diet may be helpful in the management of affected animals (11). A retrospective study looking at adverse food reactions with dermatologic signs found that while 100% of affected dogs exhibited pruritus, 3/16 also had PFD. All three dogs with PFD were GSDs, so while this data may not be extrapolated to other breeds, there may be an association between PFD and food allergy in this breed. To further support a role of adverse food reaction in the pathogenesis of PFD, as discussed above, the feeding of a novel protein diet led to lower recurrence rate after surgical excision of diseased tissue and bilateral anal sacculectomy was also performed (13). The lower incidence of recurrence was attributed to the novel protein diet. Because medications such as cyclosporine can potentially cause GI upset, I typically recommend changing to a novel protein diet during the maintenance phase of medical therapy when the pet is on less oral medications. I especially encourage the owner to perform an elimination diet trial if the dog is also exhibiting other signs of food allergies such as pruritus, or developing recurrence of lesions as the oral medications are tapered or flaring on lower doses of maintenance medications. I recommend using either novel protein diets or hydrolyzed diets for at least 8 weeks for my elimination diet trials.

Antibacterial therapy

Secondary bacterial skin infections can be a common sequelae of PFD **(Figure 6)**. Topical therapy to keep the perineal area clean is helpful to treat and/or prevent bacterial skin infection. This can include clipping excess hair and using topical antiseptics to cleanse the area, and applying topical antibiotic therapy. Oral antibiotics may also be needed depending on the extent of the infection. I prefer either cephalexin (22-30 mg/kg q12H) or cefpodoxime (5-10 mg/kg q24H) for empirical antibiotic therapy, but metronidazole (10-15 mg/kg q12H) or amoxicillin-clavulanate (14.5-22 mg/kg q12H) are also good choices. If the infection is refractory to empirical antibiotics, then culture and susceptibility testing is recommended. I also feel that adjunctive topical antibiotic therapy with mupirocin or silver sulfadiazene can also be useful as long as the patient can tolerate the application.

CONCLUSION

Perianal fistula disease is a chronic, potentially debilitating disease that was historically difficult to manage and had a high recurrence rate; it often resulted in a guarded prognosis. However, our newer understanding of its immune-mediated etiology has led to more effective therapies and an understanding of the need for chronic maintenance regimens to keep the disease in remission. My treatment of choice is cyclosporine, usually with ketoconazole, later adding tacrolimus as a long-term maintenance therapy to help prevent relapse of the disease. If there is persisting anal sac involvement, anal gland sacculectomy may be required after initial immunosuppressive or immunomodulatory therapies, but we also need to be considering the role of food allergies and the potential for elimination diet trials as part of the management regime.

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THE TOM AND JERRY SYNDROME

The current approach to seizures in cats is largely based on our knowledge of canine epilepsy, but recent evidence suggests that this could be a simplistic and possibly misleading method, as evidenced in this paper from Mark Lowrie and Laurent Garosi on one specific feline disorder.

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KEY POINTS

1. Feline audiogenic reflex seizures (FARS) – also known as the "Tom and Jerry Syndrome" – represent a condition in older cats that suffer from myoclonic seizures with occasional generalized tonic-clonic seizures.
2. The condition is thought to be degenerative, with a predisposition to Birman cats. Half of all affected cats are deaf.
3. Seizures are often induced by noise; these seem to include very high pitched, relatively quiet sounds such as the jangling of keys or typing on a keyboard.
4. Treatment with levetiracetam has been shown to be very effective for the management of myoclonic seizures.

Introduction

Information regarding feline disorders has often been obtained by extrapolating knowledge from dogs with similar conditions. No better example of this can be found than in feline seizure disorders, whereby treatment and management has mirrored what is considered appropriate for dogs with epilepsy. However, the last decade has given more focus to specific seizure disorders in the cat, and feline audiogenic reflex seizures (FARS) – also sometimes colloquially known as the "Tom and Jerry Syndrome" after the children's cartoon series – is one such disorder whose recognition may alter our management of some aspects of companion animal epilepsy in the future. A description of FARS and its setting within feline epilepsy is given in this article.

Seizure classification

Epilepsy is defined as chronic, recurring seizures and is therefore not a single disease but a group of heterogeneous disorders (1). Historically, seizures have been divided according to either the etiology or clinical type (semiology).

According to etiology

The three etiological classifications for seizures are idiopathic (or primary) epilepsy, symptomatic (or secondary) epilepsy, and reactive seizures (2). Symptomatic epilepsy is a term used to describe seizures that result from an identifiable intracranial structural lesion (*e.g.,* a brain tumor **[Figure 1]**, inflammatory or infectious brain disease, and congenital intracranial malformations such as hydrocephalus). Reactive seizures are the reaction of a normal brain to a systemic metabolic or toxic event. When the metabolic or toxic event passes, the cat does not have recurrent seizures, and therefore reactive seizures are not considered a form of epilepsy. Idiopathic (or primary) epilepsy is a term that is reserved for patients with chronic, recurring seizures that have no detectable underlying abnormality. Early studies reported that up to 87% of cats with recurrent seizures were diagnosed with an identifiable cause for the epilepsy, although inclusion criteria in these studies excluded cats with partial seizures relating to primary epilepsy (3). This figure is now disputed, with subsequent larger studies demonstrating the proportion of epileptic cats with structural or reactive seizures to be much lower, at around 10% (4).

According to semiology

Semiological classification is based on the widely accepted concept that seizures can be generalized or focal (5).

i) **A generalized seizure** is considered to arise from within both hemispheres of the forebrain (although not necessarily the entire cortex).

- **Generalized tonic-clonic seizures (GTCS)** are the most common form of generalized seizure, with clinical recognition being relatively straightforward. The cat will suddenly fall to the ground and lose consciousness, whilst showing chomping and chewing jaw movements, foaming at the mouth, paddling of the legs, and sometimes voiding urine or feces. The seizures usually last no more than a few minutes.

- **Generalized myoclonic seizures** are generalized seizures by definition, in that they involve both cerebral hemispheres and involve loss of consciousness. However, they are often so brief in nature that an objective measurement of consciousness is impossible, and observation of an episode may pass without any obvious discernible loss of awareness. Myoclonic seizures are sudden, brief, shock-like involuntary contractions resembling the effect often seen in response to an electric shock (6).

- **Generalized absence seizures** describe episodes whereby a cat loses awareness of its

"Feline audiogenic reflex seizures (FARS) is now a recognized disorder that may alter our management of some aspects of companion animal epilepsy in the future."

Mark Lowrie

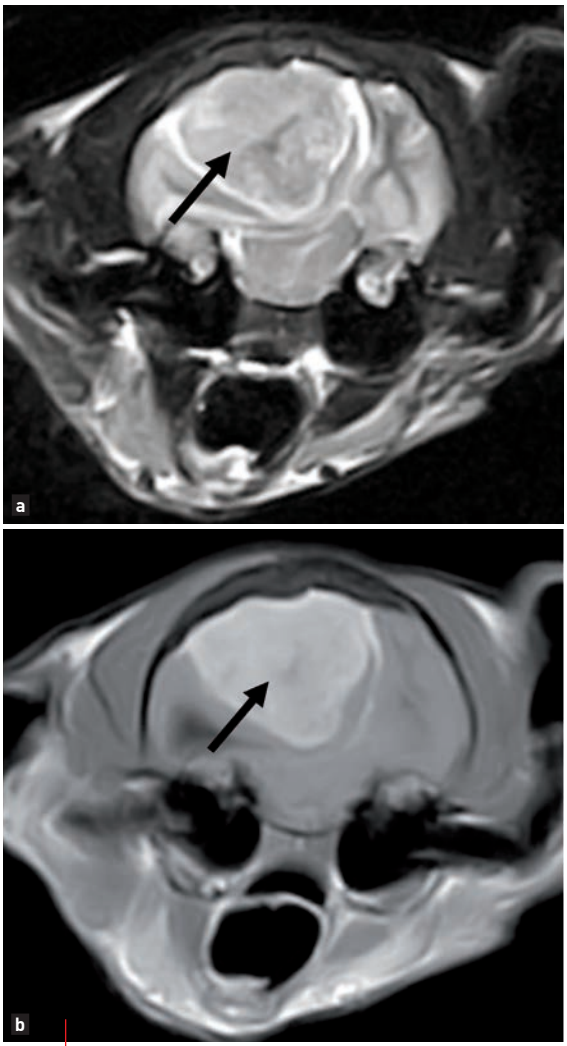

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Figure 1. Brain tumors can often cause seizures in cats. A T2-weighted **(a)** and T1-weighted **(b)** post-contrast transverse image of the brain taken at the level of the tympanic bullae in a 12-year-old cat. A large extradural mass (black arrow) is seen in the forebrain that is isointense to grey matter on T2 but hyperintense on T1 with homogenous contrast enhancement. The imaging characteristics are compatible with a meningioma.

surroundings for a transient period of time, staring vacantly into space and not responding to stimuli, such as the owner calling its name (7). These are also known as "petit mal" seizures.

ii) A **focal seizure** is thought to arise within a specific area of the forebrain and confined to one hemisphere. Focal seizures can spread within the same hemisphere, or to areas in the other hemisphere, and evolve into a generalized tonic-clonic seizure. Further classification depicts a "simple" and a "complex" form of partial seizure, in which a subjective assessment of consciousness is required:

- **Simple partial seizures** describe an unaltered consciousness, with asymmetric localized motor signs, *e.g.*, facial twitching.

- **Complex partial seizures** differ in that they involve some degree of impaired mentation.
  Two types are recognized in cats. The first are referred to as orofacial seizures, with the defining features of hippocampal pathology visible on MRI and serological evidence of voltage-gated potassium channel complex antibodies (8). This form appears similar in many ways to limbic encephalitis in people. The second type covers psychomotor seizures which are "behavioral" seizures involving the limbic system that may present as rage, aggression without provocation, fly-catching, running in circles, floor licking, vocalization, tail chasing, or star-gazing, etc. (9). *Feline hyperesthesia syndrome* has been considered by some to be within this categorization. Psychomotor seizures are controversial in the sense that they may represent a form of obsessive compulsive disorder, but no strong evidence exists to support or refute this view.

Reflex epilepsy

Reflex epilepsy is a condition in which seizures can be provoked by a stimulus such as light, sound or touch (10). Individuals with pure reflex epilepsy have seizures almost exclusively in response to specific stimuli, although spontaneous seizures may also occur (11). Audiogenic and photosensitive epilepsies have been documented in both dogs and cats (7,12-14). They are still relatively rare, although when observed it is important to recognize them as such because reflex seizures frequently require different anti-epileptic medication and management to spontaneous seizures. FARS are becoming increasingly recognized and may be more common than first thought (7).

Features of FARS

FARS represent a condition in older cats that suffer from myoclonic seizures with occasional generalized tonic-clonic seizures. There are key criteria that form the phenotype for FARS and hence a combination of these features enables a diagnosis to be formed.

"The most common type of seizure identified with FARS is a generalized myoclonic seizure; these can occur frequently, with many cats experiencing ten or more per day."
Laurent Garosi

Seizure type

The most common type of seizure identified with FARS is a generalized myoclonic seizure. These can be reported to occur frequently, with many cats experiencing ten or more per day. Although the majority of episodes are noise-induced this is not a strict criterion for diagnosis. More infrequent generalized tonic-clonic seizures (GTCS) may be observed. These can be the result of a train of noise-induced myoclonic seizures culminating in a GTCS or may be spontaneous, discrete GTCS occurring in the absence of an obvious trigger. A less common seizure observed with FARS is a generalized absence seizure, with a prevalence of 6% in the reported FARS population (7).

Signalment

FARS tend to occur in very old cats in the second decade of life, with a median onset of 15 years (7). This geriatric onset is therefore important, as it reflects the likely degenerative nature of this condition. Although any breed of cat may be diagnosed with FARS, Birman cats seem to be predisposed to this condition, with one in three cats with FARS being of this breed **(Figure 2)**. Furthermore, all Birmans that have been reported with this condition to date are of the blue or seal point variety. No sex predisposition is noted.

Clinical signs

Clinical signs other than seizures are also reported with FARS, although these tend to present two or more years following the first observed seizures. Signs include paresis, ataxia, depression, weight loss, unwillingness to jump, loss of learned behavior and head pressing. Another notable feature of FARS is that up to 50% of cats with the condition are reported to be deaf (7). This paradox currently has no explanation.

Noise triggers

The noises provoking FARS tend to be high-pitched, relatively quiet sounds, *e.g.*, a computer keyboard tapping or mouse clicking; paper or plastic bags

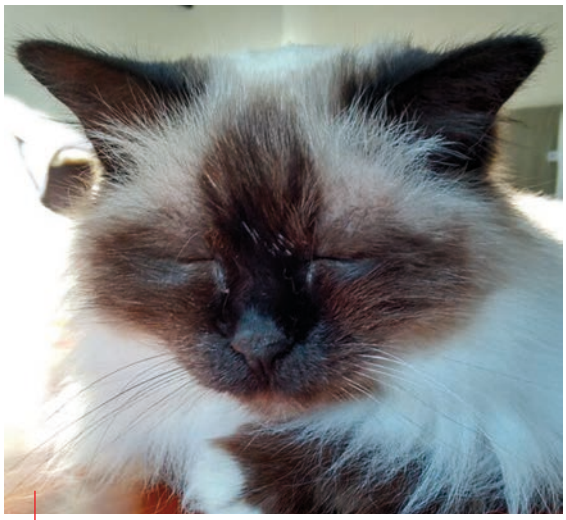

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Figure 2. FARS occur in pedigree and non-pedigree cats, but among the pedigrees, the Birman breed is over-represented. FARS is predominantly a problem of older cats – the reported median age of seizure onset is 15 years, with a range of 10 to 19 years.

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Figure 3. A domestic shorthaired cat that suffers frequent (daily) myoclonic seizures and occasional generalized tonic-clonic seizures as a result of FARS. All seizures are induced by noise, and avoidance of sound helps in reducing the episodes but does not eliminate them completely given the sensitivity this cat has to sounds in general.

crinkling; the sound of cutlery on a ceramic plate when eating or preparing food; foil crinkling; and the clinking of keys. There have even been some more unusual triggers, such as walking on a wooden floor with bare feet or squeaky shoes, and the short sharp scream of a young child. If the volume of the sound increases this has been found to increase the severity of the seizures. If the sound is persistent then this can result in repeated myoclonic seizures, occasionally culminating in a GTCS **(Figure 3)**. This phenomenon is known as *audiogenic kindling*, in which lots of small sound stimuli culminate to produce a larger response, in this case a GTCS. Repetitive sound-induced seizures (*i.e.*, audiogenic kindling) gradually induce the transference of epileptic activity from brainstem (myoclonic seizures) to forebrain structures (generalized tonic-clonic seizures), and this can accompany behavioral changes in these cats (7). The term "brainstem seizure" is therefore used to depict seizures starting in the brainstem and propagating along the limbic structures to result in the more classical forebrain seizures familiar to most veterinarians (7).

Treatment

From the few available studies in veterinary patients, successful treatment of myoclonus appears limited, a finding that mirrors the human condition. A recent study has demonstrated that levetiracetam reduced myoclonic seizure frequency by more than 50%, whereas phenobarbital had a negligible effect in the management of myoclonic seizures in cats with FARS (15). Fifty-seven cats with FARS were administered either phenobarbital (n=29) at 3-5 mg/kg q12H or levetiracetam (n=28) at 20-

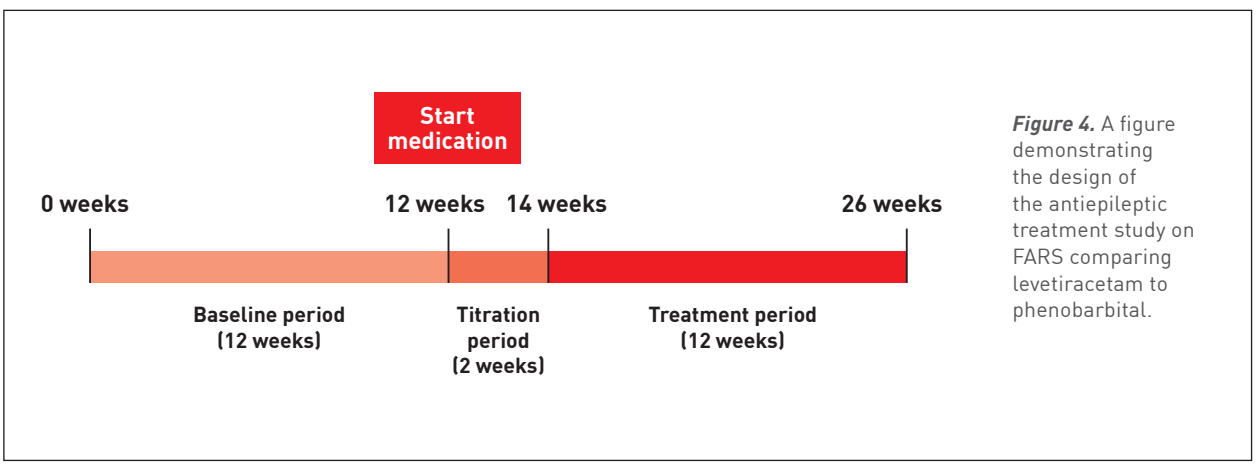

Figure 4. A figure demonstrating the design of the antiepileptic treatment study on FARS comparing levetiracetam to phenobarbital.

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Table 1. Efficacy of levetiracetam and phenobarbital in the management of feline audiogenic reflex myoclonic seizures (15).

	Levetiracetam group	Phenobarbital group	*P* Value
Number of cats achieving ≥ 50% reduction in number of myoclonic seizure days/week	28 (100%)	1 (3%)	< 0.001
Mean % reduction in number of myoclonic seizure days/week	98.8 (±4.7)	2.8 (±23.3)	< 0.001
Number of cats achieving myoclonic seizure freedom	14 (50%)	0 (0)	< 0.001
Mean % increase in myoclonic seizure-free days	95.7 (±8.8)	-57 (±54.5)	< 0.001

25 mg/kg q8H. Inclusion criteria stated that all cats had to have had at least 12 myoclonic seizure days in a 12-week baseline period prior to starting the new antiepileptic medication. Cats were monitored for 12 weeks on the antiepileptic medication **(Figure 4)** and the results are presented in **Table 1**. It was found that 100% of cats treated with levetiracetam had a reduction of at least 50% in the number of myoclonic seizure days, whereas only 3% of cats in the phenobarbital group showed a similar response. Half of the cats treated with levetiracetam had no further myoclonic seizures, whereas the phenobarbital group all continued to have myoclonic seizures. This study strongly supports the use of levetiracetam in myoclonic seizures and this finding echoes similar studies performed in people with myoclonic epilepsy. There is also the possibility that treatment with levetiracetam prevents audiogenic kindling, and as such may retard or even prevent progression of the condition. However, further studies are required to prove this (15).

CONCLUSION

Myoclonic seizure in older cats may be easily dismissed as being an age-related finding with no treatment. The work performed looking at FARS suggests that this condition is readily treatable with levetiracetam, although the long-term prognosis is guarded, as the condition will usually progress slowly over several years, leading to signs of forebrain disease developing. The hope is that further research will raise awareness among veterinarians in practice regarding this syndrome and its treatment.

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HEREDITARY ERYTHROCYTE DISORDERS

Several hereditary erythrocyte defects have now been discovered in both dogs and cats, and recent research has resulted in a great deal of new information. Urs Giger presents an overview of the current situation and offers some pointers for the diagnosis and management of these conditions.

KEY POINTS

1. A full laboratory evaluation is required to validate the diagnosis of any suspected erythrocyte disorder.
2. An inherited erythrocyte defect should be considered in animals with Coombs-negative hemolytic anemia, a lack of exposure to toxin or infection, and adequate kidney and liver function.
3. Animals with hereditary defects may adapt well, while others experience severe hemolytic crises requiring supportive therapy including transfusions.
4. Dogs with phosphofructokinase deficiency suffer from exercise intolerance and will perform poorly as field-trial dogs.

Introduction

Anemia is one of the most common clinical signs in companion animals and is a frequent finding on laboratory blood tests. Although acquired conditions (such as infections, immune disorders, toxicity, blood loss, and chronic organ failure) represent the main causes of anemia, hereditary erythrocyte defects leading to anemia are becoming increasingly important in clinical practice. Several hereditary erythrocyte defects have been clinically reported in companion animals, and much more new information, including the molecular basis of some disorders, has emerged to readily permit specific clinical diagnoses (1,2). Frequently, these causes are only considered after speculative treatments for immune and infectious diseases have failed, or if the anemia is recurring or persisting, despite the fact that in some breeds such conditions are relatively common and well recognized. This brief review focuses on the peculiarities of erythrocytes and key clinical, diagnostic and management aspects of hereditary erythrocyte defects in dogs and cats.

Canine and feline erythrocytes

Although the major structural features and functions of erythrocytes are similar among all mammals, some noticeable differences exist between canine and feline erythrocytes. Feline erythrocytes are much smaller than canine erythrocytes and thus spherocytes cannot be recognized in cats. However, the erythrocytic hemoglobin concentrations (mean cellular hemoglobin concentration, MCHC) are the same for both species. Interestingly, a few normal variations are recognized; for example, erythrocyte microcytosis is observed in many Akitas, whilst erythrocyte macrocytosis is seen in Miniature Poodles. The normal lifespan of erythrocytes in dogs and humans are similar (100-120 days), but only 70-75 days in cats.

Devoid of a nucleus and mitochondria, erythrocytes have a limited and specialized metabolism that enables them to survive in the circulation and to adequately transport oxygen. Energy is generated almost exclusively through anaerobic glycolysis (the Embden-Meyerhof pathway). The hexose monophosphate shunt reduces pyridine nucleotides and glutathione, which are necessary for degradation of oxidants, thereby preventing membrane damage and hemoglobin denaturation (oxidation). In addition, the methemoglobin or cytochrome-b5 reductase system reduces heme iron from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) form; only reduced hemoglobin can bind and carry oxygen. The Rapoport-Luebering pathway is responsible for the synthesis of 2,3-diphosphoglycerate (DPG), which influences the oxygen affinity of canine – but not feline – hemoglobin. Indeed, the erythrocytic DPG concentration is similar in dogs and humans, but very low in cats.

Dogs and cats apparently have embryonic, but no fetal hemoglobin. With the exception of some Japanese breeds, which have two hemoglobins (HbA and HbB), only one adult hemoglobin has been found in dogs. However, further studies are needed to characterize canine hemoglobin. Interestingly, cats have one α-globin and at least six different β-globins, and with each cat having

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one to four different β-globins, many hemoglobin patterns are recognized.

The erythrocyte membrane consists of a lipid bilayer, affixed to a membrane skeleton, which determines the discoid cell shape and enables easy deformability when passing through capillaries. Various transmembrane glycoproteins function as receptors or transporters. Canine and feline erythrocytes lose their Na+, K+-ATPase during late maturation in the bone marrow, owing to proteolysis (with the exception of erythrocytes from some Japanese breeds). Therefore, erythrocytes' high sodium and low potassium concentrations are similar to those of serum electrolytes. Consequently, hyperkalemia generally does not occur after intravascular hemolysis or when unseparated clotted blood is stored, unless stress reticulocytes or high-potassium erythrocytes from Japanese dogs are lysed. In fact, Akitas' erythrocytes are "leaky" *in vitro*, and pseudohyperkalemia, which is clinically unimportant, may be observed in serum samples that have not been separated immediately from the clot **(Box 1).** Finally, canine erythrocytes have been noted to be uniquely fragile under alkaline conditions, compared with erythrocytes from cats and other species, presumably because of a facilitated calcium entry under these conditions. This pH sensitivity may explain the tendency of canine erythrocytes to lyse in uncapped tubes in the laboratory and underlies the hemolytic crises seen in dogs with phosphofructokinase deficiency.

Blood groups

Erythrocyte membranes also carry several blood group antigens, and various in-clinic and laboratory typing kits are now available (3). Dogs lacking a certain blood type antigen may develop alloantibodies, after sensitization from a transfusion, which may be responsible for acute hemolytic transfusion reactions. Dogs have more than twelve blood group systems, the *DEA 1* blood group being the most important. Dogs are either *DEA 1-* or weakly to strongly *DEA 1+*. For blood transfusion, *DEA 1-* donors are preferred as they will not sensitize a *DEA 1-* patient, but blood from *DEA 1+* donors can be readily given to *DEA 1+* patients. There are other canine blood types of clinical importance, such as *DEA 4* (99.9% of dogs are *DEA 4+*) and *Dal* (seen in Dalmatians, but also Doberman Pinschers; Shi Tzus and Lhasa Apsos may be *Dal* -) (4-6). It is recommended to type any patient and donor dogs for *DEA 1*, and to crossmatch any canine patient four days after the first transfusion if further transfusions are required. Whilst a genetic marker has been found for *DEA 1*, no DNA tests are currently available.

The feline *AB* blood group system, with *A*, *B* and *AB* types, is well recognized, as it is associated with naturally occurring alloantibodies and acute hemolytic transfusion reactions and neonatal isoerythrolysis (type *A* and *AB* kittens born to type *B* queens) without prior sensitization. Thus, any patient and donor (or, for that matter, queens intended for breeding) should be blood-typed. Type *B* and *AB* should be confirmed by retyping and back typing (to confirm the strong *anti-A* antibodies in plasma/serum of any type *B* cats older than 3 months). In addition, other feline blood groups are now recognized, the latest being a red cell antigen known as *Mik*. Such antigens may produce alloantibodies causing incompatible crossmatch test results and acute hemolytic transfusion reactions without prior sensitization. It would therefore be reasonable to crossmatch cats prior to a transfusion as well as performing *AB* typing. *AB* type is relatively rare, but is frequently found in Ragdolls, and DNA tests to differentiate between *A*, *B* and *AB* have recently been developed.

Box 1. Akitas have erythrocytes which are "leaky" *in vitro* and a blood sample may demonstrate microcytosis and pseudohyperkalemia, as in the sample results shown here. The phenomenon will occur if the serum is not separated immediately at sampling, but is clinically unimportant.

Parameter	Value	Normal reference range
Hematocrit	48%	37-55%
Hb	16g/dL	12-18g/dL
MCV	**52 fl**	60-77 fl
MCHC	33%	32-36%
Sodium	148 mmol/L	146-156 mmol/L
Potassium	**9 mmol/L**	3.8-5.6 mmol/L

Classification of erythrocyte defects

Hereditary erythrocyte defects form a large and clinically heterogeneous group of diseases. Each erythrocyte disorder is observed only rarely, although some enzyme deficiencies occur frequently within certain breeds **(Table 1)**. The mode of inheritance for each disorder is autosomal recessive, with the exception of feline porphyria

Table 1. Classification of major erythrocyte defects.

Hemoglobin-related abnormalities	
Methemoglobin reductase deficiency	Various breeds of dogs. Domestic shorthair and purebred cats of various breeds – cyanosis and erythrocytosis rather than anemia are the main clinical findings
Porphyrias	Domestic shorthair and purebred cats of various breeds – erythrodontia is noted
Membrane abnormalities	
Microcytosis	Akita and Shiba Inu – not clinically important
Macrocytosis	Miniature Poodles – not clinically important
Spherocytosis	Golden Retriever and occasionally other breeds – mild anemia develops
Stomatocytosis	Alaskan Malamutes (with chondrodysplasia) and Miniature and Standard Schnauzers – mild anemia develops
Elliptocytosis	Rarely seen in dogs – mild anemia develops
Osmotic fragility	Domestic shorthair and purebred cats of various breeds; rarely seen in dogs Intermittent anemia with splenomegaly develops
Erythroenzymopathies	
Pyruvate kinase (PK) deficiency	Many canine breeds – chronic anemia with osteosclerosis develops. Abyssinian and other cat breeds – intermittent anemia develops
Phosphofructokinase (PFK) deficiency	English Springer Spaniels and rarely Cocker Spaniels, Whippets, Wachtelhund, and mixed breeds – intermittent anemia can develop, with pigmenturia after exercise, heat exposure, panting, barking
Reduced erythropoiesis	
Hereditary cobalamin malabsorption	Australian Shepherd, Beagle, Border Collies, Giant Schnauzer, Komondor – signs include pancytopenia, failure to thrive, methylmalonic aciduria due to cobalamin deficiency
Iron-resistant iron deficiency anemia (IRIDA)	Cocker Spaniel – microcytic erythrocytes develop, although dogs are not necessarily anemic

and spherocytosis, which can be inherited as either a dominant or recessive trait. While the degree of characterization is varied, many seem to be homologous to hereditary diseases in humans. These erythrocyte disorders have been classified into four groups: **(i)** heme defects and hemoglobinopathies, **(ii)** membrane abnormalities, **(iii)** glycolytic enzyme deficiencies, and possibly **(iv)** production and maturation defects; a few specific disorders are discussed in more detail below. Typically, erythrocyte defects cause hemolytic anemia which allies to all groups except for the production and maturation defects.

Although the signalment, type, and severity of the anemia and the pleiotropic effects observed may provide clues to an inherited erythrocyte defect **(Table 2)**, a full laboratory evaluation is essential in validating the diagnosis or discovering new inherited disorders. In a few breeds, more than one erythrocyte disorder has been recognized. Routine hematological laboratory tests, including complete blood count with reticulocyte count and microscopic blood smear review, as well as serum chemistry and urinalysis, are used to detect hematological abnormalities and to rule out acquired anemias. An inherited erythrocyte defect should be considered in animals with Coombs-negative hemolytic anemia (7),

Table 2. Characteristic clinical features for erythrocyte defects causing anemia.

- Younger animals
- Breed predilection, or related animals affected with an unexplained anemia
- Chronic or recurring anemias
- Negative infectious disease screen
- Negative toxin/drug screen and environmental exposure
- Negative Coombs' test
- Poorly responsive to treatment or recurring

a lack of toxin exposure or infection, and adequate kidney and liver function. A careful examination of a peripheral blood smear is pivotal in recognizing any poikilocytosis, such as elliptocytosis, spherocytosis and stomatocytosis, although most erythrocyte defects cause no change in cell shape and are historically known as non-spherocytic hemolytic anemias. The degree of reticulocytosis is often marked, even with mild anemia, and is generally proportional to the shortened survival of defective erythrocytes. Thus a bone marrow examination rarely provides new information in cases of erythrocyte defects. The signs of hemolysis may be mild owing to the chronicity, strong compensation, and low-grade hemolysis, and affected animals may adapt very well to the chronic anemia. Hyperbilirubinuria and -emia are generally noted, but may be mild due to adaptation. Low serum haptoglobin concentrations, hemoglobinemia, or hemoglobinuria, which all indicate intravascular hemolysis, have been reported, but such findings need to be carefully assessed, as they may occur artificially. Some defective erythrocytes appear extremely fragile *in vitro*, resulting in artificial lysis in blood tubes. Animals with methemoglobinemia show cyanosis (even when receiving oxygen) and may develop a secondary erythrocytosis. Furthermore, cats with porphyria exhibit erythrodontia and pigmenturia due to porphyrin accumulation.

Special laboratory tests for defining the nature of an intrinsic erythrocyte defect can be divided into general screening tests, used to characterize unknown erythrocyte disorders, and specific screening tests, for known defects in certain breeds. Both are performed only in specialized laboratories. A few panel DNA tests have been developed for specific dog breeds that cover most of the DNA disorders reported so far*.

Hemolysis resulting from erythrocyte defects may be well compensated for by marked erythropoiesis, thereby causing no or minimal clinical signs (except during a crisis) and allowing the animal to have a normal life expectancy. Furthermore, affected animals may have adapted well to the chronic anemia. In contrast, some disorders are associated with severe hemolytic crises for which animals may need to receive supportive therapy, including blood-typed and, if previously transfused, crossmatch-compatible transfusions. Cats with some erythrocyte defects develop marked splenomegaly and may be helped with splenectomy which removes a major site of erythrocyte destruction; it appears that dogs with erythrocyte defects do not benefit from this procedure. Finally, affected animals should not be used for breeding in order to prevent the further spread of these disorders. However, to maintain gene pool diversity, asymptomatic carriers with desirable traits can be safely bred to a clear animal, as long as all offspring considered for breeding are also screened by mutation-specific DNA tests.

Hemoglobin defects

In contrast to the common occurrence of thalassemia and sickle cell anemia in people, no hemoglobinopathies have been documented in dogs and cats. Isolated cases of methemoglobinemia associated with cytochrome b5 reductase deficiency have been found among various dog breeds and domestic shorthair cats. Hereditary or congenital methemoglobinemia may be suspected when a drop of blood on a filter paper remains dark. Note that hereditary methemoglobinopathies are frequently associated with cyanosis and secondary erythrocytosis rather than anemia, but affected animals are at risk of massive hemolysis when exposed to oxidant agents (such as some drugs, heavy metals, and onions or garlic). Mutations have been identified in the methemoglobin cytochrome b5 reductase gene of affected animals (8).

Porphyrias are a group of inborn errors resulting from accumulation of porphyrins due to deficient activities of specific enzymes in heme biosynthesis, so far seen in cats but not dogs. In humans, they are clinically classified as either erythroid, with cutaneous involvement, or hepatic, with acute neurovisceral attacks. Affected porphyric cats have been reported to have erythrodontia (brown discolored teeth which fluoresce pink), porphyrinuria, and mild hemolytic disorders, but show no evidence of acute life-threatening neurovisceral attacks or cutaneous lesions; they have near normal life expectancy (9). Urinary porphyrin concentrations are increased in affected cats, and based upon the urinary porphyrin pattern and enzyme deficiency they can be classified as acute intermittent porphyria (AIP, dominant) or congenital erythroid porphyria (CEP, recessive). Several mutations have been identified in the hydroxymethylbilane synthase (*HMBS*) or uroporphyrinogen III-synthase (*UROS*) gene including duplications, deletions, and missense mutations, making it the most mutated gene discovered in cats so far. Cats with discolored teeth and normal or mild hemolysis may therefore have either deficient *HMBS* or *UROS* activity, and the biochemical and molecular characterization facilitates screening of affected cats at specialist laboratories*.

Membrane defects

Elliptocytosis and spherocytosis resulting from a deficiency of the protein band 4.1 and spectrin of the cytoskeleton have been characterized clinically and at the molecular level in cross-breed dogs. Other presumed membrane abnormalities include stomatocytosis in Alaskan Malamutes and Miniature and Standard/Middle Schnauzers, spherocytosis in Golden Retrievers with gastritis, non-spherocytic anemia in Beagles, and erythrocytes with increased osmotic fragility in Abyssinian and other purebred and non-pedigree domestic cats. Except for increased osmotic fragility in cats, these membrane defects are rare and sporadic **(Figure 1)** (10).

Increased erythrocytic osmotic fragility suggests a membrane and/or ion transport defect. Chondrodysplastic Alaskan Malamute dwarfs with stomatocytosis were the first dogs to be described with fragile erythrocytes (11), but the exact mechanism is still unknown. Miniature and Standard/Middle Schnauzers with stomatocytosis have been reported to have no membrane skeletal abnormalities; interestingly, whilst the macrocytosis is severe, the anemia – based on hemoglobin measurements – is only mild (12) **(Figure 2)**.

A marked increased osmotic fragility of erythrocytes associated with intermittent anemia, severe splenomegaly, and hyperglobulinemia has been observed in Abyssinians as well as other purebred and non-purebred cats. These erythrocytes are macrocytic, extremely fragile *in vitro*, and can simply lyse when stored overnight in the refrigerator. Although the cause has not been identified, a membrane defect is suspected. Affected cats with marked splenomegaly may benefit from anti-inflammatory doses of prednisolone. If the anemia is severe and frequently recurring, and if there is massive splenomegaly, splenectomy may be helpful. However, do note that splenectomized animals are particularly prone to

"Hereditary erythrocyte defects form a large and clinically heterogeneous group of diseases, and whilst uncommon, certain disorders occur relatively frequently within some breeds."

Urs Giger

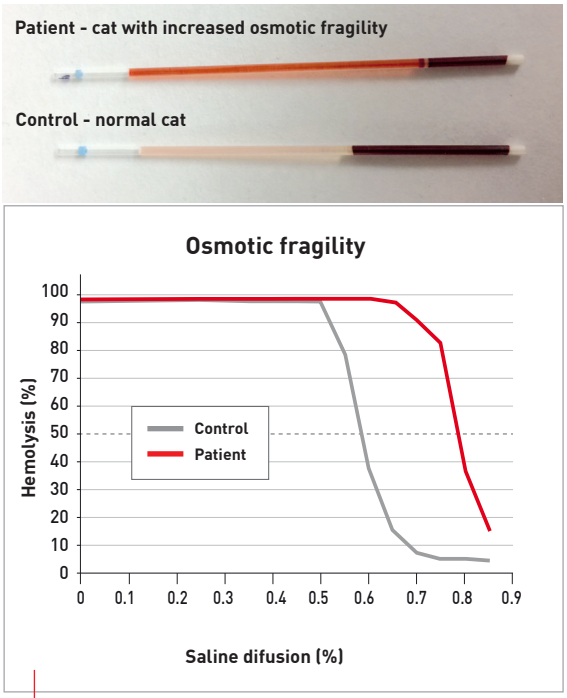

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Figure 1. Erythrocytes with increased osmotic fragility have been reported in various cat breeds, including the Abyssinian. This is seen in the above image, where a blood sample taken from an affected cat was stored in a refrigerator and the microhematocrit (PCV) assessed 24 hours later. Note the reddened plasma and lower PCV compared to a sample from a healthy cat. The fragility of the erythrocytes was assessed by the Osmotic Fragility Test measuring the degree of hemolysis against a rising concentration of saline, as shown in the graph. Normal erythrocytes lyze *in vitro* to 50% at near half-strength saline (0.6%), while the affected erythrocytes lyse at a near physiological saline concentration (0.8%).

develop sepsis for the first month post-operatively. Osmotic fragility testing is offered by some laboratories*.

Erythroenzymopathies

Deficiencies of phosphofructokinase (PFK) and pyruvate kinase (PK), the two key regulatory glycolytic enzymes, result in distinctly different

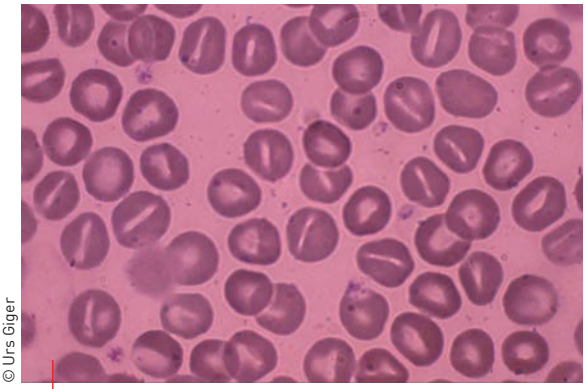

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Figure 2. Stomatocytosis in a Miniature Schnauzer; stomatocytes are erythrocytes with a slit-like central pallor, giving them the appearance of "coffee beans".

forms of hemolytic anemias in several dog breeds, while only pyruvate kinase (PK) deficiency has been seen to cause intermittent anemia in many cat breeds **(Table 3)**. Although PK deficiency was first characterized in the Basenji breed around five decades ago, the typical clinical features and biochemical abnormalities appear identical in other canine breeds and unique to dogs. In contrast, PK-deficient cats seem to have intermittent anemia which is more similar to PFK deficiency in dogs. Many animals are presumptively treated for immune-mediated hemolytic anemia for months or years prior to diagnosis, and thereby are subjected to unnecessary diagnostics and potentially harmful treatments.

Phosphofructokinase (PFK) deficiency

Despite its discovery more than three decades ago, and the availability of enzymatic and DNA screening tests, this glycolytic enzyme deficiency is still seen in field-trial English Springer Spaniels in the United States and Europe, but has also been reported in some bench/show dogs including Cocker Spaniels, Whippets, Wachtelhunds, and mixed-breed dogs. PFK deficiency is caused by one single missense mutation of the muscle-type PFK, resulting in a truncation and instability of the PFK enzyme protein in all breeds reported (other than the Wachtelhund, which has a different PFK mutation (13)).

The disorder is characterized by hemolytic crises and exertional myopathy. Sporadic dark pigmenturia resulting from severe hemoglobinuria

Table 3. Comparison of hereditary PK and PFK deficiency in dogs and cats.

Parameter	Pyruvate kinase (PK) deficiency		Phosphofructokinase (PFK) deficiency
	Dog	Cat	Dog
Anemia	Severe chronic	Intermittent	Intermittent
Triggers	Unknown – any disease or stress	Unknown – any disease or stress	Excessive panting, barking and heat; strong exercise
Regenerative erythroid response	Very strong	Mild	Strong even when not anemic
Long-bone radiography	Osteosclerosis by 1 year of age	Normal	Normal
Response to splenectomy	None	Good	None
Life expectancy	Depending on breed, 1-10 years	1-12 years	If crises are avoided, up to 12 years

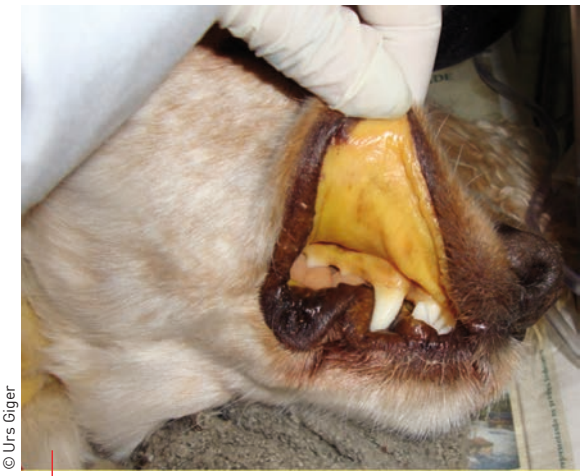

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Figure 3. An icteric English Springer Spaniel with PFK deficiency.

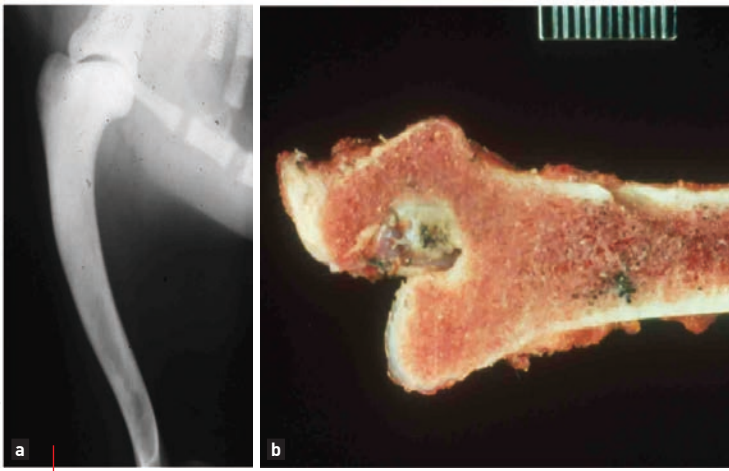

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Figure 4. Osteosclerosis associated with PK deficiency in a West Highland White Terrier. Increased density of the cortices is evident on radiography **(a)** and on post-mortem examination **(b)**.

and hyperbilirubinuria is a key feature, and commonly develops after episodes of excessive panting and barking, intense exercise, and fever or high environmental temperature. Consequently, hyperventilation-induced alkalemia results in intravascular lysis of PFK-deficient erythrocytes. During a crisis, an affected dog may become severely anemic and icteric **(Figure 3)**, and show fever, lethargy, and anorexia, which usually resolve within days. Between crises the dog will have a strong regenerative erythroid response. Furthermore, affected dogs totally lack PFK activity in muscle; therefore, they have a metabolic myopathy characterized by exercise intolerance, occasional muscle cramps, and mildly to moderately increased serum creatine kinase activity. As they cannot run hard and fast for any length of time, they will perform poorly as field-trial dogs.

Simple mutation-specific tests are offered to accurately diagnose PFK-deficient and carrier dogs by many laboratories*. For breeds without a known PFK mutation a low enzyme activity and/or high hemoglobin-oxygen dissociation curve test can be indicative. Situations triggering hemolytic crises should be avoided, such as excessive panting, barking, exercise, and heat. When a dog is experiencing a crisis, resting will help, but affected animals may require supportive therapy and occasionally blood transfusions. PFK-deficient dogs may have a normal lifespan, but have persistent hyperbilirubinuria and reticulocytosis, despite a near-normal hematocrit because of the high hemoglobin-oxygen affinity of PFK-deficient erythrocytes.

Pyruvate Kinase (PK) deficiency

Despite the severity and persistency of the anemia in PK-deficient dogs, the clinical signs, except for pallor, are mild, but triggered hemolytic crisis can occur at any age, and may be fatal. The anemia is highly regenerative, with numerous circulating metarubricytes (nucleated red blood cells) and reticulocyte counts can be as high as 90%. An unexplained progressive myelofibrosis and osteosclerosis of the bone marrow **(Figure 4)** and generalized hemosiderosis/chromatosis with associated hepatic failure can develop (14), leading to death, usually before 6 years of age, although some PK-deficient West Highland White and Cairn Terriers and Beagles have reached 10 years of age. The molecular genetic basis of PK deficiency has been identified in Basenjis, Beagles, Labrador Retrievers, Pugs, and Cairn and West Highland White Terriers (15,16), and mutation-specific DNA tests are available for these breeds, but not for others*. PK deficiency has also been reported in Miniature Poodles, Eskimo Toy Dogs, Dachshunds, and Chihuahuas, and it appears probable that the previously described non-spherocytic hemolytic anemia and osteosclerosis in Poodles was caused by a PK deficiency (17). In these breeds, a cumbersome PK-enzyme test with isozyme characterization is required to define PK deficiency, and differentiation between carriers and homozygous normal dogs based on erythrocytic enzyme activity can be difficult. Clinical signs in affected dogs are mild, likely due to chronic adaptation to severe anemia and high erythrocytic DPG concentrations facilitating easy oxygen delivery (low hemoglobin-oxygen affinity). Hepatomegaly and splenomegaly may result from severe extravascular hemolysis, extramedullary hematopoiesis and hemosiderosis/chromatosis. Iron chelation has been proposed as a treatment but has not yet been shown to be effective and safe, whilst splenectomy has not been shown to be effective.

Bone marrow transplantation has been experimentally proven effective for both enzymopathies in dogs, but because of the likely lack of a major histocompatibility complex compatible donor and the need for severe bone marrow suppression, this treatment is not offered clinically.

Feline PK deficiency

In cats, PK deficiency causes intermittent, rather than chronic anemia, with a mild to moderate regenerative erythroid response; cats do not develop osteosclerosis. Affected cats may develop bilirubin calculi in the gallbladder, hepatic failure, and mild splenomegaly. Anti-inflammatory doses of prednisolone (and splenectomy in severe cases) appears to ameliorate the clinical signs of intermittent

anemia, with the oldest affected cat reaching 11 years of age (3). Erythrocyte PK activity is severely reduced, and there is no M-type PK expression, thereby simplifying the biochemical diagnosis. It has been recognized that PK deficiency is caused by a single splicing defect resulting in a 13 base deletion since the late 1990's (18) and the disorder has been reported in Abyssinian, Somali, and several other pedigree breeds as well as domestic shorthair cats on different continents, and a DNA screening test is now offered by many laboratories. Any cat with unexplained persistent or recurring anemia following screening for toxic, infectious, and immune-mediated causes should be evaluated for PK deficiency, as this is a much more likely cause of anemia than immune-mediated hemolytic anemia.

Reduced erythropoiesis

Whereas the previous defects resulted in shortened erythrocyte survival, hemolysis, and regenerative anemias, the erythroid production and maturation disorders are not typically included when referring to erythrocyte defects. These conditions are reflected not only in a non-regenerative anemia but also in changes in other bone marrow-derived cells.

Selective cobalamin (vitamin B12) malabsorption, also known as Imerslund-Gräsbeck syndrome, resulting from a defect in the ileal intrinsic cobalamin receptor, has been reported in several breeds of dog (19,20). Giant Schnauzers and Australian Shepherds have mutations in the *AMN* gene and Beagles, Border collies and Komondors have mutations in the *CUBN* gene. Affected animals fail to thrive, and have varied degrees of cachexia, neurologic signs, leukopenia, thrombocytopenia, anemia, low serum cobalamin levels and a methylmalonic aciduria. However, prognosis is good after diagnosis; dogs respond completely to parenteral cobalamin administration every 2-4 weeks.

A severely microcytic-hypochromic anemia with low serum iron which is unresponsive to oral iron supplementation has been observed in one Cocker Spaniel and a few other dogs (21). This iron-resistant iron deficiency anemia (IRIDA) was found to be due to a defect in the *TMPRSS6* (matriptase-2) gene, which regulates the production of hepcidin and ultimately controls iron absorption and bioavailability.

**e.g.,* PennGen Laboratory, School of Veterinary Medicine, University of Pennsylvania, Philadelphia. The PennGen website also hosts the WSAVA Hereditary Disease Database where any DNA tests for a specific disease and breed are listed. The PennGen Laboratories offer specialized testing for the discovery and characterization of erythrocyte and other hereditary diseases.

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CONCLUSION

Several hereditary disorders have now been recognized and characterized in veterinary medicine. Often related to a specific breed of dog or cat, such defects can cause a wide variety of clinical signs, and it is imperative to perform full blood and urine tests if a disorder is suspected; screening to mutation-specific DNA tests have been developed to aid diagnosis. The clinical picture can vary markedly with erythrocyte disorders, from severe anemia to asymptomatic. In many cases avoidance of immunosuppressive therapy and crises-inducing situations may allow affected animals to have a good quality of life and sometimes near-normal life expectancy.

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LIQUID BIOPSY – THE FUTURE FOR CANCER DIAGNOSTICS?

Needle aspirates and tissue biopsies are commonplace in veterinary medicine but are not without their drawbacks for tumor diagnosis. Here Matthew Breen and Claire Wiley describe a new technique for the early diagnosis of canine bladder cancer, and discuss what the future may hold for liquid biopsy procedures.

Matthew Breen,

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After receiving his PhD in animal genetics in 1990, Dr. Breen was a post-doctoral researcher as part of the Human Genome Project. Following several years in Australia and the UK, Dr. Breen moved to NCSU in 2002, where he holds the post of Oscar J. Fletcher Distinguished Professor of Comparative Oncology Genetics. He has spent the past 15 years focusing his research on genomics, genome mapping and the comparative aspects of canine cancer, and his laboratory team have developed new molecular assays for diagnostic and prognostic use in veterinary medicine.

Claire Wiley,

VMD, Dipl. ACVIM (SAIM), College of Veterinary Medicine, North Carolina State University (NCSU), Raleigh, NC, USA

Dr. Wiley earned her veterinary degree and undertook a rotating internship at the University of Pennsylvania. After completing a small animal internal medicine residency at NCSU, her current focus centers on the diagnosis and treatment of lower urinary tract diseases. She is currently enrolled in a PhD program to evaluate the genetic aberrations in urothelial and prostatic carcinomas, and has a strong interest in the veterinary applications of liquid biopsies.

KEY POINTS

1. A "cancer signature" is something that can be used to indicate the presence of a cancer; ideally, such a signature should have both high sensitivity and specificity for a given cancer.
2. A liquid biopsy allows for the detection of cancer cells or cell remnants from a tumor that are circulating in the blood or deposited in the urine of a patient.
3. Early detection of a cancer provides more time to intervene with an appropriate therapy to help delay onset of clinical symptoms.
4. Advances in molecular technologies mean that liquid biopsies are starting to become available for veterinary medicine, and further tests for specific diseases are likely to be developed over the next few years.

Introduction

For many cancers, histopathologic evaluation of a biopsy of a suspicious mass has been the gold standard for diagnosis for many years. However, the biopsy process itself may be invasive, costly, and can be associated with complications to the patient. For some cancers, obtaining a solid biopsy may also increase the risk of disseminating tumor cells, leading to added concerns. In a clinical setting, identifying the presence of a specific tumor in a patient can be challenging. To complement conventional invasive procedures, clinicians have been eager to identify less invasive, safer, and more affordable alternatives to obtaining materials suitable for diagnostic evaluation. The term "cancer signature" is employed to describe an indicator of

the presence of a tumor; methods to detect such a "signature" are being urgently sought, ideally with high sensitivity and specificity. Liquid biopsy embodies these desirable characteristics by offering a non-invasive method for detecting genetic alterations in tumors through analysis of tumor cells and tumor-derived cell-free DNA (cfDNA) in plasma and urine. This approach provides opportunities for improving cancer detection and identification, and also for monitoring the impact of therapy on patients over time. Advancing quickly in human medicine, liquid biopsy is being incorporated into numerous drug development programs, and is likely to be rapidly integrated into clinical care for human patients.

What is a liquid biopsy?

Tumors release cells and DNA into surrounding tissues and fluids, thus offering the opportunity to assess the genetic composition of a solid tumor from sampling body fluids. Use of such fluids is referred to as a "liquid biopsy" (1). Although the presence of circulating cell-free nucleic acids (cfNAs) was first described almost 70 years ago, the significance was not recognized until 1994, when fragments of a driver oncogene (a mutant *RAS* gene) were identified in the blood of cancer patients (1). It is now recognized that circulating cell-free tumor DNA concentrations are higher in cancer patients than in normal controls, and that the presence of metastases is generally associated with even higher levels (2). The mechanism for release of nucleic acids into surrounding tissues is hypothesized to be associated with the fast turnover of cells and resulting apoptosis (1). Traditionally, the term "liquid biopsy" was regarded as referring to the identification of neoplastic biological materials (*e.g.*, circulating tumor cells or cfDNA) in the peripheral bloodstream (3). More recently, the definition has broadened to encompass all body fluids, including urine, cerebrospinal fluid (CSF), cavitary effusions, etc. (3).

"The new assay can detect as few as ten mutant-bearing cells in a urine sample, and so can identify bladder cancer cases at the preclinical stages of the disease."

Matthew Breen

Tumor genotyping in human medicine is becoming a routine component of the diagnostic workup of cases. Knowledge of a mass's mutational burden can help establish the type and stage of the cancer, and the aggressiveness of the disease; it may also guide treatment choice. Genotyping of tumor-associated DNA and cfDNA obtained using a liquid biopsy offers the advantage of easy, rapid, and safe access to the tumor, in contrast to traditional biopsies or fine-needle aspirates. Liquid biopsies are also increasingly being used to monitor patients for residual disease. By monitoring changes in the tumor-associated DNA and cfDNA, therapies can be adjusted based on dynamic changes in the mutation profile. Recurrences or metastases can also be detected earlier with liquid biopsies than with conventional methods (4,5).

Although tumor genotyping is common, and the use of liquid biopsy is increasing in human medicine, both approaches are still in the nascent stages in animals. However, several techniques have been described in veterinary medicine that could be referred to as "liquid biopsy". These are as follows; the CADET^{SM} *BRAF* Mutation Assay for the diagnosis and monitoring of canine transitional cell carcinoma/urothelial carcinoma (TCC/UC); cell block preparation for a variety of cancers **(Figure 1)**; polymerase chain reaction for antigen receptor rearrangement (PARR); flow cytometry for lymphoid malignancies; and the CADET^{SM} HM Assay for diagnosing canine histiocytic malignancies. This paper will focus on the new CADET Mutation Assay for TCC/UC, but brief details on the other techniques are given in **Table 1**.

Liquid biopsy for detection of canine bladder tumors

A new technique, the CADET^{SM} *BRAF* Mutation Detection Assay, has recently been developed to aid the identification of canine transitional cell carcinomas and urothelial carcinomas and subsequent monitoring of *BRAF* positive tumors. This is the world's first liquid biopsy for veterinary cancer detection and monitoring, and a short discussion on canine bladder neoplasia will help elucidate the changing face of diagnosis for such tumors.

How is TCC/UC currently diagnosed?

A common route to diagnosis of a TCC/UC is one in which a dog with lower urinary tract signs is initially managed with repeated courses of antimicrobials, and sometimes non-steroidal anti-inflammatory drugs (NSAIDs), on the assumption that the cause is non-malignant. This approach can span several months, during which a TCC/UC can develop into a more advanced state, become larger, potentially invade the muscle wall, and also have a greater chance of metastasis. When repeated treatments for the clinical signs fail, the dog is then evaluated for the presence of TCC/UC, usually via urine cytology, abdominal ultrasound, and/or cystoscopy.

When a mass is detected, a biopsy for histopathology evaluation is recommended to confirm the diagnosis of a TCC/UC and potentially indicate muscle invasion.

Table 1. Several other veterinary assays can also be considered to be liquid biopsies, as follows;

Cell blocks

It is possible to convert a liquid sample into a formalin-fixed cell block using one of several techniques. These include embedding specimens with HistoGel™ (15), surgical gel foam (16), or agarose (17), or involve formalin-fixed paraffin-embedded liquid samples (18,19). This method has several benefits over traditional cytology, including the maintenance of cell cluster architecture, the potential application of immunohistochemistry or other techniques, and preservation of samples. However the turnaround time is longer than traditional cytology or other liquid biopsy techniques.

One cell block preparation method, the cell tube block (CTB), has the potential for widespread application and can be easily prepared utilizing simple materials and equipment **(Figure 1)**. Briefly, a plain capillary tube is filled with a liquid sample and centrifuged. The tube is then broken at the liquid-solid interface and fixed in formalin for 24 hours. Formalin-fixed cell tube blocks are then embedded in paraffin and can subsequently be processed with various stains or immunohistochemistry (20).

This method relies on the fact that centrifugation causes cell layers to develop in the capillary tube, with concentrated neoplastic cells wedged between erythrocytes at the bottom and neutrophils, macrophages, and mesothelial cells at the liquid-solid interface. The absence of inflammatory cells or erythrocytes among the neoplastic cell population results in decreased background staining with immunohistochemistry (21). Note that this method of isolating neoplastic cells may also facilitate molecular characterization.

PARR

The polymerase chain reaction for antigen receptor rearrangement assay (PARR) is now used for diagnosis of lymphoma or leukemia in samples with ambiguous cytologic or histologic morphology. It can also be used for phenotyping lymphoma as B- versus T-cell, a distinction with prognostic implications. This assay uses PCR to assess the clonality of lymphocytes by evaluating the length of the immunoglobulin genes in B cells, or T-cell receptor genes in T cells (21). Although sensitivity and specificity vary by laboratory, the PARR assay is capable of detecting 1:100 neoplastic cells (21). Infectious diseases, such as *Ehrlichia* spp., can also result in a clonal population of lymphocytes (21) and decrease the specificity of PARR. In a recent study assessing 271 patients, the sensitivity and specificity of PARR for diagnosing canine lymphoma was 86.5% and 98.7% respectively (22).

This assay involves isolation of DNA from neoplastic cells obtained from a blood sample, cytology, or a histology sample. PCR is then performed using primers to amplify the variable region of T-cell receptor or immunoglobulin genes. PCR products are separated by size using a variety of methods; the detection of a single-sized PCR product suggests clonality, whereas the detection of multiple PCR products supports a reactive process.

Flow cytometry

Flow cytometry offers another molecular assay to diagnose and immunophenotype lymphoma or leukemia using liquid samples obtained either from blood, effusions, or fine-needle aspirates injected into cell culture media. Unlike with PARR, flow cytometry requires a cell suspension; cytology slides or formalin-fixed paraffin embedded specimens cannot be used. Using specific wavelengths of light to excite fluorophore-conjugated antibodies/proteins, coupled with sophisticated imaging equipment to detect the fluorescence emissions, the assay can determine various cellular characteristics. For assessing cell surface protein expression, cells are stained with antibodies conjugated to fluorescent proteins, and sorting of cells is based on their relative fluorescence. While some proteins, such as CD45, are expressed on the surface of all lymphoid cells, others are typically restricted to subpopulations of T cells (*e.g.*, CD3) and B cells (*e.g.*, CD79a, CD20). Use of these more specific reagents allows identification of the proportion of each subtype in a population of cells.

One study compared PARR to flow cytometry for diagnosis of lymphoma, as well as immunophenotype, as determined by immunohistochemistry of enlarged lymph node biopsies (23). Both PARR and flow cytometry had specificities of 100% in this study, but flow cytometry had a higher sensitivity than PARR (98% vs. 74%). This study suggests that flow cytometry may be superior to PARR for diagnosing lymphoma in enlarged lymph nodes, but since flow cytometry requires a fresh sample, PARR has obvious utility for specimens not suited to flow cytometry.

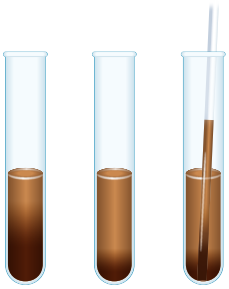
CADET^{SM} HM assay

The CADET^{SM} HM Assay is a new molecular test for distinguishing histiocytic malignancies (HM) from other similar round cell neoplasia. Some studies have shown that as many as 70% of cases initially identified as HM may be misclassified (24,25). Distinction between HM and plasma cell tumors can be particularly challenging, and this difficulty may also extend to lymphoma in routine cytological preparations. Small samples enriched for tumor cells, such as from effusions or fine-needle aspirates, may be used for the assay, and histologic samples can also be used. The assay determines the number of copies of a specific DNA sequence that are present in the tumor cells of HM cases, where a reduction in the number of copies present is consistent with a diagnosis of HM.

The assay has been validated on samples from over 500 unique, pathology-verified canine cancer specimens from HM and multiple other tumor types that can resemble HM, including lymphoma, plasma cell tumors, hemangiosarcoma, amelanotic melanoma, and mast cell tumor. The results demonstrate that this genetic signature is a highly sensitive and accurate marker for distinction between canine HM and these other tumor types, with sensitivity of 78% and specificity of 95%.

Figure 1. A schematic overview of the cell tube block (CTB) technique.

a



A capillary tube is filled with liquid or sediment (with low cellular samples).

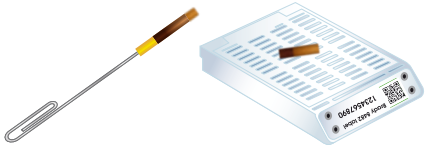
b

The capillary tube is sealed with clay; in the absence of significant blood contamination, a high-density medium (*e.g.*, percoll) may be aspirated to facilitate separation of the cell pellet from the clay.

c

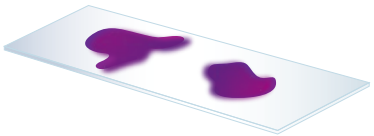
After centrifugation, the capillary tube is broken at the liquid-solid interface using a glass-writing diamond pen.

d



After fixation in 10% formalin for at least 24 hours, the CTB is pushed out of the glass capillary using a paper clip and placed in a cassette for routine paraffin embedding.

e



Paraffin-embedded CTB sections are stained with H&E or other stains and examined.

Further imaging and evaluation of local lymph nodes may be performed to assess metastasis. At the time of diagnosis over 90% of dogs present with an intermediate to high-grade invasive canine TCC/UC, and around 20% have already spread to other parts of the body (6,7). The high predominance of advanced tumors may reflect the prolonged time taken to make the diagnosis in most cases.

How is TCC/UC currently treated?

Once finally diagnosed, treatment of canine TCC/UC most commonly includes the use of chemotherapy. Where single agent therapy is used, the proportion of dogs entering remission is generally low (< 20%), although this increases to 35-50% with combined chemotherapy and cyclooxygenase inhibitors. Dogs treated with single agent NSAID therapy have a median survival time of approximately 6-7 months, whereas a combination of cytotoxic chemotherapy (typically mitoxantrone) and an NSAID yields median survival times closer to 10 months. While less common than drug-based intervention, surgery and radiation therapy are also used. Recent data suggest that the addition of full-course intensity-modulated and image-guided radiation therapy is associated with a 60% response rate, and a median survival time of > 21 months (8).

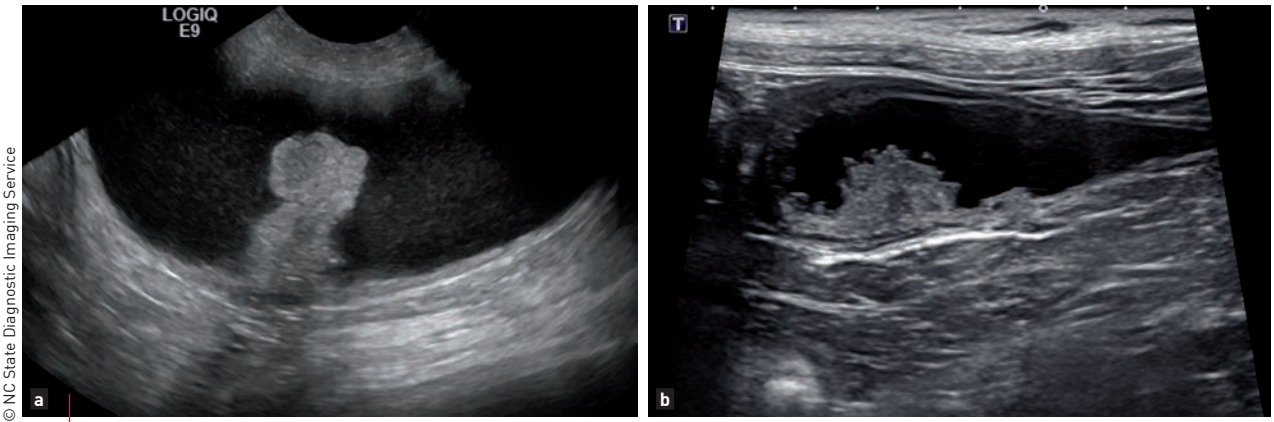
What is the challenge for TCC/UC diagnosis?

Abnormal epithelial cells in urine sediment, or in samples obtained by traumatic catheterization, prostatic wash, and/or fine-needle aspiration are used to support the diagnosis of canine TCC/UC (9). However, cytological analysis of epithelial cells may be misleading. For example, benign epithelial cells can resemble malignant cells with variation in cell size (10). Fine-needle aspiration of tumor tissue carries the risk of disseminating tumor cells along the needle tract (11). Currently, clinical diagnosis of canine TCC/UC requires comprehensive diagnostic workup, including complete blood counts, serum biochemistry, urinalysis, diagnostic imaging, clinical pathologist examination of tumor cells, and histopathology of a biopsy specimen.

Since most TCCs/UCs remain undiagnosed until at an advanced clinical stage, the diagnosis has a guarded or poor prognosis. Detection of the tumor earlier in the disease course would allow appropriate intervention sooner, which would be expected to improve quality of life and extend survival. Among a survey of 400 trainee and boarded diplomates of the American College of Veterinary Internal Medicine, a commonly highlighted unmet need was the availability of a reliable, non-invasive, diagnostic test for the detection of canine TCC/UC (manuscript in preparation).

What is the new opportunity for early detection of canine TCC/UC?

In two recent independent studies, performed by research teams at NCSU (12) and the National Institutes of Health (NIH) (13), a single mutation in exon 15 of the canine *BRAF* gene was detected in pathology-verified tumor biopsy specimens of canine TCC/UC. This single mutation results in one amino



© NC State Diagnostic Imaging Service

Figure 2. Both polypoid cystitis and TCC/UC can have similar ultrasonographic appearances. **Figures 2a and 2b** depict bladder masses obtained from two geriatric female spayed dogs with dysuria, hematuria, pyuria, and bacteriuria. Both images show lobulated masses near the apex of the bladder. **(a)** The *BRAF* mutation was not detected in the urine of this dog, and subsequent histopathology of the mass was consistent with a benign polyp. In the second dog **(b)**, the urine tested positive for the *BRAF* mutation, and cytology was consistent with TCC/UC. If a mass is detected in the urinary tract, advanced diagnostics such as histopathology or the CADET℠ *BRAF* Mutation Detection Assay are recommended to distinguish benign lesions from carcinoma.

acid change (valine to glutamic acid) in the *BRAF* protein in tumor cells. This change, located in the activation segment of the kinase domain of the gene, results in a mutated protein with increased kinase activity that signals cell proliferation, leading to the development of a tumor. The *BRAF* mutation has not been detected in non-neoplastic bladder tissues, including inflammatory bladder tissue and polyps (12) **(Figure 2)**. Where a dog has a TCC/UC, cells from the mass, ranging from very early to late in the course of disease, are shed into the urine. The NCSU team developed a rapid and highly sensitive test to detect the presence of this mutation in these cells (14) which in turn led to the refinement and commercialization of the world's first liquid biopsy for a veterinary cancer in the form of the CADET℠ *BRAF* Mutation Detection Assay*, as described in **Figure 3**.

The overall sensitivity of the assay to detect a canine TCC/UC in a free-catch urine specimen is 85%. While other canine cancers do present with the same *BRAF* mutation at a very low frequency (12), these have not yet been detected in urine specimens of such patients. The specificity to detect a canine TCC/UC is currently > 99%. Importantly, the assay is not affected by the presence of bacteriuria or hematuria, so it provides a highly effective means to detect the presence of malignant TCC/UC cells where other assays fail.

How can liquid biopsy aid veterinarians?

Aid to diagnosis

With its high sensitivity and specificity, this new liquid biopsy has been adopted widely across the USA as an aid to diagnosis of canine TCC/UC. The assay is based on the identification and quantification of both wild type and mutated *BRAF* alleles recovered from cells exfoliated into urine during urination. A comparison between the level of *BRAF* wild type and *BRAF* mutant alleles provided a quantitative measure of cells recovered from the urine samples. Importantly, in all cases that have had a biopsy of a visible mass for pathology evaluation, there is 100% correlation between the presence of a *BRAF* mutation detected in free-catch urine and subsequent confirmation of TCC/UC via biopsy. In contrast, this test does not have false positives; in studies involving hundreds of controls, a *BRAF* mutation has not been detected in specimens from dogs that were shown not to have a TCC/UC. However, the test does not indicate the location of the TCC/UC; imaging of the region will help identify tumor location and may contribute to management decisions.

* The CADET℠ series of assays are developed and commercialized by Sentinel Biomedical (www.SentinelBiomedical.com).

"As more data become available, liquid biopsy assays, especially those with high specificity and sensitivity, may eventually surpass conventional tissue biopsies"

Claire Wiley

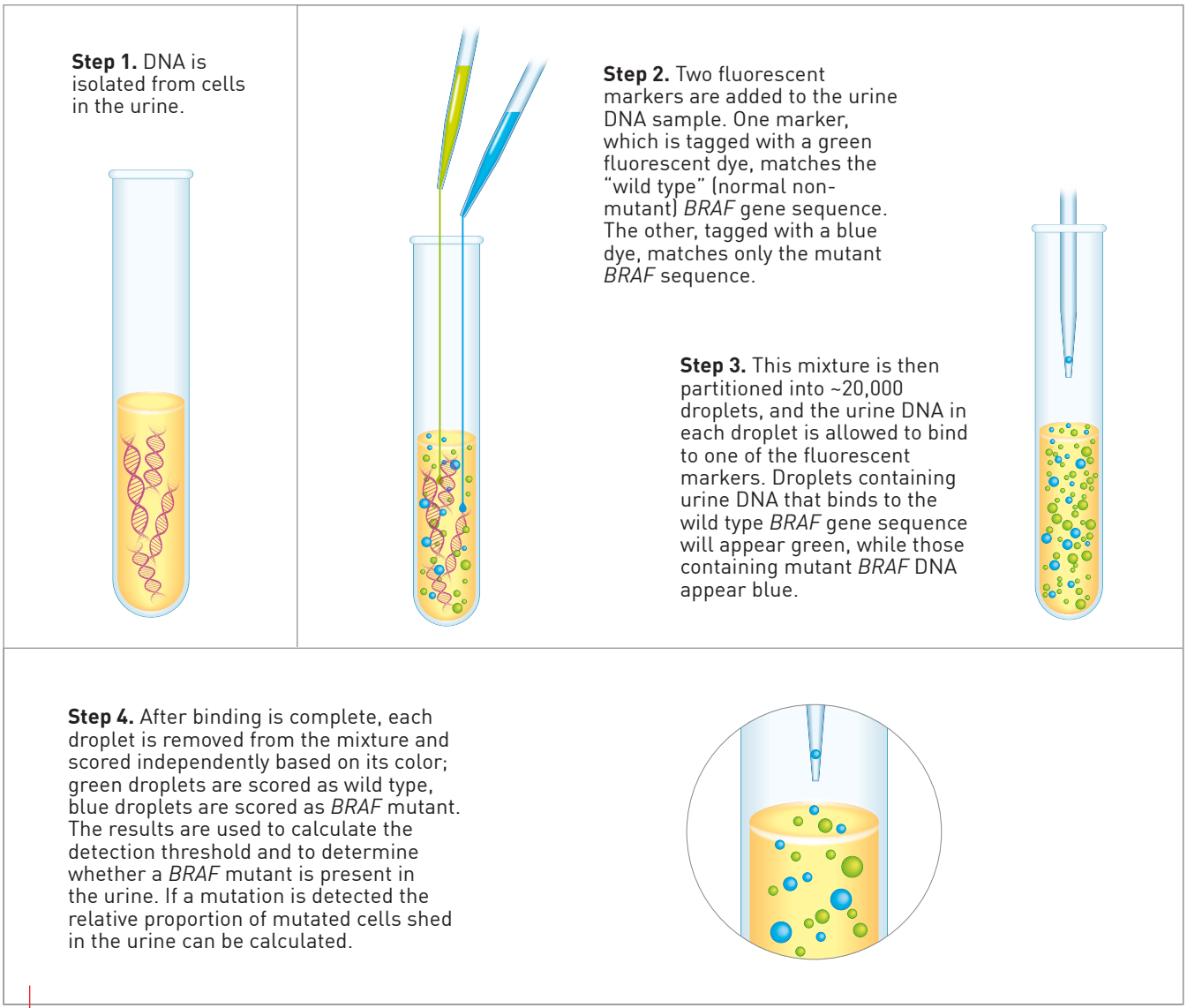


Figure 3. Schematic representation of the steps involved from specimen collection to results for the CADET[SM] *BRAF* Mutation Detection Assay. Cells shed into the urine are assessed for the presence and proportions of cells that harbor a single base change in exon 15 of the canine *BRAF* gene. Since this mutation is present in 85% of dogs with a confirmed TCC/UC and has not been detected in urine from dogs with non-malignant urinary tract lesions, the assay is > 99% specific to the presence of a TCC/UC.

Monitoring by serial liquid biopsy

Once a dog has been diagnosed with a *BRAF* positive canine TCC/UC, the assay may also be used to monitor changing levels of the mutational load during treatment over time. Early provisional data has shown that while NSAIDs such as piroxicam may have a minor impact on reducing the level of the *BRAF* mutation shed into urine, conventional chemotherapeutic agents, such as mitoxantrone, may be associated with a progressive and substantial reduction in the *BRAF* mutation levels over the course of treatment (manuscript in preparation). Temporally matched ultrasonography of dozens of cases revealed a progressive reduction in the size of a bladder mass/wall thickness, with alleviation of clinical signs. These data indicate that large changes in the *BRAF* mutational level detected in the urine over time may be used as an indicator of alteration in tumor size and proliferation. Conversely, a marked increase in levels of the *BRAF* mutation during treatment might suggest that proliferation of the tumor is not being impacted by the treatment. For patients where the level of the *BRAF* mutation has initially been substantially reduced during treatment, and then noted to rise, this may be suggestive of an increase in proliferation, indicative of relapse. While more research is needed to confirm these findings, these combined data provide an early insight into the use of this liquid biopsy as a means to monitor residual disease in the patient, both during treatment and remission.

Screening of high risk breeds

The new assay can detect as few as ten mutant-bearing cells in a urine sample, and thus can identify TCC/UC cases at very early, preclinical stages of the disease. This is a characteristic

feature of an effective early screening assay; *i.e.*, detecting the presence of an emerging cancer as early as possible in the course of the disease provides more time for the most appropriate intervention to combat the disease. The assay is now being used to screen urine of dogs from breeds with an increased risk of developing TCC/UC (*e.g.*, Beagle, Scottish Terrier, Shetland sheepdog, West Highland White Terrier). This enables owners of dogs that test positive at very low levels to follow up with their veterinarians and seek the most appropriate treatment early in the disease course, with the hope to improve both quality and duration of the dogs' lives.

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CONCLUSION

With the new advances in molecular technologies, liquid biopsies are starting to become available for veterinary medicine. Such methods are designed to be complementary to other diagnostics, although as more data become available it is possible that liquid biopsy assays, especially those with remarkably high specificity and sensitivity, may eventually surpass conventional tissue biopsies. Liquid biopsy is also being used as a monitoring tool to assess changes in the level of malignant cells, which may be an indicator of the efficacy of therapy, as well as a means to identity impending relapse. In addition to aiding diagnosis, it is expected that, as in human medicine, these new molecular-based tests will soon be available to guide therapeutic choices in veterinary medicine.

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BREED PREDISPOSITIONS FOR UROLITHIASIS

Uroliths are a relatively common problem in cats and dogs with lower urinary tract disease. Understanding the prevalence of different types of uroliths, as well as breed and gender predilections, can help veterinarians make the best clinical decisions and recommendations when managing their patients.

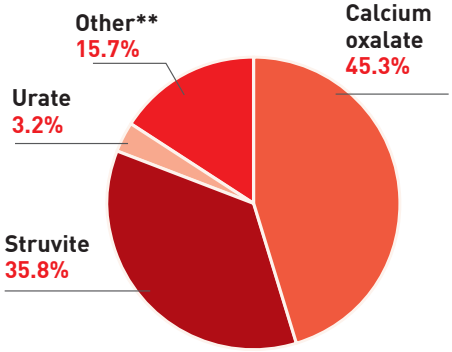
This short report summarizes the key findings from data associated with all feline and canine urinary bladder calculi analyzed at the Canadian Veterinary Urolith Centre between February 1, 1998 and November 30 2014. Canine submissions represented 78.9% (75,674) and feline submissions 21.1% (20,183) of the 95,857 uroliths analyzed. Urolith composition was determined using quantitative analysis methodology. Significant findings are identified below, including the prevalence of different urolith types, trends in the proportion of each of those urolith types, and breed and gender predispositions (1,2).The study identified breeds at increased risk compared to a reference population rather than breed association with urolithiasis.

Genetic mutations associated with canine cystine, urate and xanthine urolithiasis have been identified in several dog breeds, explaining some of the breed predispositions noted (3-5). A potential calcium oxalate susceptibility gene has been identified in Miniature Schnauzers, and similar genetic factors may account for calcium oxalate predispositions in other breeds (6). Genetic variants causing cystinuria in cats have recently been identified (7) and genetic determinants predisposing to other types of uroliths are suspected (but have not yet been identified) in this species; more research in this area is needed.

Identified trends in urolith composition between 1998-2014:

- Calcium oxalate: ↑
- Struvite: ↓
- Urate: ↓
- Cystine: ↑
- Mixed: ↑
- Silica: ↓
- Calcium phosphate carbonate: ↓

(Note: Since 2014, canine cystine have surpassed urate submissions.)



The chart shows the average percentage for each type of urolith analysed between 1998 and 2014.

The study identified gender associations with certain types of uroliths, as follows:

- **Males:** calcium oxalate, urate, cystine, silica, calcium phosphate apatite.
- **Females:** struvite, calcium phosphate carbonate, compound.

65% of the canine urolith submissions were from the following breeds:

- Mixed Breed
- Shih Tzu
- Miniature Schnauzer
- Bichon Frisé

Canine breeds at higher risk for cystine urolithiasis*:

- Scottish Deerhound 88%
- Newfoundland 56%
- Mastiff 52%
- Basenji.....47%
- Whippet.....44%
- French Bulldog 32%
- Great Dane.....27%
- Pit Bull.....26%
- Bulldog 24%
- Bull Mastiff 24%
- English Bulldog 21%
- Miniature Pinscher.....6.3%
- Dachshund 4%
- Chihuahua 3.5%

Compared to Mixed Breed ..0.32%

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Canine breeds at higher risk for struvite urolithiasis*:

84% of the breeds identified at risk are medium to giant breed dogs.

(Note: Struvite uroliths in dogs are most often infection-induced.)

- Saint Bernard 92%
- Labrador Retriever.....81%
- Golden Retriever.....77%
- Rottweiler 72%
- Chow Chow.....69%
- Scottish Terrier 69%
- Corgi 68%
- Boxer 68%
- Cocker Spaniel 67%
- German Shepherd 67%
- Bernese Mountain Dog.....64%
- Border Collie 64%
- Australian Shepherd 62%
- Beagle 57%
- Pug.....55%
- Pekingese 54%
- Shih Tzu 46%

Compared to Mixed Breed...42%

Canine breeds at higher risk for urate urolithiasis*:

- Dalmatian 94%
- American Bulldog 72%
- Black Russian Terrier 62%
- Giant Schnauzer 43%
- English bulldog 37%
- Bulldog 37%
- Pit Bull 34%
- Yorkshire Terrier 6.0%
- Havanese 4.2%
- Pug 3.4%
- Chihuahua 3.2%
- Jack Russell Terrier.....3.2%
- Pekingese.....3.1%
- Shih Tzu 2.5%
- Dachshund 2.2%
- Miniature schnauzer.....1.8%

Compared to Mixed Breed...1.2%

Canine breeds at higher risk for calcium oxalate urolithiasis*:

74% of the breeds identified at risk are small breed dogs

- Wire Fox Terrier..... 81%
- Fox Terrier 79%
- Miniature Pinscher..... 73 %
- Pomeranian ..... 72 %
- Schnauzer..... 71%
- Maltese 71%
- Cairn Terrier ..... 71 %
- Chihuahua 68%
- Portuguese Water Dog ..... 69 %
- Papillon..... 69%
- Kerry Blue Terrier 69%
- Miniature Schnauzer 65%
- Doberman Pinscher 64%
- Lhasa Apso 62%
- Yorkshire Terrier 62%
- Jack Russell Terrier 60%
- Standard Poodle 59%
- Miniature Poodle ..... 57 %
- Boston Terrier 54%
- Keeshond..... 54 %
- Havanese 50%
- Cavalier King Charles Spaniel 47%
- Bichon Frisé 43.4%

Compared to Mixed Breed...41%

87% of the feline urolith submissions were from the following breeds:

- Domestic Shorthair (DSH)
- Domestic Mediumhair (DMH)
- Domestic Longhair (DLH)

The study identified gender associations with certain types of uroliths, as follows:

- **Males:** calcium oxalate, urate, calcium phosphate apatite, dried solidified blood calculi.
- **Females:** struvite.

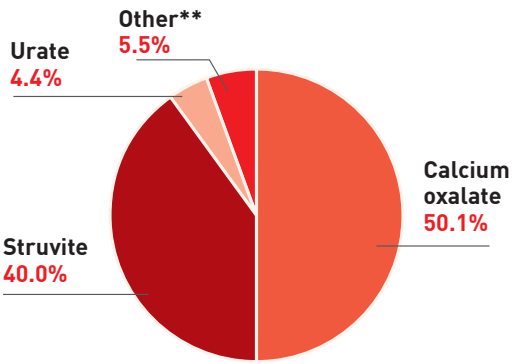
Identified trends in urolith composition between 1998-2014:

- Calcium oxalate: stable
- Struvite: ↓
- Urate: ↑
- Compound & Mixed: ↑

Feline breeds at higher risk for calcium oxalate urolithiasis*:

- Tonkinese 83%
- Burmese.....80%
- Himalayan 69%
- Devon Rex 69%
- Persian 68%
- Siamese 59%

Compared to DSH 49%



The chart shows the average percentage for each type of urolith analyzed between 1998 and 2014.

Feline breeds at higher risk for urate urolithiasis*:

- Egyptian Mau 80%
- Ocicat 44%
- Birman 29%
- Siamese 16%

Compared to DSH 4.2%

Feline breeds at higher risk for struvite (magnesium ammonium phosphate hexahydrate) urolithiasis*:

- Domestic Longhair 48%

Compared to DSH 41%

* The % beside each breed is the % of uroliths of this mineral type amongst the uroliths submitted from this breed.

** Other includes: cystine, xanthine, silica, calcium phosphate, potassium magnesium pyrophosphate, dried solidified blood calculi, compound, mixed.

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