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Serum 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D are decreased in dogs with sinonasal aspergillosis

Arnaud Snoeck [a,*], Jared A. Jaffey [b,**], Frédéric Billen [a], Nina F. Rodrigues [a], Stéphanie Peeters [c], Caroline Le Goff [c], Etienne Cavalier [c], Dominique Peeters [a], Cécile Clercx [a]

[a] Department of Companion Animal Clinical Sciences, Faculty of Veterinary Medicine, University of Liège, Belgium
[b] Department of Specialty Medicine, College of Veterinary Medicine, Midwestern University, United States
[c] Department of Clinical Chemistry, CHU de Liège, University of Liège, Center for Interdisciplinary Research on Medicines, Belgium

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ABSTRACT

Canine sinonasal aspergillosis (SNA) is a poorly understood disease and remains a challenge to treat. Hypovitaminosis D is associated with many infectious diseases in humans and Vitamin D (VitD) deficiency in experimental mice decreases resistance to *Aspergillus fumigatus*. The objective of this study was to determine whether dogs with SNA have different VitD metabolite concentrations compared to healthy dogs (HD) and dogs with other nasal conditions and if those concentrations change after cure for SNA dogs. Twenty-two dogs with SNA, 12 HD, 9 dogs with lymphoplasmacytic rhinitis (LPR) and 10 dogs with nasal neoplasia (NN) were included. Serum 25-hydroxyvitamin D_2 ($25(OH)D_2$), 25-hydroxyvitamin D_3 ($25(OH)D_3$), 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$), 3-epimer-25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$) concentrations were measured by a certified liquid chromatography tandem mass spectrometry method at time of diagnosis or revisit. Twelve SNA dogs were available for serial blood collection until cure. Serum 25(OH)D and $24,25(OH)_2D_3$ were lower in dogs with SNA (mean ± standard deviation; 23 ng/ml ± 7.3 and 10.2 ng/ml ± 4.2, respectively) than in HD (34.1 ng/ml ± 7.5; $P = 0.007$ and 18.2 ng/ml ± 5.4; $P = 0.002$) while there was no difference among the other groups. Cured SNA dogs had higher serum 25(OH)D concentrations (27.7 ng/ml ± 9.4) compared to before treatment (23.1 ng/ml ± 7.7; $P = 0.0002$). These results further support the rationale that VitD may play a role in the complex SNA pathophysiology. Whether lower VitD status contributes to the development of the disease or is a consequence of it is unknown.

Introduction

Sinonasal aspergillosis (SNA) is a common cause of chronic nasal disease in dogs, most often related to the opportunistic fungus *Aspergillus fumigatus* (Talbot et al., 2014). Most affected dogs are young adult to middle-aged animals from mesaticephalic and dolichocephalic breeds, for reasons that remain unknown (Peeters and Clercx, 2007). In humans, most *Aspergillus* spp. related diseases appear in immunocompromised hosts (Latgé and Chamilos, 2019). In contrast, SNA classically develops in immunocompetent dogs (Peeters and Clercx, 2007). Results from transcriptomic studies on nasal mucosae of SNA dogs suggest that dysregulation of the local immune system may explain the failure to clear the fungus (Mercier et al., 2012; Peeters et al., 2007, 2006; Valdes et al., 2020; Vanherberghen et al., 2012).

There is increasing evidence in the last decade showing that Vitamin D (VitD) plays a role in both innate and adaptive immunity (Charoenngam and Holick, 2020). Many observational studies in both humans and dogs found a link between hypovitaminosis D and the

Abbreviations: CDC, Centers for Disease Control and Prevention; CRP, C-reactive protein; DEQAS, Vitamin D external quality control assessment scheme; HD, Healthy dogs; HRCT, high-resolution computed tomography; LC-MS/MS, Liquid chromatography tandem mass spectrometry; LPR, Lymphoplasmacytic rhinitis; NN, nasal neoplasia; SNA, Sinonasal aspergillosis; VDSCP, Vitamin D standardization certification program; VitD, Vitamin D; VMR, Vitamin D metabolite ratio; $1,25OH_2D$, 1,25-dihydroxyvitamin D; $24,25OH_2D_3$, 24,25-dihydroxyvitamin D_3; $25OHD_2$, 25-hydroxyvitamin D_2; $25OHD_3$, 25-hydroxyvitamin D_3; 3-epi-$25OHD_3$, 3-epimer-25-hydroxyvitamin D_3.

* Correspondence to: Avenue de Cureghem 1, Liège 4000, Belgium.
** Corresponding author.
E-mail addresses: arnaud.snoeck@uliege.be (A. Snoeck), jjaffe@midwestern.edu (J.A. Jaffey).

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higher prevalence or worse outcome of various diseases (Charoenngam and Holick, 2020; Clarke et al., 2021; Liu et al., 2022). Such association has been shown in human chronic rhinosinusitis (Mostafa et al., 2016) and aspergillosis (Kreindler et al., 2010). However, the causality of hypovitaminosis D on the development of these disorders is debated. Interestingly, experimental studies highlight that VitD deficiency in mice increases pulmonary susceptibility to *A. fumigatus* infection (Li et al., 2014) and exacerbate sinonasal inflammation induced by *Aspergillus fumigatus* sensitization (Mulligan et al., 2017). Moreover, in chronic rhinosinusitis in humans, alterations in the sinonasal local metabolism of VitD have been reported with suppressed ability of epithelial cells to convert 25-hydroxyvitamin D (25(OH)D) to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (most active form), which is associated with more severe disease (Schlosser et al., 2016). The clinical implications of these findings in dogs with naturally occurring SNA are unknown.

Many therapeutic options for SNA have been proposed in the literature (Sharman et al., 2010). Noninvasive techniques using endoscopically placed catheters to infuse enilconazole or clotrimazole are currently most commonly used showing high overall success rate ranging from 89.5 % to 100 % (Billen et al., 2010; Saunders et al., 2003; Vangrinsven et al., 2018; Zonderland et al., 2002). However, approximately 40 % of dogs require more than one treatment to achieve a cure (Sharman et al., 2010; Vangrinsven et al., 2018). Since the treatment of SNA in dogs remains a challenge and the pathophysiology is poorly understood, novel insights that could lead to development of novel therapeutic targets are needed.

Systemic inflammation decreases 25(OH)D concentrations (Clements et al., 2020; Reid et al., 2011) and is therefore an important factor to consider when investigating VitD in diseases. C-reactive protein (CRP) is a commonly used biomarker to indicate the magnitude of inflammatory activity in dogs (Kjelgaard-Hansen et al., 2013). The measurement of serum CRP concentrations may provide insight into whether alterations in VitD status may be related to a target disorder or a consequence of systemic inflammation.

Given that some evidence suggests a potential role of VitD in the immune response to *Aspergillus* spp. The aim of this observational study was to explore serum VitD metabolite concentrations in dogs with SNA, other nasal diseases including lymphoplasmacytic rhinitis (LPR) and nasal neoplasia (NN), and healthy dogs (HD). Objectives of this study were 1) to compare serum concentrations of various VitD metabolites in dogs with SNA to HD and dogs with LPR or NN; and 2) to determine whether serum concentrations of VitD metabolites in SNA dogs change from the time of diagnosis to when cure is achieved. We hypothesized that dogs with SNA would have lower serum 25(OH)D concentrations than HD. Additionally, we hypothesized that there would be no change in VitD metabolites in SNA dogs from time of diagnosis to when cures were achieved. This hypothesis was based on the premise that lower VitD concentrations may predispose dogs to the SNA rather than being solely a consequence of the disease process itself.

Materials and methods

Criteria for selection of cases

A selection of client-owned dogs presented at the companion animal hospital of the University of Liège (Belgium) for blood donation or with a definitive diagnosis of SNA, LPR, or NN between September 2016 and October 2022 were included in this prospective cohort study with convenience sampling. Exclusion criteria included dogs that were pregnant or lactating, that had any other known concomitant disease or were administered with VitD or calcium supplements. In addition, dogs presented with SNA and a concomitant foreign body were excluded. This study was approved by the animal ethical committee of the University of Liège (file 1854). Informed owner consent was obtained for each dog.

Group definitions

Diagnosis of SNA was made based on the presence of compatible clinical signs, per-endoscopic identification of fungal plaques with turbinate destruction (Fig. 1) and either a positive fungal culture for *A. fumigatus* or a positive *A. fumigatus* polymerase chain reaction (PCR). Additional diagnostic procedure included high-resolution computed tomography (HRCT). Dogs were treated either by a minimally invasive endoscopic debridement followed by a 15 min enilconazole infusion as previously described (Vangrinsven et al., 2018) or by surgical debridement (Claeys et al., 2006) followed by 15 min enilconazole infusion in case of cribiform plate lysis. The procedure was performed by two board-certified small animal internists with extensive experience (C. Clercx and F. Billen). Completeness of debridement after the procedure was systematically documented in the report as complete (no visible fungal plaques) or partial. Serial examinations were repeated once every 3–6 weeks until cure. Dogs with SNA were determined to be cured at the scheduled visit in which no fungal plaques were identified on endoscopic examination in conjunction with being subclinical (i.e., absence of clinical signs related to SNA).

Dogs were considered healthy based on a collective interpretation by a board-certified small animal internist of medical history, physical examination, complete blood count and serum biochemical profile. Healthy dogs were matched with the canine SNA typical morphotype (mesocephalic/dolichocephalic) and age (2–6 years).

Diagnosis of LPR was made based on clinical signs, rhinoscopic (unilateral or bilateral mild to moderate turbinate lysis with congestion and oedema of the mucosa, mucous to mucopurulent secretions, and failure to demonstrate a foreign body, neoplasia, or fungal plaques in the nasal cavities) and histopathological findings analyzed by a board-certified anatomical pathologist.

Diagnosis of NN was made based on clinical signs, rhinoscopic visualization of a tumor in the nasal cavities and histopathological findings analyzed by a board-certified anatomical pathologist. HRCT was performed in some cases.

Sample and data collection

Medical records of all dogs were encoded on a database (SAP®) and included age, sex, neutering status, breed, bodyweight, history, clinical signs, duration of clinical signs, endoscopy report, histopathological and PCR results, follow-up information. We define decreased appetite as a reduction in expected total daily maintenance food intake.

Blood samples were collected from the jugular vein and immediately transferred into clot activating tubes and then centrifuged at room temperature. Serum was collected, aliquoted in cryotubes and stored at −80°C for batch analysis of 25-hydroxyvitamin D_2 ($25(OH)D_2$), 25-hydroxyvitamin D_3 ($25(OH)D_3$), 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$), 3-epimer-25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$), and CRP concentrations.

Vitamin D metabolite and C-reactive protein

Serum samples were evaluated at the department of clinical chemistry of the University of Liège for batched analysis of VitD metabolites and canine specific CRP concentrations. The analyzed VitD metabolites included $25(OH)D_2$, $25(OH)D_3$, $24,25(OH)_2D_3$ and 3-epi-$25(OH)D_3$. Quantification of VitD metabolites was achieved by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described (Fabregat-Cabello et al., 2017). Briefly, 100 µl of serum was processed for protein precipitation before being injected in the LC-MS/MS system. Our analysis was validated by using reference material 972a from the National Institute of Standard and Technology (Gaithersburg, USA). The suitability of the methodology has been verified extensively by comparison with both a reference method and certified reference materials (Fabregat-Cabello et al., 2017). The lower limit of quantification (LOQ)

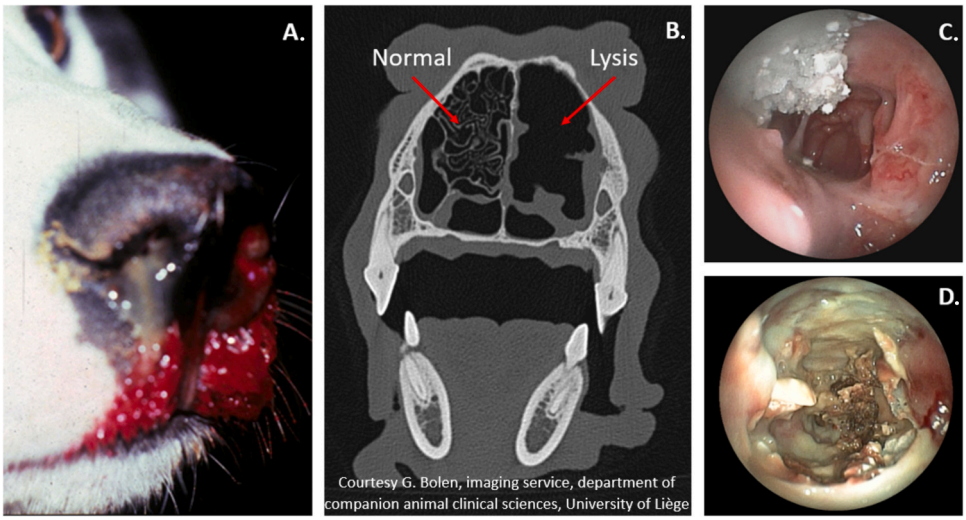

Fig. 1. Representative images of (A) clinical, (B) high-resolution computed tomography (HRCT), and (C, D) rhinoscopic abnormalities from dogs with sinonasal aspergillosis at baseline. (A) Left unilateral epistaxis and a right unilateral mucopurulent nasal discharge; (B) Transverse image (bone window) illustrating unilateral sinonasal aspergillosis; (C) Rhinoscopic visualization of a fungal plaque (white material) and severe nasal turbinates lysis (cavernous aspect); and (D) Rhinoscopic visualization of necrotic material and severe nasal turbinates lysis.

for metabolites measurement were < 0.98 ng/ml, < 1.8 ng/ml, < 0.26 ng/ml, < 1.3 ng/ml for $25(OH)D_2$, $25(OH)D_3$, $24,25(OH)_2D_3$, 3-epi-$25(OH)D_3$, respectively. The laboratory is certified by the Centers for Disease Control and Prevention (CDC) according to the Vitamin D Standardization Certification Program (VDSCP) for the 25(OH)D analysis and participates to the Vitamin D External Quality Control Assessment Scheme (DEQAS) program for the $24,25(OH)_2D_3$ and 3-epi-25(OH)D. 25(OH)D corresponds to the sum of $25(OH)D_2$ and $25(OH)D_3$. The VitD Metabolite Ratio (VMR), a parameter considered as a good indicator of the catabolic clearance of VitD (Makris et al., 2021), was calculated with the following formula: ($24,25(OH)_2D/25(OH)D_3$) x 100. This value is expressed as a percentage.

Serum CRP was analyzed on serum using a commercial canine-specific CRP sandwich ELISA kit (Cusabio, Houston, Texas). The lower limit of quantification was 0.3 µg/ml. Standard curves was generated using kit-provided canine standards. Mean absorbance was used to calculate concentration. The optical density of the samples was determined with the ETI-Max 3000 (DiaSorin, Saluggia, Italy) set to a wavelength of 550 nm. Background absorbance was subtracted from sample absorbance.

Statistical analysis

Statistical analyses were completed using R Statistical Software (v4.2.2; R Core Team 2022). The normality of the data was assessed through comparison of means and medians, QQ-plots and the Shapiro-Wilk test. The homogeneity of the variance was verified with the Levene and Fisher test for the ANOVA and non-paired *t*-test respectively. CRP and VMR did not follow a normal distribution in at least one group.

Sample size calculation was performed based on evaluation of data presented at the 29th European Congress of Veterinary Internal Medicine – Companion Animals (Research Communications of the 29th ECVIM-CA Congress, 2020). This retrospective analysis showed a significant decrease in serum 25(OH)D concentrations in dogs with SNA compared to HD. Post hoc analysis revealed that 10 individuals in each group (SNA and HD) were required to detect the identified effect size (effect size d=1.12) with a power of 80 %.

Serum concentrations of VitD metabolites were compared among the four groups using a one-way ANOVA followed by the Tukey test for pairs comparisons. Comparisons of the VMR and CRP between groups were made using the Kruskall-Wallis test. Differences in serum VitD metabolite concentrations from SNA diagnosis to cure were assessed with a paired *t*-test. Differences in serum 25(OH)D concentrations between dogs with or without a decreased appetite were assessed with a non-paired *t*-test. A Spearman test was used to investigate a correlation between serum $25(OH)D_3$ or CRP and either the number of treatments until cure or the duration of clinical signs before presentation. Likewise, Spearman rank test was used to determined associations between serum $25(OH)D_3$ and CRP. A *P*-value ≤ 0.05 was considered significant for all analyses.

Results

Animals

Details about age, sex, neutering status, body weight and breed of

Table 1
Signalment details in the different groups.

Variables	SNA (n = 22)	LPR (n = 9)	NN (n = 10)	HD (n = 12)
Age (years)	6.5 ± 2.8	6.7 ± 3.5	9.3 ± 3.1	5.8 ± 3.2
Sex	13 males (8 neutered) 9 females (1 neutered)	3 males (1 neutered) 6 females (6 neutered)	3 males (2 neutered) 7 females (7 neutered)	10 males (6 neutered) 2 females (1 neutered)
Body weight (kg)	33.1 ± 12.7	15.7 ± 8.6	30.3 ± 13.8	32.7 ± 5.8
Breeds	Golden Retriever (n = 8) Crossed breed (n = 2) Husky (n = 2) Border Collie (n = 2) American Staffordshire Terrier (n = 2) Other breeds (n = 6)	Husky (n = 2) Other breeds (n = 7)	Labrador Retriever (n = 3) Golden Retriever (n = 2) Other breeds (n = 5)	Crossed breed (n = 3) Golden Retriever (n = 2) Border Collie (n = 2) Other breeds (n = 5)

SNA, Sinonasal aspergillosis; LPR, Lymphoplasmacytic rhinitis; NN, Nasal neoplasia; HD, Healthy dogs
Note: Mean ± standard deviation.

dogs in the different groups are presented in Table 1. Twenty-four dogs with SNA were initially enrolled. Subsequently, 2 dogs were excluded because of concomitant nasal foreign body, leaving 22 dogs with a final diagnosis of SNA that were included. Golden Retriever was the main breed (8/22) represented in this group. The most common clinical signs included mucopurulent nasal discharge (17/22), epistaxis (16/22), sneezing (13/22), nasal hyperkeratosis (7/22), nasal planum depigmentation (9/22), facial discomfort (4/22). At the time of diagnosis, 18 % of dogs presented with systemic clinical signs including lethargy (4/22) and decreased appetite (3/22). Duration of clinical signs before presentation ranged from 3 to 72 weeks (median of 12 weeks). Rhinoscopy was performed in all dogs. Nasal turbinate destruction and fungal plaques were endoscopically observed in all dogs. Unilateral SNA was diagnosed in 77 % of cases (17/22), bilateral SNA in 23 % (5/22). Additionally, *A. fumigatus* was detected either by a fungal culture (11/22) or an A. *fumigatus* PCR (11/22). HRCT was performed in 45 % (10/22) of cases, with evidence of cribriform plate lysis in two cases. Two dogs with severe burden of disease and cribiform lysis were treated initially by surgical debridement rather than endoscopy to avoid prolonged anesthesia time and potential risk related to intense nasal flushing. Among these two dogs, one was deemed cured at the next scheduled visit, while the other was lost for follow-up. Endoscopic treatment was performed in the remaining SNA dogs (20/22). Complete perendoscopic debridement was achieved in 60 % of the cases (12/20). Four dogs were lost to follow-up before confirmation of cure. First endoscopic treatment success rate was 40 % (8/20) and overall treatment success rate was 100 % (18/18). Out of the 9 dogs that needed a second therapeutic procedure, 7 were cured after the second endoscopy while 2 required a third balneation before cure could be confirmed. Twelve SNA dogs were sampled at both time of diagnosis and cure. The median number of days from diagnosis (i.e. baseline before treatment) to cure was 33 days (range, 22 – 196 days).

Nine, ten and twelve dogs with LPR, NN, and HD, respectively were included. Nasal neoplasms included nasal carcinoma (6/10), melanoma (1/10) and chondrosarcoma (3/10). Clinical signs of dogs with NN included epistaxis (10/10), sneezing (9/10), stertor (4/10), mucopurulent nasal discharge (3/10), facial deformity (2/10), epiphora (1/10) and decreased appetite (1/10). Duration of clinical signs before diagnosis ranged from 1 to 28 weeks (median, 12 weeks). Dogs diagnosed with LPR were presented with serous to mucopurulent nasal discharge (9/9), sneezing (8/9) and epistaxis (1/9). Duration of clinical signs before presentation ranged from 6 to 146 weeks (median, 22 weeks).

All dogs were fed with a commercially available diet.

Vitamin D metabolites and C-reactive protein

Serum 25(OH)D_2 was below the LOQ (<1.3 ng/ml) for all dogs. 3-epimer-25(OH)D_3 was detected in only one dog from the HD group (1.52 ng/ml). Results for 25(OH)D_3, 24,25$(OH)_2D_3$ analyses are summarized in Table 2. Mean serum 25(OH)D concentration (i.e., combination of 25(OH)D_2 and 25(OH)D_3) was significantly lower in dogs with SNA compared to the HD ($P = 0.007$; effect size d= 1.5). Similar results were obtained for 24,25$(OH)_2D_3$ between SNA and HD ($P = 0.002$; effect size d= 1.7). There were no differences in 25(OH)D or 24,25$(OH)_2D_3$ between SNA dogs and any other nasal group. Similarly, there were differences in VitD metabolites between HD and dogs with other nasal diseases outside of SNA. No differences in VMR were identified between any groups.

Serum 25(OH)D concentrations in SNA dogs significantly increased from the time of diagnosis to when cures were achieved ($P = 0.0002$). In contrast, this effect was not observed for 24,25$(OH)_2D_3$ concentrations and VMR before treatment and after cure ($P = 0.16$ and $P = 0.45$, respectively) (Fig. 2). Serum 25(OH)D_3 concentrations at time of SNA diagnosis did not correlate with the number of treatments needed to achieve a cure ($P = 0.32$, rho=0.19) (Fig S1). Serum 25(OH)D_3 concentrations at diagnosis did not correlate with the duration of symptoms in both SNA ($P = 0.83$, rho=0.04), RLP ($P = 0.58$, rho=0.21) and NN ($P = 0.75$, rho=-0.12) groups (FigS2a). Similarly, no correlation was found between CRP concentrations and the duration of symptoms in both SNA ($P = 0.26$, rho=-0.25), RLP ($P = 0.85$, rho=0.07) and NN ($P = 0.46$, rho=-0.29) groups (Fig S2b). No difference in 25(OH)D concentrations was observed between SNA dogs with or without a decreased appetite ($P = 0.44$).

Results of CRP in the four groups are summarized in Table 2. Serum CRP concentrations did not differ between groups ($P = 0.08$). There was a weak and clinically unsignificant inverse association between serum concentrations of CRP and 25(OH)D ($P = 0.05$; rho= −0.28) (Fig S3) (Cohen, 1992).

Discussion

In this observational study, using a certified LC-MS/MS method for VitD metabolites, we showed that SNA dogs have lower 25(OH)D and 24,25$(OH)_2D_3$ compared to HD while this effect was not observed for other nasal conditions (LPR and NN). Serum 25(OH)D concentration increased after SNA cure. The causality of these observations is unknown.

In dogs, many recent investigations have linked low serum 25(OH)D_3 concentrations to gastrointestinal (Titmarsh et al., 2015), cholangiohepatic (Ambrosini et al., 2022; Jaffey et al., 2020b), infectious (Jacobs et al., 2021; Jaffey et al., 2021; Martori et al., 2021), urinary (Miller et al., 2020), tumoral (Wakshlag et al., 2011; Weidner et al., 2021) and immune-mediated (Mick et al., 2019) diseases. However, most studies have used an immunoassay-based technique for 25(OH)D quantification. Unfortunately, such techniques present many issues including different affinities for 25(OH)D_2, 25(OH)D_3, important and variable cross reactivity with 24,25$(OH)_2D_3$ and matrix/patient variation (Lee et al., 2015; Makris et al., 2021). The LC-MS/MS technique does not suffer from these limitations and also allows for accurate measurement of other metabolites for a better understanding of some metabolic variation (Fritz et al., 2017; Makris et al., 2021). In this study, we used a LC-MS/MS technique (Fabregat-Cabello et al., 2017) with a simultaneous separation and quantification of four VitD metabolites: 25(OH)D_2, 25(OH)D_3, 24,25$(OH)_2D_3$ and 3-epi-25(OH)D_3.

Table 2
Vitamin D metabolites, VMR and CRP concentrations in the different groups.

Variables	All dogs	SNA (n = 22)	LPR (n = 9)	NN (n = 10)	HD (n = 12)	*P*-value
25(OH)D_3 (ng/ml)	27 ± 10	23 ± 7.3	27.4 ± 13.7	26.5 ± 10	34.1 ± 7.5	**0.01**[a]
24,25$(OH)_2D_3$ (ng/ml)	13.6 ± 6.4	10.2 ± 4.2	14.3 ± 8.7	14.8 ± 6.1	18.2 ± 5.4	**0.003**[b]
VMR (%)	50 [41.7–58.8]	41.7 [35.7–52.6]	52.6 [47.6–62.5]	55.6 [47.6–58.9]	55.6 [45.5–55.6]	0.06
CRP (µg/ml)	2 [1.1–3.1]	2.5 [1.2–3.7]	2.7 [2.4–3.7]	1.4 [0.4–2.5]	1.4 [0.7–2.6]	0.08

SNA, sinonasal aspergillosis; LPR, lymphoplasmacytic rhinitis; NN, nasal neoplasia; HD, healthy dogs; VMR, vitamin D metabolite ratio; CRP, C-reactive protein.
Note: Mean ± standard deviation, Median [interquartile range].
[a]Pairwise comparison: Lower serum 25(OH)D concentrations in SNA dogs than in HD ($P = 0.007$).
[b]Pairwise comparison: Lower serum 24,25$(OH)_2D_3$ concentrations in SNA dogs than in HD ($P = 0.002$).

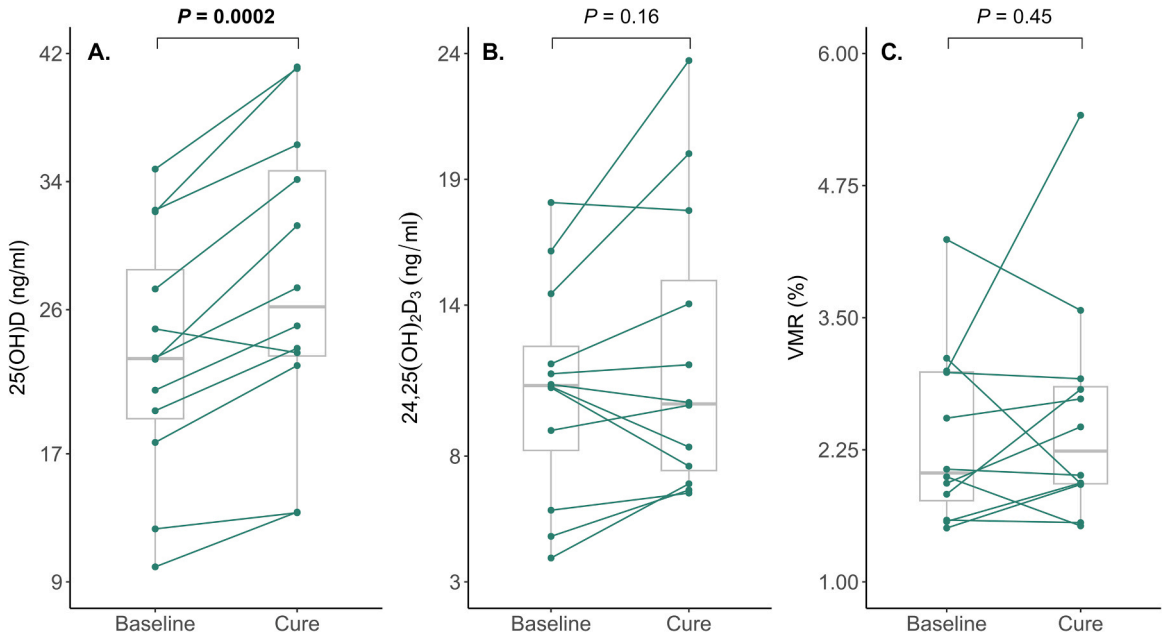

Fig. 2. Box and whisker plot comparing serum (A) 25-hydroxyvitamin (OH)D, (B) 24,25-dihydroxyvitamin $(OH)_2D_3$, and (C) vitamin D metabolite ratio (VMR) in dogs with sinonasal aspergillosis at baseline (i.e., at diagnosis before treatment) and after treatment at the time of cure. Enclosed circles represent individual dog data. The top and bottom of the boxes represent the 25th and 75th quartiles, respectively with the horizontal line representing the median.

In humans, 25(OH)D is considered as the metabolite of choice to determine VitD status (Makris et al., 2021). We found that dogs with SNA have lower 25(OH)D concentration than HD suggesting that SNA dogs had a lower VitD status. Similar associations have been reported in dogs with other fungal diseases (Hernandez et al., 2023; Jacobs et al., 2021). One possible explanation is that VitD serum 25(OH)D concentration decreases secondary to an inflammatory insult (Clements et al., 2020; Reid et al., 2011; Selting et al., 2016; Weidner et al., 2021). Our results suggest that systemic inflammation may not be a strong contributing factor in dogs with SNA because CRP concentrations did not differ between groups and there was just a weak and clinically irrelevant association between CRP and 25(OH)D concentrations. This presumption is tempered by the fact that our sample size was small, which may have limited detection of significant results. Additionally, we cannot definitively rule out that localized inflammation may still contribute to a decrease in VitD metabolites in dogs with SNA, without a CRP increase. A second possible mechanism for lower 25(OH)D concentrations is that dogs, unlike humans and other mammals, cannot produce endogenous VitD cutaneously so they rely solely on dietary intake (Clarke et al., 2021). As a result, decreased food intake in ill dogs may contribute to lower serum 25(OH)D concentrations compared to HD. This hypothesis is unlikely in dogs with SNA as only 14 % of them in our study had a decreased appetite at time of diagnosis and there was no difference in 25(OH)D concentrations between dogs with and without a decreased appetite among this group. Despite the decreased appetite recognition by the owner being straightforward, it remains subjective. A more complete nutrient profile assessment with intake quantification may bring additional information. Concentrations of 25(OH)D in SNA dogs increased after cure, which may suggest that another factor linked to the disease is responsible for this lower VitD status. However, despite being statistically significant, there was a mean change of only 4.6 ng/ml. The clinical relevance of this change is unknown and despite the observed post-cure increase in 25(OH)D suggests that low VitD metabolites in dogs with SNA are, at least in part, a consequence of the disease, we should not exclude that VitD status may play a role in disease susceptibility. Overall, itis challenging to conclusively determine whether low VitD metabolites in dogs with SNA are a cause of the disease, a consequence of the disease or a combination of both. A long-term follow-up after cure may help determine whether a more substantial difference in 25(OH)D levels emerges over time.

Since dolichocephalic and mesocephalic median to large breeds of dogs are predisposed to SNA, one could speculate that VitD levels could be genetically different according to the breed. In humans, it has been shown that polymorphism in genes involved in the metabolism (enzymes), catabolism (enzymes), transport (Vitamin D Binding Protein (VDBP)) and binding of VitD to its receptor (Vitamin D Binding Receptor (VDR)) may partially explain the variability of some disease susceptibility from one person to another (Liu et al., 2022; Rhead et al., 2016). While the genetic effect explaining the variability in 25(OH)D concentration in humans is modest (10.5 %) (Jiang et al., 2018; Revez et al., 2020), it might be of bigger importance in dogs due to the inbreeding and artificial selection (Marsden et al., 2016). Interestingly, one study (Sharp et al., 2015) reported a 26 % lower median 25(OH)D concentration in Golden Retrievers compared to German Shepherd and since Golden Retrievers are more prevalent in the population of dogs diagnosed with SNA (Schuller and Clercx, 2007), it is tempting to speculate that there might be a link. Indeed, an immune dysregulation has been suggested to be involved in the canine SNA pathophysiology (Peeters et al., 2007, 2006; Valdes et al., 2020; Vanherberghen et al., 2012). VitD may be one possible explanation as it has been demonstrated to have immunomodulatory effects in both humans and dogs (Charoenngam and Holick, 2020; Jaffey et al., 2020a, 2018a, 2018b). In mice model of Aspergillosis, VitD deficiency alone results in sinonasal changes in local immunity (Mulligan et al., 2017) with exacerbated inflammation and defective resistance to *A. fumigatus* infection (Li et al., 2014). We could not evaluate the breed effect on VitD metabolites concentrations because of the low number of included HD. Future studies on big cohorts of HD are needed to evaluate a genetic effect on VitD status of various breeds.

Despite the 25(OH)D concentrations being lower in SNA dogs compared to HD, it is not possible to conclude whether these dogs are in VitD insufficiency since no universally accepted cut-off points have been established in dogs. Accordingly, we cannot exclude that the level of 25 (OH)D in dogs with SNA is still in an optimal range. With the LC-MS/MS technique, we were able to separate and quantify other VitD metabolites to better understand metabolic variations. The $24,25(OH)_2D_3$

metabolite in humans is a biomarker of VitD catabolism (Makris et al., 2021). Measuring this metabolite allows for the calculation of the VMR. In humans, this value has been suggested as a more reliable marker for VitD status than 25(OH)D alone (Castillo-Peinado et al., 2022; Fabregat-Cabello et al., 2017; Ginsberg et al., 2021; Tang et al., 2017) as it overcomes the differences induced by factors influencing 25(OH)D such as ethnicity and changes in VDBP (Berg et al., 2015; Ginsberg et al., 2021). In our study, the 25(OH)D results show a lower VitD status in SNA dogs while the VMR did not differ between groups, failing to support this lower VitD status. However, the VMR, adapted to assess the VitD status in humans might not be a good indicator in dogs. Indeed, as previously reported (Spoo et al., 2015; Weidner et al., 2017), we identified that $24,25(OH)_2D_3$ concentrations in dogs seem to be much higher than in humans (Makris et al., 2021) for still unknown reasons. In humans, the normal reference interval for VMR is between 4.3 % and 14.3 % (Tang et al., 2017), while in our study, the mean VMR in the HD was 52.6 % indicating that this pathway is 4–12 times more active in dogs than in humans. This could suggest a potential alternative role of this metabolic pathway in dogs and warrants further investigations.

VitD exists in two forms, VitD2 and VitD3, which exhibit only minor differences in their structure (Makris et al., 2021). In line with others (Hurst et al., 2020a, 2020b) we did not detect in any dog the presence of $25(OH)D_2$, which is unsurprising since commercial dog food is supplemented with VitD3. The 3-epi-$25(OH)D_3$ was detected in only one dog while another study reported 52 % of 117 HD had quantifiable concentrations of 3-epi-$25(OH)D_3$ (>1.6 ng/ml) (Hurst et al., 2020a). Such discrepancy is also observed in human reports (Makris et al., 2021) and the physiological importance and role of the epimeric pathway is still unclear (Kubiak et al., 2018). Epimers of VitD are less biologically active and have been suggested as a protective mechanism against VitD toxicity (Kubiak et al., 2018). Therefore, it might be possible that we did not detect 3-epi-$25(OH)D_3$ in our study because the 25(OH)D levels of included dogs were not high enough to activate epimerization.

Our study had several limitations. We included relatively small populations of dogs in each group, which could have led to a type II error. Several factors that might influence VitD status have been described in dogs, such as age (da Fonseca et al., 2020; Wakshlag et al., 2011), sex, diet, neuter status, breed (Sharp et al., 2015), activity level (Spoo et al., 2015). However, their importance is not well established and controversial (Alizadeh et al., 2022). By matching our HD to the SNA population, we aimed to limit the effect of certain confounding factors that could have mediated associations between VitD concentration and the outcome. Future studies using certified LC-MS/MS should be conducted in the future to assess their effect on VitD status.

In this study, we investigated VitD status by measuring various circulating metabolites, which may not reflect local disturbances in VitD metabolism. Many cell types (including immune cells) express the VDR (Cartwright et al., 2018; Charoenngam and Holick, 2020) and are capable of producing 1-hydroxylase (Adams et al., 2014), an enzyme that converts the 25(OH)D into $1,25(OH)_2D$. In humans, the sinonasal inflammation, triggered by A. *fumigatus*, is capable of impairing local VitD metabolism causing a decrease in local levels of 1-hydroxylase and thus $1,25(OH)_2D$, without reducing the circulating $1,25(OH)_2D$ (Mulligan et al., 2022, 2017). More subtle dysregulations might also occur by impairing the cellular response to $1,25(OH)_2D$. As an example, an autocrine VitD signaling loop switches off pro-inflammatory programs of human lung helper 1 T-cells (Chauss et al., 2022). This program has been suggested as being abnormal in human patients with COVID-19, who subsequently show an exaggerated pro-inflammatory response with collateral tissue damages (Chauss et al., 2022). Such context-dependent investigations of VitD molecular mechanisms are needed in SNA patients to both explain the epidemiological association and determine if these mechanisms could be potentially exploited therapeutically by using VitD as an adjunct or prophylactic treatment. Lastly, the NN group included dogs with several types of neoplasm. This heterogeneity may have masked potential differences within specific types of NN and SNA.

Conclusion

This study showed that SNA dogs have lower serum concentrations of 25(OH)D and $24,25(OH)_2D_3$ compared to HD. No differences in VitD metabolites were observed between HD and those with other nasal conditions (i.e., LPR and NN) nor were differences found between SNA dogs and dogs with LPR or NN. While these findings suggest a potential association between VitD status and SNA, they do not establish a causal relationship. Further studies should be conducted to investigate the causality and the role of VitD in the sinonasal immunity against *A. fumigatus*.

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CRediT authorship contribution statement

Peeters Dominique: Conceptualization. **Cavalier Etienne:** Writing – review & editing, Validation, Resources. **Clercx Cécile:** Writing – review & editing, Supervision, Resources, Investigation, Conceptualization. **Snoeck Arnaud:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Billen Frédéric:** Resources, Investigation. **Jaffey Jared A.:** Writing – review & editing, Conceptualization. **Peeters Stéphanie:** Validation, Investigation. **Rodrigues Nina F.:** Resources, Investigation. **Le Goff Caroline:** Validation, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Regulatory body approval

File number 1854 accepted by the Animal Ethical Committee from the University of Liège.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2025.106318.

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