Vitamin D metabolism and disorders in dogs and cats

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Abstract
Vitamin D plays an important role in regulating calcium metabolism and in the development and maintenance of skeletal health of companion animals. There is also a growing interest in understanding the role vitamin D plays in non-skeletal health in both human and veterinary patients. This review provides an update of our current understanding of vitamin D biology in dogs and cats and gives an overview of how vitamin D metabolism can be assessed in companion animals. Congenital and acquired vitamin D disorders are then summarised before the review concludes with a summary of recent studies which have explored the role of vitamin D in the development and outcomes of non-skeletal diseases of dogs and cats.

Introduction
Vitamin D plays an important role in regulating calcium metabolism and in the development and maintenance of skeletal health of companion animals. In addition, and paralleling a similar phenomenon in human medicine, there is growing interest in understanding the role of vitamin D has on non-skeletal health outcomes in dogs and cats (R. J. Mellanby, 2016). Here, we review vitamin D metabolism in dogs and cats and discuss both congenital and acquired vitamin D disorders. We conclude with an overview of our current understanding of the role vitamin D plays in the non-skeletal health of dogs and cats.

Metabolism and role of vitamin D on skeletal health
The importance of vitamin D on skeletal health has been known for almost a century (Elder & Bishop, 2014). The classic experiments of Sir Edward Mellanby, which involved supplementing dogs fed a restricted diet with either linseed or cod liver oil, demonstrated that cod liver oil, but not linseed oil, could protect dogs from developing rickets (E. Mellanby, 1976). The anti-rachitic factor in cod liver oil was later discovered to be vitamin D (Elder & Bishop, 2014). Dogs and cats, unlike other mammals such as humans, cattle and sheep, cannot produce vitamin D cutaneously so are reliant on their diet to obtain vitamin D (How, Hazewinkel, & Mol, 1994a, 1994b, 1995; Hurst, Homer, Gow, et al., 2020). Vitamin D is available in two forms, namely vitamin D$_2$ (ergocalciferol) and D$_3$ (cholecalciferol), with most pet foods supplemented with vitamin D$_3$ (Parker, Rudinsky, & Chew, 2017). Where the term ‘vitamin D’ is used in this review we refer to cholecalciferol/vitamin D$_3$ unless otherwise stated.

Following intestinal absorption, vitamin D$_2$ and D$_3$ enter the circulation and are predominantly bound to the vitamin D binding protein (VDBP), with a small percentage also bound to albumin (Bikle et al., 1986; Herrmann, Farrell, Pusceddu, Fabregat-Cabello, & Cavalier, 2017); other vitamin D metabolites are also bound in this
manner. Less than 1% of vitamin D circulates as free (unbound) and is readily available for cellular utilisation (Bikle, Malmstroem, & Schwartz, 2017; Schwartz et al., 2018). It is possible that vitamin D which is bound to the VDBP can also exert a biological effect as several target organs of 25(OH)D express megalin, a transmembrane protein which mediates internalisation of VDBP-bound metabolites. Vitamin D$_{2/3}$ are prohormones that are subsequently activated by sequential hydroxylation steps by the action of cytochrome P450 (CYP) enzyme family (Jones, Prosser, & Kaufmann, 2014). Vitamin D$_{2/3}$ is first hydroxylated at C25 in the liver to 25-hydroxyvitamin-D$_{2/3}$ (25(OH)D$_{2/3}$) (calcifediol), primarily catalysed by 25-hydroxylases CYP2R1 in the endoplasmic reticulum of the liver and to a lesser extent by CYP27A1 in the liver mitochondria (Cheng, Levine, Bell, Mangelsdorf, & Russell, 2004; Zhu, Ochalek, Kaufmann, Jones, & Deluca, 2013). Hydroxylation at C1$\alpha$ in the proximal tubule of the kidney converts 25(OH)D$_{2/3}$ to the most hormonally active form, 1$\alpha$,25-dihydroxyvitamin-D$_2$/$D_3$ (1,25(OH)$_2$D$_2$/$D_3$) (calcitriol). Hydroxylation at C1$\alpha$ occurs in the kidney mitochondria by the action of 1$\alpha$-hydroxylase CYP27B1 (Zehnder et al., 1999). This enzyme has been detected in other tissues and cell types, and evidence of local production of 1,25(OH)$_2$D$_2$/$D_3$ was a major contributor to identifying extraskeletal roles of vitamin D (Adams & Hewison, 2012; Hewison et al., 2007; Zehnder et al., 2001). The regulation of CYP27B1 is tightly controlled via parathyroid hormone (PTH) (Brenza & DeLuca, 2000; Murayama et al., 1998; Murayama et al., 1999) and fibroblast growth factor 23 (FGF23) (Shimada et al., 2004), as well as a negative feedback loop whereby 1,25(OH)$_2$D acts on itself to suppress CYP27B1 and induce its own catabolism by promoting CYP24A1 (Boyle, Gray, & DeLuca, 1971; Murayama et al., 1999). CYP24A1 can induce C23 and C24 hydroxylation of 1,25(OH)$_2$D and 25(OH)D (Beckman et al., 1996; Jones et al., 2014; Prosser & Jones, 2004). C24 hydroxylation of 1,25(OH)$_2$D results in a five-step process which culminates with calcitriol being excreted in the bile (G. S. Reddy & Tserng, 1989). C24 hydroxylation of 25(OH)D$_3$ forms 24,25-dihydroxyvitamin-D3 (24,25(OH)$_2$D$_3$). 24,25(OH)$_2$D$_3$ was thought to be an inactive catabolic product of 25(OH)D$_3$, however studies have now shown that it exerts biological activity independent of the Vitamin D Receptor (VDR) (Boyan, Hurst-Kennedy, Denison, & Schwartz, 2010; Boyan et al., 2016; C. Martineau et al., 2018; St-Arnaud, 2010).

Vitamin D metabolites can also be metabolised via the C3-epimerization pathway. C3-epimerization results in the hydroxyl group at position C3 of the A-ring of the vitamin D metabolite structure being converted from the alpha to beta orientation, forming stereoisomers of the original metabolite (the only difference between the molecules is the spatial arrangement of the hydroxyl group at C3). All major vitamin D intermediate metabolites are susceptible to epimerization in this way and the process is irreversible; is independent on the presence of a hydroxyl group at the C1$\alpha$ and C25 positions (Brown et al., 2005); and epimers can be further metabolized by hydroxylases at C1$\alpha$ and C25 as in the standard pathway (Bailey, Veljkovic, Yazdanpanah, & Adeli, 2013; Kamao et al., 2004; Tuckey, Tang, Maresse, & Delaney, 2019). This means that, not only can...
25(OH)D, 1,25(OH)₂D and 24,25(OH)₂D all be epimerized to 3-epi-25(OH)D, 3-epi-1,25(OH)₂D and 3-epi-24,25(OH)₂D respectively, but 3-epi-25(OH)D can be C1α and C24 hydroxylated by their respective CYP enzymes (Bailey et al., 2013; Kamao et al., 2004; Kamao et al., 2001; Rhieu et al., 2013). The enzyme responsible for C3-epimerization has yet to be identified but the process can occur in a range of extrarenal tissues and cell types and it employs enzymes distinct from those classically involved in vitamin D metabolism (Astecker, Reddy, Herzig, Vorisek, & Schuster, 2000; Masuda et al., 2000; Siu-Caldera et al., 1999).

The principal role of 1,25(OH)₂D, acting alongside parathyroid hormone (PTH) and calcitonin, is to maintain circulating calcium concentrations within a tight reference range (R. J. Mellanby, 2016; Tornqvist-Johnsen et al., 2020). 1,25(OH)₂D exerts its actions on target cells and tissues following binding to the VDR which is widely expressed in most canine tissues (Cartwright et al., 2018). The VDR-1,25(OH)₂D complex then heterodimerises with the retinoic acid receptor, retinoid X receptor (RXR). This complex exerts genomic actions as a transcription factor to regulate target genes that contain a vitamin D response element in their promoter, which then influence numerous physiological processes (Elder & Bishop, 2014). Alternatively, 1,25(OH)₂D can bind to the plasma membrane VDR to induce rapid response biological actions, for example, the stimulation of intestinal calcium transport, which are non-genomic actions of 1,25(OH)₂D (Bikle, 2014; Haussler, Jurutka, Mizwicki, & Norman, 2011). Calcium homeostasis is achieved mainly by the ability of 1,25(OH)₂D to increase intestinal absorption of calcium (Bikle, 2014). 1,25(OH)₂D can also increase calcium reabsorption in the distal renal tubule and mobilises the release of calcium from the skeleton in conjunction with parathyroid hormone (PTH) during periods of hypocalcaemia (Christakos, Dhawan, Verstuyf, Verlinden, & Carmeliet, 2016). When circulating concentrations of calcium decline, increased plasma PTH concentrations lead to an increase in renal CYP27B1 activity, which in turn raises circulating 1,25(OH)₂D concentrations. The epimeric forms of 1,25(OH)₂D also appear to have a role in calcium homeostasis. For example, 3-epi-1,25(OH)₂D₃, the most biologically active epimer, can suppression PTH secretion to a similar degree as the parent metabolite (Bailey et al., 2013; Bikle, 2014; Brown et al., 2005). Despite this, 3-epi-1,25(OH)₂D₃ has low calcaemic effects (Fleet, Bradley, Reddy, Ray, & Wood, 1996; Rhieu et al., 2013) and anti-proliferative activity of ~30% of that of the non-epimeric form (Bailey et al., 2013; Kamao et al., 2004); it also exhibits reduced affinity for binding to the VDBP and the VDR (Kamao et al., 2004; Kamao et al., 2001; Masuda et al., 2000; Nakagawa et al., 2001; Norman et al., 1993). This reduced affinity results in reduced ability to induce calcium transport (Al-Zohily, Al-Menhal, Gariballa, Haq, & Shah, 2020).

**Measurement of vitamin D metabolites**

Although a wide range of vitamin D metabolites can be measured using a variety of instrumentation and technologies, the primary vitamin D metabolites measured in both human and veterinary clinical cases are
25(OH)D and 1,25(OH)\(_2\)D (Su, Narla, & Zhu, 2014). 25(OH)D has a half life of 2-3 weeks and is widely regarded as the most accurate measurement to assess vitamin D status (Rumbeiha et al., 1999). 25(OH)D serves as a reservoir for the generation of the more biologically active 1,25(OH)\(_2\)D. 25(OH)D can be measured by a wide range of assays including chemiluminescent immunoassay, enzyme immunoassay, radioimmunoassay, high-performance liquid chromatography (HPLC) and the widely considered gold standard technique of liquid chromatography tandem mass spectrometry (LC-MS/MS) (Fritz, Navetta, Wolford, & Colangelo, 2017; Jenkinson et al., 2016).

Quantification of vitamin D metabolites, even of 25(OH)D, is not straightforward. The vitamin D pathway is a highly complex and dynamic system involving a number of structurally very similar compounds which may interfere with analysis; not only that, but the metabolites circulate predominantly bound the VDBP and at low concentrations, meaning they are particularly challenging to identify, isolate and accurately quantify.

Immunoassay-based techniques can be integrated into fully-automated laboratory systems allowing for rapid analysis in a high-throughput clinical chemistry laboratory setting. They offer good sensitivity for 25(OH)D and require minimal sample volume, however selectivity continues to be one of their major limitations (Altieri et al., 2020; Couchman & Moniz, 2017; Volmer, Mendes, & Stokes, 2015). Cross reactivity with different vitamin D metabolites, especially 24,25(OH)\(_2\)D, occurs in many of the immuno-based assays. Lack of selectivity between 25(OH)D\(_2\) and 25(OH)D\(_3\), and unequal cross reactivity of the two metabolites can cause bias and have a significant impact depending on the sample being analysed. Furthermore, vitamin D metabolites must be released from the VDBP in order to be measured, which is difficult to achieve in automated immunoassays in which strong organic solvents cannot be used (Altieri et al., 2020; Couchman & Moniz, 2017). Therefore, samples in which variation in the VDBP levels exists (during pregnancy or cases of renal disease for example) are known to impact on the performance of these assays.

LC-MS/MS is considered the gold-standard method for analysing vitamin D metabolites (Bikle, 2018; Dirks et al., 2018; Garg, 2018). This method does not suffer from those limitations outlined above for immunoassays and has the capability to detect and quantify multiple, highly similar analytes simultaneously within one sample, enabling the profiling of several metabolites of the vitamin D pathway. Using LC-MS/MS facilitates the effective release of vitamin D metabolites from the VDBP during sample preparation by the use of strong organic solvents. High levels of selectivity are achieved in LC-MS/MS methods by the chromatographic resolution of individual analytes and detection based on specific mass-to-charge (m/z) ratios and fragmentation patterns, meaning that D\(_1\) and D\(_2\) metabolites can be distinguished and quantified individually.
Isomeric and isobaric metabolites such as the C3-epimers complicate the bioanalytical assessment of vitamin D metabolites even by LC-MS/MS. The C3-epimers have the same molecular mass as the non-epimer metabolites, meaning they are not distinguished in the mass spectrometer and many LC-MS/MS assays do not separate them. However, optimisation of the LC parameters and the use of specialist LC columns can ensure that sufficient resolving power can be achieved and the C3-epimers can be chromatographically separated for accurate quantitation. Although low levels of 3-epi-25(OH)D have been recently detected in dogs (Hurst, Homer, Denham, et al., 2020), cats have much higher concentrations (Sprinkle, Hooper, & Backus, 2018), which, given the unconfirmed biological activity of the C3-epimers, if not resolved from 25(OH)D may overestimate 25(OH)D concentration.

As LC-MS/MS has the capability to assess multiple metabolites from a single sample, assays which include the measurement of other vitamin D metabolites are now being reported. Since 1,25(OH)_{2}D has the most impact on calcium homeostasis, there are clinical indications for its measurement, notably in dogs with hypercalcaemia caused by calcitriol exposure. A range of assays have been reported for the quantification of 1,25(OH)_{2}D, including immunoassays and LC-MS/MS methodologies (Jenkinson et al., 2016). Serum concentrations of 24,25 dihydroxyvitamin D, the degradation product of 25(OH)D, have been quantified in dogs and found to be present in higher concentrations than other species (Spoo et al., 2015). 24,25 dihydroxyvitamin D can only be measured by LC-MS/MS, which also provides concomitant quantification of 25(OH)D, thus allowing calculation of the 25(OH)D: 24,25 dihydroxyvitamin D ratio, also known as the vitamin D metabolite ratio (VMR).

Free 25(OH)D concentrations have recently been quantified in dogs for the first time using an enzyme-linked immunosorbent assay (ELISA). In 117 healthy dogs, the median and inter-quartile range of free 25(OH)D concentrations detected was 15.2 (12.5 – 23.2) pmol/L (Hurst, Homer, Denham, et al., 2020). Further studies are required to ascertain whether total or free 25(OH)D concentrations is the best assessment of vitamin D status in companion animals. This is likely to have important clinical implications. For example, in people, VDBP levels change significantly during pregnancy influencing the concentration of vitamin D metabolites including 25(OH)D (Fernando et al., 2020) highlighting the merits of measuring free 25(OH)D (Ananthakrishnan, 2016).

One of the main challenges regarding vitamin D analysis in both human and veterinary samples is the lack of standardization between laboratories (Binkley et al., 2017). Harmonization of 25(OH)D testing has been historically challenging but has improved recently through the development of standard reference materials (SRM) for some metabolites (Tai et al., 2017) and reference method procedures (RMPs) (Stepman, Vanderroost, Van Uytfanghe, & Thienpont, 2011; Tai, Bedner, & Phinney, 2010; Tai & Nelson, 2015) by the
Vitamin D Standardization Program (VDSP) and External Quality Assessments (EQAs) such as the Vitamin D External Quality Assessment Scheme (DEQAS), a global quality assurance scheme for laboratories measuring vitamin D metabolites (Durazo-Arvizu et al., 2019), enables participating laboratories to validate and continuously monitor their assay performance in comparison to both National Institute of Standards and Technology (NIST) RMPs and other laboratories using the same method. These programs have considerably reduced the variability between laboratories (Binkley et al., 2017; Carter et al., 2018). SRMs will be required for other vitamin D metabolites and there are currently no EQAs specific for the assessment of vitamin D in veterinary samples (DEQAS assesses the analysis of vitamin D metabolites in human serum samples).

### Congenital vitamin D disorders

Congenital disorders of vitamin D homeostasis are rare in companion animals and can be classified into three main types (Figure 1) (Dittmer & Thompson, 2011). Vitamin D dependent rickets type 1A (VDDR-1A) refers to animals with CYP27B1 deficiencies which leads to impaired conversion of 25(OH)D to the most metabolically active vitamin D metabolite 1,25(OH)₂D (Grahn, Ellis, Grahn, & Lyons, 2012). In contrast, vitamin D dependent rickets type 1B is caused by mutations in CYP2R1 gene leading to failure of vitamin D to be converted to 25(OH)D (Teshima et al., 2019). Both type IA and 1B are inherited as autosomal recessive traits (Grahn et al., 2012). The main consequences of these disorders are hypocalcaemia which can be severe enough to cause seizures and skeletal abnormalities; generalised skeletal pain can also be a feature. Feline VDDR-1A has been reported (Geisen, Weber, & Hartmann, 2009; Grahn et al., 2012), while type 1B has been reported in a cat (Teshima et al., 2019). The disorders can be managed, with varying degrees of success, mainly through 1,25(OH)₂D supplementation.

The third group of genetic vitamin D disorders is vitamin D dependent rickets type 2A (VDDR-2A), also called hereditary vitamin-D resistant rickets (HVDRR), which involves mutations in the VDR gene. In this rare condition, hypocalcaemia, secondary hyperparathyroidism and increased concentration of 1,25(OH)₂D are typical biochemical changes alongside skeletal changes and accompanying pain which is consistent with rickets (LeVine et al., 2009). Alopecia may also be a feature of VDR gene mutations. Treatment is challenging and based around high dose calcium and 1,25(OH)₂D supplementation. VDDR-2A has been reported in the veterinary literature in cats (Godfrey, Anderson, Barber, & Hewison, 2005; Schreiner & Nagode, 2003; Tanner & Langley-Hobbs, 2005) and a Pomeranian dog (LeVine et al., 2009).

Provisional diagnosis of these congenital vitamin D disorders is usually based on compatible clinical signs in young patients, who have a dietary history which demonstrates adequate vitamin D intake. Definitive diagnosis is more challenging. A genetic test for VDDR-2A is available for Pomeranian dogs (Laboklin, UK).
Experimentally, the dysfunction of the vitamin-D receptor in VDDR-2A has been proven by the inability of skin fibroblasts, which express VDR, to effectively bind 1,25(OH)₂D (Godfrey et al., 2005; Tanner & Langley-Hobbs, 2005). Additionally, entire DNA sequencing of the CYP27B1 gene has also been described as a means of diagnosing VDDR-1A (Teshima et al., 2019).

**Acquired vitamin D disorders – deficiency**

Acquired vitamin D disorders can result from either an excess or deficiency of vitamin D. An important cause of vitamin D deficiency in companion animals is the consumption of a diet which is deficient in vitamin D. The European pet food industry (FEDIAF) nutritional guidelines state that the minimum recommended vitamin D allowance for adult dogs is 55.2 IU and for adult cats 25 IU per 100g dry matter. One study demonstrated that 25(OH)D concentration was highly variable, including some instances of hypovitaminosis D, in healthy dogs fed a variety of different commercial diets, possibly implying that some commercial diets might contain inadequate quantities of vitamin D (Sharp, Selting, & Ringold, 2015). However, a recent study demonstrated that the majority of proprietary dog foods tested contained vitamin D concentrations within the manufacturers’ stated range (Kritikos et al., 2018). Consequently, most cases of hypovitaminosis D occur in dogs and cats fed improperly prepared homemade diets (Dodd S, 2019; Hutchinson et al., 2012; Malik, Laing, Davis, Allan, & Wigney, 1997; Sharp et al., 2015; Tal, Parr, MacKenzie, & Verbrugghe, 2018). The hypovitaminosis D state may result in hypocalcaemia, secondary hyperparathyroidism and possible skeletal abnormalities, typically rickets in young animals and osteomalacia in older dogs and cats (Hall, 2020). In rickets, the classical skeletal changes include impaired mineralisation of physeal and epiphyseal cartilage with lesions typically involving the fastest growing bones such as the radius, tibia, metacarpals and metatarsals. Clinically this manifests as a stiff, lame gait, deformed limbs, pain on palpation of bones and muscle weakness. Seizures may also occur due to hypocalcaemia (Dittmer & Thompson, 2011). On radiographic and post-mortem examination, typical changes include widening of the physeal growth plate, metaphyseal flaring, poor skeletal mineralisation and potentially pathological fractures (Dittmer & Thompson, 2011).

Low vitamin D status has been reported in cats and dogs with gastrointestinal disorders (compared to healthy controls), especially in dogs with a protein losing enteropathy (PLE) (Gow et al., 2011; S. Lalor et al., 2014; R. J. Mellanby, Mellor, et al., 2005), as well as dogs with exocrine pancreatic insufficiency (Barko & Williams, 2018) acute pancreatitis (Kim et al., 2017) and both dogs and cats with liver disease (Kibler, Heinze, & Webster, 2020; Schulze, Rothuizen, van Sluijs, Hazewinkel, & van den Ingh, 2000).

**Acquired vitamin D disorders – excess**
Vitamin D excess and associated hypercalcaemia can occur in cats and dogs through the consumption of diets containing disproportionately high concentrations of vitamin D. This typically occurs as a consequence of inadvertent consumption of vitamin D containing rodenticides or medications, or through the administration of inappropriately vitamin D enriched commercial diets (Cortinovis, Pizzo, & Caloni, 2015; Crossley, Bovens, Pineda, Hibbert, & Finch, 2017; DeClementi & Sobczak, 2018; Y. Nakamura et al., 2004). As with vitamin D minimum values, the (FEDIAF) nutritional guidelines also state the safe maximum values and European legal limit of vitamin D that canine and feline commercial diets should contain: for cats a maximum of 3000 IU/100 g DM is safe for all feline life stages (EU legal limit 227 IU/100g DM), while in dogs this is 320 IU/100g DM (EU legal limit 227 IU/ 100g DM). Home-made and commercial raw diets have been shown to contain vitamin D concentrations above the recommended maximum (Freeman & Michel, 2001). Although dogs can tolerate oral vitamin D doses above the recommended allowance of 55.2 IU/ 100g DM without major ill-effect (Young & Backus, 2016), significant over-fortification of foodstuff with vitamin D can lead to debilitating hypercalcaemia in both dogs and cats (Bischoff & Rumbeiha, 2018). This can occur due to the presence of excessive supplementary vitamin D (R. J. Mellanby, Mee, Berry, & Herrtage, 2005; Wehner et al., 2013) or consumption of a diet high in vitamin D rich ingredients. Historically, increased concentrations of vitamin D have been observed in cat foods which has been attributed to their high content of oily fish (J. G. Morris, 1996; J. G. a. E. Morris, K.E., 1996).

Hypervitaminosis D secondary to rodenticide consumption is well recognised and unfortunately, is likely to be an increasing problem as some rodenticide manufacturers switch to vitamin D containing products (DeClementi & Sobczak, 2018; Gerhard & Jaffey, 2019). Consumption of vitamin D containing medication is also an increasingly well recognised cause of hypervitaminosis D in companion animals. This can arise due to the ingestion of oral vitamin D supplements or unintentional consumption of ointments containing vitamin D analogues, now widely prescribed for psoriasis treatment (Fan et al., 1998; Ho, Ellison, Edwards, & Bates, 2020; Saedi, Horn, Muffoletto, & Wood, 2007). There have been reported cases of dogs developing hypervitaminosis D as a consequence of licking owner’s skin covered in topical ointments containing 1,25(OH)₂D or vitamin D analogues or through consumption of detached 1,25(OH)₂D containing skin plaques (Fujita et al., 2017; K. Nakamura et al., 2016).

Clinical disease occurs in hypervitaminosis D states when vitamin D metabolite concentrations become sufficiently increased to cause hypercalcaemia, resulting in clinical signs such as polydipsia, polyuria, lethargy and inappetence. Acute renal failure and long term complications caused by widespread soft tissue mineralisation can occur in the most severely affected individuals (DeClementi & Sobczak, 2018; Morita et al., 1995). Two dogs diagnosed with hypervitaminosis D had serum 25(OH)D concentrations around 400 nmol/L.
(reference range 17-140 nmol/L) when measured in DEQAS accredited laboratory (R. J. Mellanby, Mee, et al., 2005). Where there is known exposure to potentially toxic doses of vitamin D, immediate treatment involves induction of emesis followed by administration of activated charcoal, in conjunction with supportive and symptomatic care, typically involving intravenous fluid therapy (DeClementi & Sobczak, 2018). Intravenous lipid emulsion has been recently reported as a method to successfully lower serum 25(OH)D concentrations in a dog with vitamin D toxicity (Perry, McMichael, Rick, & Jewell, 2016). Both experimentally and clinically, bisphosphonates have been successfully used to treat hypervitaminosis D, with intravenous pamidronate or zolendronate the most widely utilised (Rumbeiha et al., 1999; Schenk, Lux, Lane, & Martin, 2018). Whilst bisphosphonates are effective and generally well tolerated in companion animals (Hostutler et al., 2005), complications such as osteonecrosis are increasingly well recognised (Larson, Oakes, Epperson, & Chew, 2019; Lundberg, Roady, Somrak, Howes, & Fan, 2016). The lipid soluble properties of vitamin D can lead to sequestration of the vitamin into fat deposits in the body. Consequently, it can take several months for the serum 25(OH)D concentrations to return to within the reference interval following a hypervitaminosis D episode (Gerhard & Jaffey, 2019). Long term sequelae such as soft tissue mineralisation are well recognised complications of prolonged hypercalcaemia secondary to hypervitaminosis D (Crossley et al., 2017).

Endogenous over-production of active vitamin D metabolites is another mechanism for the development of hypervitaminosis D. This classically occurs in patients with granulomatous diseases where a dysregulated immune response results in the excessive production of 1,25(OH)₂D from 25(OH)D, typically by macrophages, without the regulation of negative feedback. This syndrome has been reported in dogs with sterile granulomatous lymphadenitis (R. J. Mellanby et al., 2006), granulomatous inflammation following placement of a biological implant (Linde, Kelleher, & Perry, 2018), Angiostrongylus vasorum infections (Boag, Murphy, & Connolly, 2005) and Mycobacterium avium subspecies hominissuis infection (Hobi, 2015). While in cats, multiple infectious diseases have been associated with this syndrome: blastomycosis (Stern, Chew, Schissler, & Green, 2011), Mycobacterium infection (Baral et al., 2006), feline infectious peritonitis (Savary, Price, & Vaden, 2000), Toxoplasmosis (Savary et al., 2000), Nocardia infection (Mealey, Willard, Nagode, & Helman, 1999), Cryptococcosis (Savary et al., 2000) and rhinitis caused by Actinomyces (Savary et al., 2000). Excessive production of 1,25(OH)₂D has also been postulated to be important in driving hypercalcaemia in dogs with autoimmune diseases such as immune mediated polyarthritis (Adamany & Dhumeaux, 2016). Successful treatment of the underlying condition typically resolves the increases in systemic 1,25(OH)₂D concentrations and associated hypercalcaemic state (Adamany & Dhumeaux, 2016).

Role of vitamin D on non-skeletal health
The vital role of vitamin D in the maintenance of bone health and calcium homeostasis has been widely acknowledged since the discovery of vitamin D nearly a century ago. Since the more recent discovery of VDR expression in tissues beyond the intestines, kidney and bone, many studies have investigated the non-skeletal health benefits of vitamin D in both human and veterinary patients (Holick, 2007; R. J. Mellanby, 2016).

**Gastrointestinal Diseases**

In patients with PLE, total hypocalcaemia is not an uncommon biochemical abnormality and historically this has been attributed to a reduction in protein bound calcium, secondary to hypoalbuminaemia (Jacobs, Collins-Kelly, Lappin, & Tyler, 1990; Jergens, Moore, Haynes, & Miles, 1992). However, the wider availability of ionised calcium assays has generated a greater awareness that many dogs with a PLE have reductions in ionised as well as protein bound calcium (Kull, Hess, Craig, Saunders, & Washabau, 2001). Whilst a blunted PTH response due to concurrent hypomagnesemia has been postulated to be an important driver of hypocalcaemia in some cases of PLE (Kimmel, Waddell, & Michel, 2000), there is a growing consensus that low concentrations of 25(OH)D play an important role in the development of the hypocalcaemic state associated with PLE in dogs (Allenspach, Rizzo, Jergens, & Chang, 2017; Gow et al., 2011). A positive correlation was identified between 25(OH)D concentrations, serum albumin and ionised calcium levels in dogs with inflammatory bowel disease (IBD) in one study (Gow et al., 2011). This study also demonstrated that dogs with PLE had markedly lower 25(OH)D concentrations compared to: a healthy control population, dogs with intestinal inflammation who were normoalbuminemic and hospitalised dogs without gastrointestinal signs. Furthermore, serum 25(OH)D concentration has been found to be negatively correlated with duodenal histopathology scores (Wennogle, Priestnall, Suarez-Bonnet, & Webb, 2019) and inversely correlated with canine inflammatory bowel disease activity index (Gow et al., 2011). Similarly, dogs with a chronic inflammatory enteropathy (CIE) and low serum 25(OH)D concentrations had higher canine chronic enteropathy clinical activity index scores compared to CIE dogs with normal serum 25(OH)D concentrations (Wennogle et al., 2019). In some dogs with a PLE, the hypovitaminosis D and secondary hypocalcaemia can be so severe that neurological signs, including seizures, can be a significant clinical complication of the intestinal disease (Whitehead, Quimby, & Bayliss, 2015; Woods, 2019). Additionally, the severity of the hypovitaminosis D state has been shown to correlate with adverse clinical outcomes in dogs with a PLE (Allenspach et al., 2017; H. Titmarsh, A. G. Gow, et al., 2015).

The cause of hypovitaminosis D in PLE is likely to be multifactorial. Malabsorption of fat soluble vitamin D (especially in dogs with lymphangiectasia) is widely considered to be the most important cause; however, if this was the sole aetiology, malabsorption of other fat-soluble vitamins, such as vitamin K, might be expected to occur simultaneously (Kimmel et al., 2000). Although this has not been specifically investigated, significant vitamin K deficiency would be a rare complication of PLE given the absence of coagulopathies caused by a
deficiency in vitamin-K dependant clotting factors. Contrarily, patients with PLE have been described as hypercoagulable (Goodwin, Goggs, Chan, & Allenspach, 2011). Spontaneous bleeding has been reported in two cats with lymphoplasmacytic enteritis, which the authors hypothesised could be attributed to hypovitaminosis K secondary to malabsorption (Edwards & Russell, 1987). However, vitamin K was not measured, activated partial thromboplastin time (APTT) and prothrombin time (PT) were only assessed in one cat and the presence of malabsorption was presumed due on weight loss; neither cat was hypoalbuminaemic and cobalamin was not measured.

Other factors that may contribute to the depletion of vitamin D in PLE patients include: ongoing systemic inflammation, reduced dietary intake of vitamin D and increased vitamin D metabolism. Additionally, 1,25(OH)₂D has immunomodulatory effects which may support mucosal health (Kellermann et al., 2020). Due to this immunomodulatory role it is also possible that patients with PLE develop this disease in part due to their 25(OH)D deficiency (Ananthakrishnan, 2016). A human study demonstrated that women who had low 25(OH)D levels were at an increased risk of the development of Crohn’s disease (Ananthakrishnan, 2016). Finally, magnesium is required for the hydroxylation step necessary to produce 1,25(OH)₂D in the renal tubules. Therefore, concurrent hypomagnesemia in dogs with PLE may consequently reduce the amount of 1,25(OH)₂D available (Kimmel et al., 2000). Protocols for correction of hypovitaminosis D associated with chronic enteropathy have not been well established in human or veterinary medicine. Although parenteral administration of calcitriol would be logical, due to the concerns about impaired gastrointestinal absorption of fat soluble vitamins, in people with inflammatory bowel disease enteral supplementation is often utilised and is beneficial (Gubatan & Moss, 2018). The optimal dose of calcitriol and the duration of treatment is also uncertain (Gow et al., 2011).

Pancreatic and Hepatic Diseases

Dogs with pancreatic and hepatic disorders have also presented with low serum concentrations of 25(OH)D. Dogs with weight loss and exocrine pancreatic insufficiency (EPI) were found to have significantly lower serum 25(OH)D concentrations than dogs with EPI and stable weight (Barko & Williams, 2018). Additionally, concentrations of three fat soluble vitamins (A, E and D) remained reduced in dogs with EPI even after pancreatic enzyme supplementation (Barko & Williams, 2018). The reason for this is uncertain, hypothesised explanations include; long term dietary fat malabsorption which is refractory to pancreatic enzymes supplementation, depletion of stored vitamin D due to the loss of adipose tissue associated with weight loss, or on-going reduced dietary intake secondary to the EPI. Dogs with acute pancreatitis had lower 25(OH)D status than healthy dogs (Kim et al., 2017). In addition, dogs with acute pancreatitis which survived had significantly higher serum 25(OH)D concentrations than dogs who died (Kim et al., 2017). This positive
correlation between reduced vitamin D status and prognosis has also been identified in human studies (Bang et al., 2011; S. V. Reddy, Ramesh, & Bhatia, 2013). Vitamin D deficiency and rickets has occasionally been reported in dogs with liver diseases likely as a consequence of impaired intestinal absorption of vitamin D (Schulze et al., 2000). Additionally, many cats with cholestatic liver disease, especially hepatic lipidosis, had suboptimal serum 25(OH)D levels, although the clinical importance of this remains uncertain given that that ionised calcium concentrations remained normal (Kibler et al., 2020).

**Infectious Diseases**

Vitamin D status has been found to be lower in patients with a wide variety of infectious diseases. Dogs with clinically active leishmaniasis (Rodriguez-Cortes et al., 2017), blastomycosis (O’Brien, McMichael, & Le Boedec, 2018), babesiosis (Dvir, Rosa, Handel, Mellanby, & Schoeman, 2019) and Spirocerca lupi (Rosa, Schoeman, Berry, Mellanby, & Dvir, 2013) infections had lower 25(OH)D status than healthy dogs. Similarly, cats with mycobacteria and feline immunodeficiency virus infections had lower 25(OH)D concentrations than healthy cats (S. M. Lalor et al., 2012; H. F. Titmarsh, S. M. Lalor, et al., 2015). A negative relationship between 25(OH)D concentrations and disease severity has also been reported in dogs with babesiosis and Spirocerca lupi infections (Dvir et al., 2019; Rosa et al., 2013).

**Immune mediated diseases**

The role of vitamin D in immune mediated and inflammatory diseases was explored, in part, due to the identification of the VDR on the surface of many cell types, including leukocytes (Provvedini, Tsoukas, Deftos, & Manolagas, 1983). Consequently, dogs with a range of immune mediated diseases, including immune mediated haemolytic anaemia, thrombocytopenia and polyarthritis, have been found to have altered vitamin D homeostasis (Mick, Peng, & Loftus, 2019) and reduced concentrations of 25(OH)D. Additionally, low serum 25(OH)D concentrations were also predictive of a poorer clinical outcome in dogs with immune mediated diseases (Mick et al., 2019). Dogs with acute polyradioloneuritis had lower circulating 25(OH)D concentrations than dogs with idiopathic epilepsy (Laws, Kathrani, Harcourt-Brown, Granger, & Rose, 2018). Interest in the relationship between autoimmune diseases and vitamin D has been fuelled by the growing evidence that vitamin D metabolites can influence the canine immune cell function and phenotype ex-vivo, typically switching innate immune cells from a pro-inflammatory to a more anti-inflammatory response (Jaffey, Amorim, & DeClue, 2018a, 2018b). For example, a recent *in-vitro* study using blood from healthy shelter dogs identified that the concentrations of tissue necrosis factor-α significantly decreased, while interleukin-10 significantly increased, following incubation with 1,25(OH)₂D (Jaffey, Bessette, Tao, Bradley-Siemens, & Thompson, 2020).
Cardiovascular

Circulating 25(OH)D concentrations were significantly lower in dogs with congestive heart failure compared to unaffected dogs even though the two groups had a similar dietary vitamin D intake (Kraus et al., 2014). 25(OH)D concentrations were also found to be lower in dogs with chronic valvular disease stage B2 and C/D disease than dogs with stage B1 disease (Osuga et al., 2015). In cats, both 25(OH)D₃ and a C-3 epimer of 25(OH)D were lower in cats with cardiomyopathy compared to healthy cats, although, when patient age was taken into account this did not reach statistical significance since older cats had significantly lower 25(OH)D₃ concentrations (Ware et al., 2020).

Oncology

The connection between vitamin D status and neoplasia has been extensively investigated in human medicine, with one study finding that serum 25(OH)D concentrations greater than 40 ng/mL resulted in a reduced risk of rectal, colonic and breast cancer (Welsh, 2012). Several studies have shown that vitamin D metabolites have anti-proliferative effects on canine cancer cell lines in-vitro (Kaewsakhorn et al., 2005; Kunakornsawat et al., 2002; Rassnick et al., 2008). Vitamin D status of dogs with cancer has frequently been reported to be lower than control populations. For example, dogs with mast cell tumours had lower 25(OH)D concentrations than healthy dogs despite having no significant differences in vitamin D oral consumption (Wakshlag et al., 2011). Dogs with three different types of cancer (mast cell tumour, lymphoma and osteosarcoma) all had altered vitamin D metabolism compared to the healthy control population (Weidner et al., 2017). Another study found that relative risk of cancer in dogs increased with decreasing 25(OH)D concentrations (Selting, Sharp, Ringold, Thamm, & Backus, 2016). However, other studies have failed to find a difference in vitamin D homeostasis in dogs with and without cancer including the observation that 25(OH)D concentrations were not significantly different between dogs with an osteosarcoma and control dogs (Willcox, Hammett-Stabler, & Hauck, 2016).

Urinary tract disease

In dogs with chronic kidney disease (CKD), lower levels of serum 25(OH)D have been identified (Galler et al., 2012; Parker, Harjes, et al., 2017). In addition, low concentrations of 1,25(OH)₂D have also been reported in both cats and dogs with CKD. This is thought to be due to a combination of lower availability of 25(OH)D, reduced CYP27B1 (1α-hydroxylase) activity due to renal damage and urinary loss of vitamin D (Cortadellas, Fernandez del Palacio, Talavera, & Bayon, 2010; Parker, Harjes, et al., 2017). The decline in 1,25(OH)₂D concentration is considered important in the development of secondary hyperparathyroidism in CKD, leading to interest in the potential therapeutic merits of 1,25(OH)₂D supplementation in companion animals with renal failure (Barber & Elliott, 1998; Cortadellas et al., 2010; de Brito Galvao, Nagode, Schenck, & Chew, 2013). One unpublished study found that judicious administration of 1,25(OH)₂D may confer a survival benefit in dogs with
IRIS stage 3 and 4 providing calcium, phosphate and PTH can be closely monitored (Polzin, 2005b). A recent study found that administering an extended release form of 25(OH)D to dogs with CKD resulted in an increase in vitamin D metabolites, including 1,25(OH)₂D, while avoiding hypercalcaemia (Parker, Rudinsky, Benedict, Beizaei, & Chew, 2020). However, the benefits of long-term 25(OH)D supplementation on the progression of CKD, and the patient’s quality of life, remained undetermined in this study (Parker et al., 2020).

Vitamin D homeostasis disorders may increase the risk of other renal disorders, with a recent study suggesting that altered vitamin D catabolism may predispose some dogs to the development of calcium oxalate uroliths (Groth, Lulich, Chew, Parker, & Furrow, 2019). In dogs with non-azotaemic protein losing nephropathy (PLN), a significant positive correlation between serum albumin and concentrations of both 25(OH)D and 24,25(OH)₂D was observed (Miller, Rudinsky, Klamer, Chew, & Parker, 2020). Additionally, dogs with PLN had significantly lower serum 25(OH)D, 1,25(OH)₂D and 24,25(OH)₂D concentrations compared with the control population (Miller et al., 2020).

**Inflammation**

Several studies have shown an inverse relationship between biomarkers of inflammation and vitamin D status in companion animals. For example, serum 25(OH)D concentrations in dogs diagnosed with a CE have been shown to negatively correlate with circulating neutrophil, monocyte and serum pro-inflammatory cytokines (H. F. Titmarsh, A. G. Gow, et al., 2015). Furthermore, two studies have observed that vitamin D status in dogs with a CE negatively correlated with severity of intestinal inflammation (H. F. Titmarsh, A. G. Gow, et al., 2015; Wennogle et al., 2019). Hospitalised cats with neutrophil counts above the reference range had lower serum 25(OH)D concentrations than cats with neutrophil counts below the upper limit of the reference range (Titmarsh et al., 2016). Dogs with blastomycosis also had a negative relationship between vitamin D status and neutrophil counts (O’Brien et al., 2018), and CRP was significantly lower in a population of healthy dogs with a serum 25(OH)D concentration above 100 ng/mL compared to dogs with a 25(OH)D concentration below 100 ng/mL (Selting et al., 2016). However, the widely reported negative association between vitamin D status and inflammation is not an absolute consensus, with one study reporting an increase in both c-reactive protein and 25(OH)D concentrations in racing sled dogs (Spoo et al., 2015).

**Dermatology**

Serum 25(OH)D concentrations in dogs with atopic dermatitis were not significantly different from healthy dogs (Kovalik, Thoday, Berry, van den Broek, & Mellanby, 2012). However, atopic dogs which responded well to prednisolone had higher serum 25(OH)D concentrations (Kovalik et al., 2012). Oral vitamin D treatment has been shown to improve clinical outcomes in an atopic dermatitis treatment trial with an increase in serum...
25(OH)D correlating to a reduction in pruritus and skin lesions (Klinger et al., 2018). In this study paricalcitol, a VDR agonist, was also trialled in a small number of dogs, however unacceptable side effects including hypercalcaemia and polyuria/polydipsia resulted in withdrawal of treatment (Klinger et al., 2018).

Critical care

Several studies have identified a relationship between low vitamin D status and adverse clinical outcomes in hospitalised cats and dogs. A study of hospitalised cats found that cats which died within 30 days of initial assessment had significantly lower 25(OH)D than cats which survived (H. Titmarsh, S. Kilpatrick, et al., 2015). Further analysis revealed that the relationship between vitamin D status and mortality risk was not linear, as cats within the lowest 25(OH)D tertile had a significantly higher mortality risk than cats in the upper or middle tertile (H. Titmarsh, S. Kilpatrick, et al., 2015). Low vitamin D status has also been associated with anaemia in hospitalised cats (Titmarsh et al., 2020). Similarly, vitamin D status in critically ill dogs that survived was found to be significantly higher than in dogs that died (Cazzolli, Prittie, Fox, & Lamb, 2019). Critically ill dogs and dogs with sepsis had significantly lower serum 25(OH)D concentrations compared to healthy control dogs, and 25(OH)D concentration was an independent predictor of in-hospital and 30 day survival (Jaffey, Backus, McDaniel, & DeClue, 2018). Experimental induction of inflammation is known to reduce 25(OH)D concentrations in dogs (Holowaychuk et al., 2012).

Future directions

Whilst numerous studies have linked low vitamin D status to adverse non-skeletal health outcomes in cats and dogs, there is still significant uncertainty over the importance of vitamin D in shaping clinical outcomes in non-skeletal disorders. The optimal way to supplement vitamin D also remains uncertain, both in terms of the route of administration (enteral versus parenteral) and the vitamin D metabolite of choice (25(OH)D, 1,25(OH)₂D or cholecalciferol). The relationship between low 25(OH)D concentrations and the development or outcomes of non-skeletal disorders cannot be considered to be invariably causative and may, in fact, be due to reverse causation. For example, serial measurements of circulating 25(OH)D concentrations in experimental and human patients has shown that inflammation and infections can result in lower vitamin D status (Nonnecke et al., 2014; Reid et al., 2011; Waldron et al., 2013). A recent study which longitudinally tracked both markers of inflammation and 25(OH)D concentrations in dogs undergoing elective surgery have recently shown that the post-operative increase in acute phase proteins is mirrored by modest declines in total, but not free, serum 25(OH)D concentrations (Clements et al., 2020). To disentangle the relationship between vitamin D and non-skeletal health outcomes, human focussed studies have embraced mendelian randomisation approaches as well as examining the non-skeletal health benefits of long term vitamin D supplementation in large scale clinical trials (Davies, Holmes, & Davey Smith, 2018; Kupferschmidt, 2012). Mendelian...
randomisation techniques use genetic variation as a natural experiment to investigate the causal relations between potentially modifiable risk factors and health outcomes in observational data (Davies et al., 2018). This approach has allowed the health consequences of genetically determined low lifetime vitamin D status to be studied in large human populations. Human mendelian randomisation approaches have been highly informative in strengthening the link between low vitamin D status and development of diseases such as multiple sclerosis (Mokry et al., 2015; Rhead et al., 2016). The complementary approach of investigating the non-skeletal health benefits of vitamin D through supplementation trials, despite their complexities and caveats, are also being highly informative in the human healthcare setting. For example, recent large trials have not shown any clear benefit in reduction of incidence of cardiovascular or cancer (Manson, Cook, et al., 2019; Scragg et al., 2018; Scragg et al., 2017). However, trials are revealing that vitamin D supplementation can be helpful in respiratory tract disorders, notably in patients with low 25(OH)D concentrations (Jolliffe et al., 2019; A. R. Martineau et al., 2017), and in improving survival times in patients diagnosed with cancer (Keum, Lee, Greenwood, Manson, & Giovannucci, 2019; Manson, Bassuk, Buring, & Group, 2019). The global COVID-19 pandemic caused by SARS-CoV-2 has stimulated a flurry of interest into the role of vitamin D in both the treatment and prevention of this infection, with some studies postulating that a depleted vitamin D status could be associated with a worse outcome (Bilezikian et al., 2020). Whilst these approaches may be challenging to undertake in the veterinary sector, long term studies of healthy animals of known vitamin D status are necessary to further define the relationship between 25(OH)D concentrations and non-skeletal health outcomes in dogs and cats.

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