Canine Sinonasal Mycosis

A retrospective and prospective study of different treatment options, their successes and influence of different variables

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6 September 2013

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Abstract

This study reviews treatment outcome of commonly used treatment techniques of dogs with Sinonasal Mycosis (SNM). Treatment options considered are:

Treatment 1: Removal of local abnormalities (e.g. corpus alienum); removal mycotic plaques by rhinoscopy, followed by the local application of clotrimazole cream (1%).

Treatment 2: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation.

Treatment 3: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%).

Treatment 4: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus with placement of tubes to administer enilconazole (1%) twice daily for 10-14 days.

Treatment 5: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation, removal of mycotic plaques followed by local application of clotrimazole cream (1%).

Influence of different variables on treatment failure will be examined. Variables considered are: age, gender, ipsilateral versus bilateral affection, affection of the frontal sinus, severity of the infection and enlargement of the mandibular lymph node.

Furthermore, a prospective case control study was done on 4 new patients with SNM in order to determine specific changes in blood values, signs of underlying disease and specific properties of the fungus itself.

Study design:

Retrospective (n = 51) and prospective study (n=4).

Methods:

Retrospective study: Medical reports of 51 dogs treated for sinonasal mycosis were obtained via research of the clinical database of the Faculty of Veterinary Science, Medicine of Companion Animals at Utrecht University for the time period March 2005 to March 2013. First treatment outcome and influence of variables described above were evaluated.

Prospective case control study: Clinical examination, blood analyses, CT, rhinoscopy and biopsies were performed at 4 new patients with SNM at Department of Clinical Sience of Companion Animals, Faculty of Veterinary Medicine, Utrecht University. Mycotic plaques were collected, pH was measured, samples were cultured and DNA was isolated and typed. Determination of a resistance profile was started.

Results:

Retrospective study: When all topical treatment options were combined together, treatment success was 52.9% after first treatment and 78.4% after two treatments.

No statistical difference was found in treatment outcome (recurrence of infection) over the entire follow-up between the different treatment groups 1-5.

Treatment failure was not associated with age, gender, SNM in the frontal sinus, ipsilateral versus bilateral SNM infection, severity of the infection or enlargement of the mandibular lymph node.
Significant proof that recurrence of SNM will not occur within 6 weeks after being treated with treatment option 4 ($p= 0.049$) was found. Recurrence rates of a combined group consisting of treatment 2 and 5 (to enlarge the sample size) showed a significant ($p= 0.039$) reduction in recurrence rate within 6 weeks when compared with other treatments.
Relative risks and odds ratios calculated in this study to compare treatment outcomes and influence of different variables are not reliable because calculated results are not significant, 95% confidence intervals are wide and they cross the value 1. This indicates that the results are unreliable.
Prospective case control study: Mycotic plaques collected in 4 patients were all typed as *Aspergillus fumigatus*. The pH of fresh collected fungus was between 7.9 and 8.2. Histological evaluation of biopsies shows that the fungus grows only superficially on the mucosa. The lamina propria was not infiltrated. Determination of a resistance profile was not completed while writing this report.
Three patients were treated with treatment option 3 and one patient was treated with treatment option 5. One patient was euthanized because of clinical deterioration. Recurrence of clinical signs of SNM was seen in two dogs (patient 3 and 4, 50% of total). However, recurrence of SNM in patient 4 was not proven after he was euthanized. Slight hypo-hypoalbuminemia was seen in three out of four patients.

Contents

Introduction

Chronic nasal discharge is common in dogs. Chronic sneezing, depigmentation of the nasal planum and (unilateral) nasal discharge are symptoms that may indicate an abnormality in the nasal cavities, sinuses and/or nasopharynx. A fungus infection in the nasal cavity and frontal sinus may be one of the causes of these clinical signs. (Benitah, 2006; Peeters & Clercx, 2007; M. J. Sharman & Mansfield, 2012). Research has shown that 7 to 34% of dogs with chronic nasal discharge is caused by fungal rhinosinusitis (M. J. Sharman & Mansfield, 2012). Fungi that can cause such a clinical picture are *Aspergillus fumigatus*, *Aspergillus flavus, Aspergillus niger* or *Penicillium spp*. Of these fungi *A. fumigatus* is by far the most demonstrated in patients suspected of a sinonasal fungal infection (Benitah, 2006; Peeters & Clercx, 2007; Quinn, Markey, Carter, Donnelly, & Leonard, 2002; M. J. Sharman & Mansfield, 2012; N. Sharp, Sullivan, & Harvey, 1992). Sinonasal mycosis (SNM) is diagnosed by a thorough history, clinical signs, physical examination and diagnostic imaging, such as computed tomography (CT) and rhinoscopy. Sinonasal Aspergillosis (SNA) should not be confused with the rare systemic form of Aspergillosis. The latter disease is mainly seen in German Shepherds, the nasal region is usually not involved and it is caused by *A. terreurs* instead of *A. fumigatus* (M. J. Sharman & Mansfield, 2012). SNM is characterized by destruction of the nasal conchae (see figure 3 and 4). In an advanced stage there may also be damage to the frontal bone, the periorbital tissue and cribriforme plate (ethmoid). Bone destruction is caused by toxins produced by *A. fumigatus* and by the inflammatory process of the patient. (Peeters & Clercx, 2007). Clinical signs seen in dogs with SNM is: chronic nasal discharge (mostly mucopurulent). This usually starts unilaterally, but may eventually occur bilaterally. Moreover there are signs of pain at the nose and sinuses, depigmentation and ulceration of the nasal planum Changes at the nasal planum are most likely (caused by toxins of the fungus) (Nelson et al., 2009; Peeters & Clercx, 2007; M. J. Sharman & Mansfield, 2012; N. Sharp et al., 1992). Often there is increased air flow through the nasal sinus because of increased destruction of conchal structures. Furthermore epistaxis, sneezing, reversed sneezing, loss of appetite and depression is reported (Peeters & Clercx, 2007; M. J. Sharman & Mansfield, 2012; N. Sharp et al., 1992). In severe cases deformities of the skull can be seen caused by hyperostosis of the frontal bone. Sometimes epiphora secondary to SNM in the orbit and brain damage is seen if the ethmoid bone is no longer intact (Peeters & Clercx, 2007). SNM can occur secondary to an underlying primary cause. Primary causes are as a foreign body in the nasal sinus, previous trauma to the nose, neoplasia or a oronasal fistula. Removal of this primary cause will in most cases resolve the secondary fungal infection. Although, an opportunistic fungal infection can be the primary cause which is more challenging to treat. In this study, we focus on the latter group.

In order to determine the extent of the infection and subsequently to determine which treatment is most appropriate for the individual patient, diagnostic imaging such as computed tomography (CT) and rhinoscopy are necessary. CT evaluation of the skull is necessary to determine if the frontal sinus is affected and if the cribriform plate and the orbita is intact. These factors will influence the best treatment technique. For instance, one hour flushing with clotrimazole under pressure is not possible if the cribriform plate is not intact; this will lead to

severe complications or even death. Trepanation should be performed if the frontal sinus is affected (Benitah, 2006; Peeters & Clercx, 2007).

A. fumigatus is a ubiquitous prevalent fungus, an infection can be acquired by inhalation of spores (Quinn et al., 2002). *A. fumigatus* can be found in the nose, throat and pharynx of healthy animals and humans (Nelson et al., 2009; Peeters & Clercx, 2007; Quinn et al., 2002; M. J. Sharman & Mansfield, 2012; N. Sharp et al., 1992). It is still unclear why only a subset of the dogs exposed to *A. fumigatus* will develop clinically relevant disease (Peeters & Clercx, 2007). The fungus infection is relatively more prevalent among dogs of mesocephal and dolichocephal races and is seen more among young or middle aged dogs (Peeters & Clercx, 2007; Quinn et al., 2002; M. J. Sharman & Mansfield, 2012; N. Sharp et al., 1992). Rarely it is also seen in cats. It is thought that a compromised immune system plays a role in the development of the fungus infection, but this is not evidence-based.

Several treatment protocols are described with variable success rates (Burbidge et al., 1997; Claeys, Lefebvre, Schuller, Hamaide, & Clercx, 2006; Clercx, 2006; Mathews et al., 1998; Schuller & Clercx, 2007; M. Sharman et al., 2010; M. J. Sharman & Mansfield, 2012; N. Sharp et al., 1992; Sissener, Bacon, Friend, Anderson, & White, 2006; Zonderland et al., 2002). In many cases treatment should be repeated to achieve a higher success rate and recurrence is not uncommon. To treat SNM, topical antimycotics are preferred to systemic antifungals because of poor efficacy of the latter (Benitah, 2006; Nelson et al., 2009; Peeters & Clercx, 2007; Quinn et al., 2002; M. J. Sharman & Mansfield, 2012; N. Sharp et al., 1992).

This study investigates five different topical treatment options to cure SNM in dogs (see research goals). Three of them include one hour contact time with clotrimazole solution, one of them administered through surgically placed catheters in the frontal sinus. Two of the treatment options were followed by local application of clotrimazole cream. One treatment option consists only of removal of mycotic plaques followed by local application of clotrimazole cream (1%). Another treatment option is trepanation of the frontal sinus with placement of tubes to administer enilconazole twice daily for 10-14 days.

Literature results

Few studies described topical clotrimazole treatments in dogs with sinonasal Aspergillosis (Burbidge et al., 1997; Hayes & Demetriou, 2012; Mathews et al., 1998; M. Sharman et al., 2010; M. Sharman, Lenard, Hosgood, & Mansfield, 2012; Sissener et al., 2006). Burbridge et al. (1997) showed that noninvasive intranasal infusion with one hour contact time appears to be an effective treatment for sinonasal Aspergillosis in dogs, their sample size was small however (n=5) (Burbidge et al., 1997). Mathews et al. (1998) compared the effect on outcome of topical administration of clotrimazole (1%) of surgically placed versus non-surgically placed catheters in 60 dogs. In 65% of the cases there was resolution of clinical disease after one treatment with clotrimazole through non-surgical placement of catheters. A higher percentage of success (87%) was seen after multiple treatments (Mathews et al., 1998). In a

retrospective study of Sharman et al. (2010), first treatment success after commonly used treatments was examined. Thirty nine of the 85 dogs (45.8%) treated with a topical treatment had resolution of disease. Treatment success was associated with younger age. After multiple topical treatments 69.4% of patients were cured. When considering only non-invasive treatment of a one hour clotrimazole (1%) flush inserted via catheters placed in the nares, 40% (18 of 45) of the dogs were cured after first treatment. Seventy percent (7 of 10) of the dogs that were treated with a short 5-minute clotrimazole (1%) soak followed by administration of clotrimazole cream (1%) were cured after first treatment. Trepanation of the frontal sinus for temporary placement of the catheter and a one-hour flush with clotrimazole solution was performed in 24 dogs. Two dogs were soaked with enilconazole instead of clotrimazole. Fifty percent of these dogs were considered cured after first treatment. No statistical difference between different treatment groups was found in this study (M. Sharman et al., 2010).
Pomrantz et al. (2010) repeated rhinoscopic evaluation to assess the effectiveness of intranasally administered clotrimazole in 23 dogs. When the frontal sinus was affected, trepanation was performed. All dogs had rhinoscopic follow-up examination 1 to 4 months after treatment. In 48% (11 of 23) of the dogs no fungal plaques were seen during rhinoscopic follow-up evaluation and were classified as treatment success. Persistent fungal disease was found in 52% (12 of 23). Three of seven dogs (42.8%) were cured after second treatment and one of three dogs (33.3%) were free of disease after 3 treatments. Overall the efficiency of intranasal administration of clotrimazole solution could be confirmed in 15 of 17 dogs (1-3 treatments). Delayed recurrence was seen in three dogs, all of them had involvement of the frontal sinus. Treatment success was 67% (Pomrantz & Johnson, 2010).

Combined clotrimazole flush and depot therapy was examined by Sissener et al (2006). In this study fourteen dogs were treated by frontal sinus trepanation with a short 5-minute flushing of 1% clotrimazole solution followed by administration of 1% clotrimazole cream. Twelve of the fourteen dogs (85.7%) had no clinical signs after treatment or had only signs of mild rhinitis during follow-up period of six months (Sissener et al., 2006).

For several years the standard treatment for sinonasal aspergillosis was an enilconazole emulsion administered by surgically placed catheters into the frontal sinus and nasal chambers (Benitah, 2006; N. Sharp et al., 1992). Enilconazole is less toxic and irritating to mucous membranes than clotrimazole, especially in low concentrations (Peeters & Clercx, 2007). Enilconazole was administered twice daily for 7-14 days (N. Sharp et al., 1992; N. J. Sharp, Sullivan, Harvey, & Webb, 1993). Sharp et al. (1992, 1993) described that enilconazole cured 90% of the dogs with nasal aspergillosis (N. Sharp et al., 1992; N. J. Sharp et al., 1993). However in some dogs treatment was followed by a six week course of ketoconazole orally (N. J. Sharp et al., 1993).
Long term outcomes of dogs with sinonasal aspergillosis treated with non-invasive treatments of topical enilconazole were examined in a study of Shuller et al. (2007). Fifteen dogs were treated with an infusion of 1% enilconazole emulsion through a blindly placed catheter and twelve dogs were treated with a 2% emulsion of enilconazole infused through endoscopically

placed catheters in the frontal sinus. Fifty percent of the patients were asymptomatic throughout the follow-up period of 38±17 months. The other 50% had only mild clinical signs which were interpreted as chronic lymphoplasmacytic rhinitis/sinusitis and episodes of bacterial infection (Schuller & Clercx, 2007).

Distribution of clotrimazole and enilconazole has been examined in multiple studies (Burrow, Baker, White, & McConnell, 2013; Hayes & Demetriou, 2012; M. Sharman et al., 2012). Hayes et al. (2012) reported that clotrimazole cream persists in the frontal sinus and is distributed effectively in normal canine cadavers. Retention time is probably shorter in living dogs because of head movements and sneezing. Drainage of cream would probably be faster in animals with conchal atrophy (Hayes & Demetriou, 2012). Based on Hayes & Demetriou (2012), this study assumes that administration of a clotrimazole cream prolongs drug contact time in comparison with a clotrimazole solution. Because of this, treatment option 5 fundamentally differs from option 2 (both consist of a one hour flush with clotrimazol solution, however option 5 ends with administration of clotrimazole cream), which is why they are considered as separate treatments. However, because of the small sample size and the same treatment principle, their data will be pooled together as well.
A Computed Tomography (CT) study of the distribution of clotrimazole cream installed in the frontal sinus by trepanation in canine cadavers was performed by Burrow et al. (2011). Sinus filling was excellent in 10 of 12 dogs (83.3%) (22 sinuses) and filling of caudal nasal cavities was excellent in all dogs (Burrow et al., 2013).

Distribution of clotrimazole and enilconazole solutions were assessed with CT scan performed 5 minutes after treatment by Sharman et al. (2012). He reported that distribution of clotrimazole (1%) and enilconazole (10%) solutions in all regions of the nasal cavity and frontal sinuses was achievable using temporary trepanation of the frontal sinus. However distribution results varied considerably and retention was poor in 10 of the 18 regions assessed.

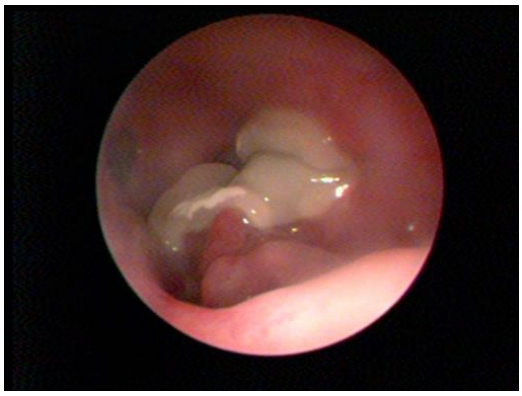

Fig. 1: Rhinoscopic image of fungus in the caudal sinus nasalis (patient 4).

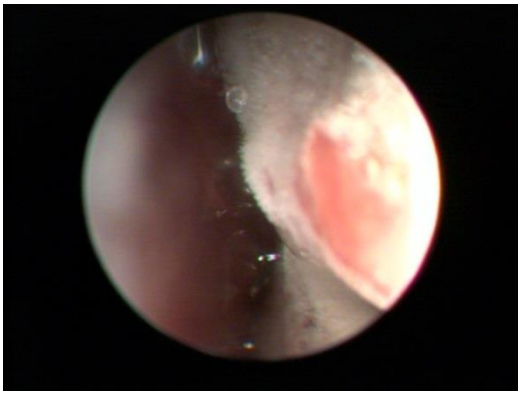

Fig. 2: Rhinoscopic image of fungus on the mucosa of the sinus nasalis (patient 2).

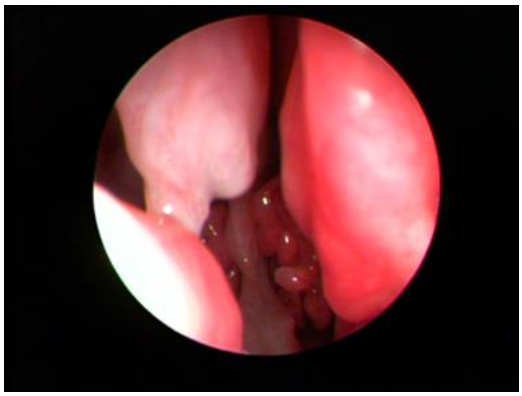

Fig. 3: Rhinoscopic image of destruction of the nasal conchae and septum defect on the left (patient 1).

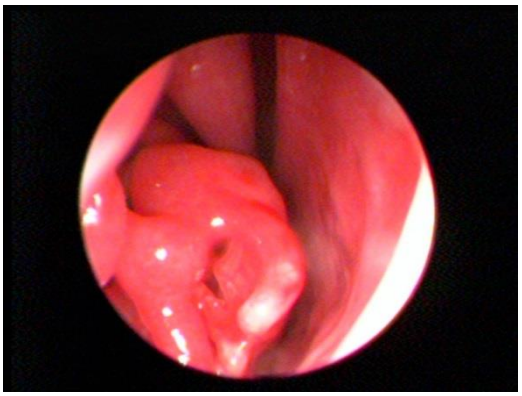

Fig. 4: Rhinoscopic image of destruction of the nasal conchae (patient 1).

Research goals: Retrospective study

Because treatment of SNM is still difficult and it is not clear which treatment technique is most effective, the goal of this research is to determine which treatment is most successful. Which treatment has the least chance of recurrence of SNM?
Treatment techniques investigated in this study are:

Treatment 1: Removal of local abnormalities (e.g. corpus alienum); removal mycotic plaques by rhinoscopy, followed by the local application of clotrimazole cream (1%).
Treatment 2: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation.
Treatment 3: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%).
Treatment 4: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus with placement of tubes to administer enilconazole (1%) twice daily for 10-14 days.
Treatment 5: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation, removal of mycotic plaques followed by local application of clotrimazole cream (1%).

The treatment techniques above were selected because they are the most common form of treatment for canine SNM in the Netherlands. All treatment techniques had been performed at the Department of Clinical Science of Companion Animals, Faculty of Veterinary Medicine Utrecht University and will be discussed in detail in material and methods.
Furthermore, we determined the influence of different variables on treatment failure or success. Variables that are examined are: age, gender, ipsilateral versus bilateral disease, disease of the frontal sinus, severity of the infection and enlargement of the mandibular lymph node.

Material and methods: Retrospective study

Medical reports of dogs treated for sinonasal mycosis were obtained via research of the clinical database of the Department of Clinical Science of Companion Animals, Faculty of Veterinary Medicine Utrecht University for the time period March 2005 through March 2013. Requirements that the patients had to meet in order to be included in the study are: confirmed diagnosis of sinonasal mycosis by either computed tomography and rhinoscopy, pathology or fungal culture. They have to have been treated with one of the treatment options described above and their medical records need to be complete. Patients treated with one of the treatment options described above in combination with systemic antifungals were excluded from this study.
Treatment success was defined as resolution of nasal mucopurulent/purulent discharge, epistaxis, sneezing and other clinical signs such as pain of the head or nose and lethargy. Treatment failure was defined as recurrence of clinical symptoms described above and confirmed by rhinoscopic visualization of mycotic plaques or fungal culture.
Patients with only mild serous nasal discharge without evidence of fungal recurrence were classified as treatment success.
A subdivision was made for recurrence within 6 weeks, within 12 weeks, after 12 weeks and over the entire follow-up. With the entire follow-up is meant the whole period between first visit to the Departement of Clinical Sciences of Companion Animals with symptoms of SNM until April 2013. This subdivision was made because recurrence within 6 or 12 weeks is likely due to unsuccessful treatment whereas recurrence after e.g. one year is more likely due to reinfection.

Statistics

Data was calculated by hand and by using the Statistical Package for Social Siences (IBM SPSS version 21.0.0.0). To calculate the significance of first treatment failure and determine if there is an association (relationship) between treatment option and recurrence of SNM, Chi-squared tests or Fishers exact tests were used. The same tests were used to determine if there is an association between different variables and recurrence over the entire follow-up (Fishers exact test was used when expected frequencies were less than 5 (Field, 2009; Petrie & Watson, 2006)).

Relative risks and odds ratios with their 95% confidence intervals were calculated for each treatment option to determine which treatment option is best by determining treatment failure (i.e. recurrence during follow-up) after first treatment. (Table 4).
Because lack of a control group (i.e. patients that did not receive treatment after diagnosis of SNM), each treatment option was compared with the total of all other treatment options (e.g. option 1 is compared with option 2+3+4+5+(2+5)). Each treatment option was also compared to each other treatment option individually (e.g. the relative risk of option 1 to option 2 equals option 1 divided by option 2: 20.00/53.125 = 0.376 which means that option 1 has a 0.376 greater chance of recurrence than option 2). (Table 5).

Influence of different variables on recurrence of SNM after treatment was determined by calculating odds ratios with their 95% confidence intervals using logistic regression tests. Although the odds ratio is a reasonable estimate of the relative risk if prevalence of disease is small, they were both calculated.

Significance was defined as $p < 0.05$ (Field, 2009; Petrie & Watson, 2006).
Variables investigated were: age, gender, ipsilateral versus bilateral disease, disease of the frontal sinus, severity of the infection and enlargement of the ipsilateral mandibular lymph node.

Note that all calculations are about the risk of recurrence, not about the success rate of the therapy. Odds ratios and relative risks indicate how much greater the risk of recurrence after treatment with a particular therapy or influence of another variable is.

Explanation of association, odds ratio, relative risk and confidence interval
With association is meant that there is a significant relationship between a treatment option and its outcome (i.e. a treatment option has consistently lower recurrence rates when compared with other treatment options) (Field, 2009).

An odds ratio is the ratio between the probability that an event occurs and the likelihood that it will not occur (Field, 2009; Petrie & Watson, 2006). In this study the odds ratio is defined as the probability that SNM will reoccur after a treatment (or because of other variables such as age, gender etc.), divided by the probability that it will not reoccur. The results of the odds ratio should be interpreted in terms of change in odds. If the value is greater than one, it indicates that the odds of the outcome occurring increase. If the value is less than one, it indicates that the odds of the outcome occurring decrease (Field, 2009).

Relative risk is a measure of the strength of association between disease and exposure to a factor (Petrie & Watson, 2006). This means that in this study, it indicates the strength of the association between recurrence of SNM after first treatment and the chosen treatment option. It is calculated by the risk in the exposed group divided by the risk of the unexposed group (Petrie & Watson, 2006). For example in this case, dogs with recurrence after treatment option 1 divided by dogs with recurrence after not being treated with treatment option 1 (all treatments except option 1, Table 4) or dogs with recurrence after being treated with treatment option 1 divided by dogs with recurrence after treatment option 2 etc. The relative risk value indicates how much greater the chance of recurrence with treatment X is compared to the chance of recurrence with treatment Y. A relative risk of 1 therefore means that the chance of recurrence is equal for both treatments.
(Table 5).

The best way to conclude something about a population is to investigate every individual of that population. This, however, is too impractical to do. Therefore a sample is taken from the

population, and any conclusion drawn from the sample is then generalized for the population. This means that the validity of the conclusion hinges on how well the sample represents the population. In order to calculate with the sample, the mean of the sample is calculated, and the conclusions drawn from it are then generalized for the population mean. The confidence interval is a range of values that represents the precision of the sample mean. The population mean should fall within this range with a degree of certainty. This study used 95% confidence intervals to investigate different values; it is 95% certain that the population mean lies within this interval. If the confidence interval is narrow this means that the mean represents the true mean of the population well. However calculated confidence intervals in this study are wide, the sample mean is a poor estimate of the population. The sample mean could be very different from the true mean (Field, 2009; Petrie & Watson, 2006).

Description of the implementation of the different treatment options

***Treatment 1**: Removal of local abnormalities (e.g. corpus alienum); removal of mycotic plaques by rhinoscopy, followed by local application of clotrimazole cream.*
Under general anesthesia rhinoscopy is performed. Local foreign bodies that might be present and mycotic plaques are removed by surgical hook and suction. Further local clotrimazole-cream (1%) will be applied at the place where the fungus was located using a urinecatheter attached to a syringe to do so.
This treatment technique is only used in mild cases of SNM, when there are only a few mycotic plaques, most of them primarily caused by a corpus alienum.
In this research 5 dogs were treated with this treatment technique.

***Treatment 2**: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation.*
Under general anesthesia rhinoscopy is performed and mycotic plaques are removed by surgical hook and suction. The dog is positioned in sternal recumbency and has to be intubated with a cuffed endotracheal tube. Then the tip of a 20- French Foley catheter is inserted through the mouth and placed at the junction between the hard and the soft palate in the caudal nasopharynx. The nasopharynx will be occluded by inflating the balloon. After, the Foley catheter is clamped. Moist surgical gauzes are counted and placed in the pharynx to avoid leakage of the clotrimazole-solution into the trachea. Next, the side holes of two 18-French Foley catheters (used to flush) are clipped off and one is placed in each nostril into the dorsal sinus nasalis. Within these Foley catheters we yellow urine catheters are placed, also with the side holes cut off, though which the clotrimazole will be administered. Each catheter is connected to a 50 ml infusion syringe with clotrimazole-solution. After that the balloons are inflated which close off the nostrils and so prevent leaking out of the nose. Clotrimazole solution (1%) is flushed into the nasal cavities through the catheter until the nasal cavity and frontal sinus are sufficiently filled and leakage along the Foley catheters or mouth is noticed (approximately 25 ml in each nostril in medium to large sized dogs). Then the catheters are clamped. The dog is positioned 15 minutes in sternal recumbency and then rotated every 15 minutes to left lateral recumbency, right lateral recumbency and dorsal recumbency to ensure drug contact with all the sinonasal surfaces. Every 15 minutes more clotrimazole solution is added if possible (with a maximum of 75 ml per nasal cavity) to ensure maximal drug contact. At the end of the infusion (after 1 hour) the dog is positioned in sternal recumbency with the head pointed downwards. The catheters in the nostrils are removed to drain the nose for 15 minutes. At the end of the procedure the 20-French Foley catheter of the nasopharynx and the gauzes in the pharynx are removed. The oral cavity is checked and fluid is removed by suction before recovery from anesthesia. For this treatment procedure CT evaluation is necessary to determine if the frontal sinus is not affected and if the cribriform plate is intact. If the sinus frontalis is affected, preferred therapy are treatment 3 and 4 because these provide direct access to mycotic plaques by trepanation.

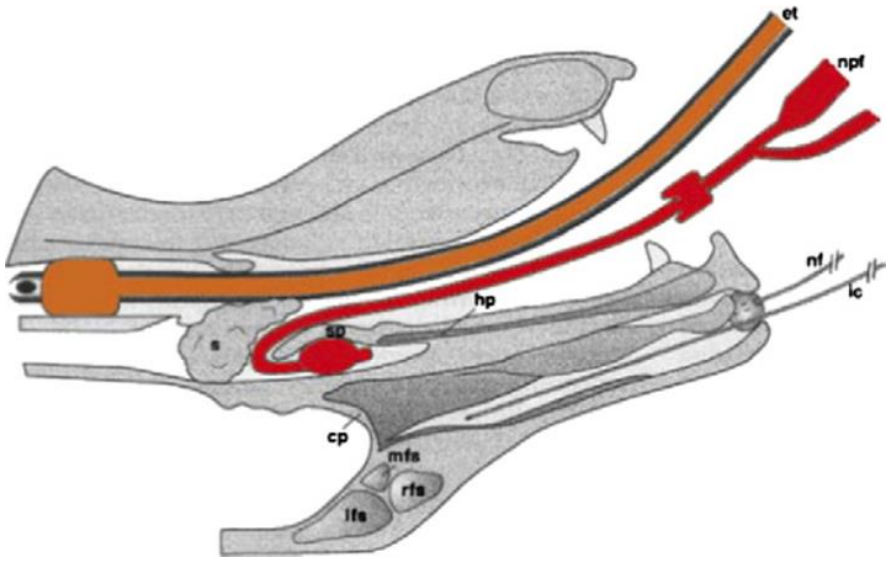

Fig. 5: Saggital section shows the position of the endotracheal tube (et), nasopharyngeal Foley catheter (npf), Pharyngeal sponges (s), infusion catheter (ic) and rostral nasal Foley catheter in relation to the hard palate (hp), soft palate (sp), cribriform plate (cp), rostral frontal sinus (rfs), medial frontal sinus (mfs), and lateral frontal sinus (lfs). (From Mathews KG, Davidson AP, Koblik PD, et al. Comparison of topical administration of clotrimazole through surgically placed versus nonsurgically placed catheters for treatment of nasal aspergillosis in dogs: 60 cases (1990-1996). J.Am. Vet Med Assoc 1998,213(4):503) (Peeters & Clercx, 2007)

Treatment 2 is frequently used at Utrecht University, sometimes combined with clotrimazole cream (this is treatment 5) which will be discussed below. In this research 32 dogs were treated with this treatment technique.

Treatment 3: *Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%).*

The dog is positioned in sternal recumbency and intubated with cuffed endotracheal tube. Under general anesthesia rhinoscopy is performed and mycotic plaques are removed by surgical hook and -suction.

First rhinoscopy is performed and mycotic plaques are removed by surgical hook and suction. After removal of all mycotic plaques through the nose, trepanation of the sinus frontalis is performed.

Landmarks for trepanation of the frontal sinus are the zygomatic process of the frontal bone laterally and the bony margin of the orbital rim ventrally. These make a fictitious triangle with the midline of the skull medially. The location of the trepanation is in the center of this triangle (Benitah, 2006; N. Sharp et al., 1992). The trepanation is performed with a surgical hand drill. In order to determine if trepanation is necessary unilaterally or bilaterally and if the cribriform plate is intact, a CT scan should be performed. When only one sinus frontalis is affected we only make one trepanation. Mycotic plaques in the sinus frontalis are removed

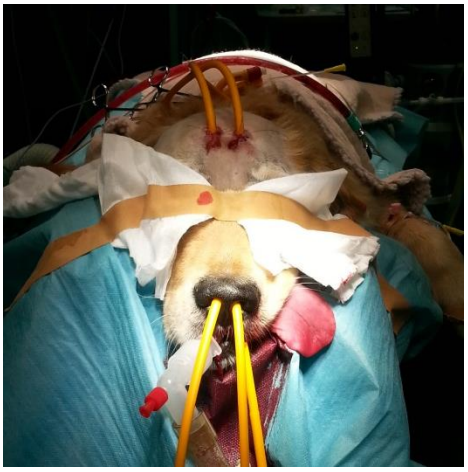

Fig. 6: Bilateral trephanation of the frontal sinus with placement of the catheters

under rhinoscopic guidance. Then, the side holes of an 18-French Foley catheter are cut off and a sterile urine catheter, also with the tip cut off, is placed in this Foley catheter. The Foley catheter is placed into the frontal sinus through the trepanation hole and the balloon is inflated with air. The tip of a 20-French Foley catheter is placed in the nasopharynx as described before, the balloon is inflated and the Foley catheter is clamped. Moist surgical gauzes are placed in the pharynx to avoid leakage of the clotrimazole solution into the trachea. An 18-French Foley catheter is placed in the nostril of the ipsilateral side to prevent leaking out of the nose. If the frontal sinus is infected bilaterally, follow the same instructions for the contralateral side. (Figure 2). If it is not, cut off the tips of an 18-French Foley catheter and a urine catheter and place the latter into the Foley catheter. This catheter is placed in the nostril into the dorsal meatus and the balloon is inflated to prevent leaking out of the nose. Both urine catheters are connected to a 50 ml infusion syringe with clotrimazole-solution (1%). In this last case one side is flushed from the sinus frontalis directed rostrally and the other side is flushed from the nose directed caudally. Flushing protocol (as described above, see treatment 2) should be followed (one hour flushing in total). After removal of the 18-French Foley catheters and draining the nose for 15 minutes, clotrimazole-cream is left into the frontal sinus. Subcutis and skin are closed with interrupted sutures of 3-0 to 4-0 absorbable suture material (Monocry®). The oropharynx suctioned free of fluids before recovery from anesthesia.
This treatment technique is used when the frontal sinus is affected by the fungus. In this retrospective study only two dogs received this treatment. However it is used in several new patients with affected frontal sinuses. This technique is preferred over treatment option 4 because it is less invasive and stressful for the dogs and their owners. For that reason I wanted to include this technique in this research.

Treatment 4: *Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus with placement of tubes to administer enilconazole (1%) twice daily for 10-14 days.*
Under general anesthesia rhinoscopy is performed and mycotic plaques are removed by surgical hook and suction. The dog is positioned in sternal recumbency and has to be intubated with a cuffed endotracheal tube. Trepanation is performed as described above (see treatment 3). Under rhinoscopic guidance mycotic plaques in the frontal sinus are removed by surgical hook and suction. Thereafter a silicone tube is placed into the frontal sinus through the trepanation hole and fixed with two sutures (PDS 3-0) in the subcutis and skin. The tube is fixed on the back of the head by means of a butterfly and two sutures, and can also be fixed to a specially designed collar. Enilconazole should be administered twice daily for 10-14 days. At the Faculty of Veterinary Medicine at Utrecht University, Imaverol® is used. This contains

100mg/ml enilconazole. Dilute 1 ml Imaverol® into 9 ml of warm sterile NaCl before administering through the tubes. After treatment, the tubes are removed and subcutis and skin are closed with interrupted sutures of 3-0 to 4-0 absorbable suture material (Monocry®).
This treatment technique is administered to patients with affected frontal sinus(es), severe bone destruction or if the cribriform plate is not intact.
Whether tubes are placed unilaterally or bilaterally should be determined by CT evaluation.
In the past this treatment was frequently used. Nowadays, treatment option 3 is preferred over this treatment technique because it is less invasive and stressful for the dogs and their owners. However, this is only possible if the cribriform plate is intact and if there is no severe bone destruction. If the cribriform plate is damaged or there is severe bone destruction whereby a connection has formed between the frontal sinus and the brains, flushing under pressure should not be performed. Trepanation of the frontal sinus and placement of tubes to administer enilconazole twice daily for 10-14 days is the only treatment option.
Since the patients are conscious during daily treatment, some dogs do not cooperate when administering enilconazole. Most dogs are admitted to the veterinary hospital during the treatment phase. Nine dogs were treated with this treatment protocol.

Treatment 5: *Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation, removal of mycotic plaques followed by local application of clotrimazole cream (1%).*
Treatment technique 5 is almost identical to treatment technique 2, the only difference is that in technique 5 the one hour flush with clotrimazole solution (1%) is followed by local administration of clotrimazole-cream (1%). See treatment 2 for clotrimazole flushing protocol.
Three patients were treated with treatment option 5.

Severity score

In order to investigate the influence of the severity of the infection on treatment outcome, a severity score was designed. This severity score is based on a score designed by J. Zonderland et al. (Zonderland et al., 2002), with some additions. The total severity score is calculated by summing up the score obtained for each separate part. The outcome is divided into four groups.

Severity score	
Signs of painful nose or muzzle	0 to 2
Depigmentation of the nasal planum/alar fold	0 to 1
Hyperkeratosis of the nasal planum	0 to 1
Ulceration nasal planum	0 to 1
Episodes of epistaxis	0 to 2
Computed tomography turbinate destruction	0 to 2 (score for both left and right side)
Rhinoscopic turbinate destruction	0 to 2 (score for both left and right side)
Destruction of nasal septum	0 or 2
Involvement of the frontal sinus	0 or 2 (score for both left and right side)
Severity of intranasal or sinusal fungal plaques	0 to 2
Destruction of frontal bones	0 or 2
Destruction of cribriform plate	0 or 2

Table 1. Severity score for calculating severity group.

Groups severity score	
Score	**Severity of the infection**
3 – 4	1. Mild
5 – 7	2. Moderately severe
8 – 11	3. Severe
12 – 17	4. Very severe

Table 2. Severity group.

Results: Retrospective study

In the time period March 2005 to March 2013, 78 dogs were diagnosed with SNM at the Faculty of Veterinary Science, Medicine of Companion Animals. Twenty-seven dogs were excluded from the study because they did not match the inclusion criteria. The remaining 51 patients were included in the study and statistical analyses were performed. Affected breeds are: the Golden retriever (n=5), German Shepherd Dogs (n=4), Belgian Shepherd Tervuren (n=3), Flatcoated retrievers (n=3), Labrador retrievers (n=3), Jack Russel Terriers, , Border Collies (n=2), Rottweilers (n=3), Bull Mastiffs (n=3), Bull terriers (n=2), Greater Swiss Mountain dogs (n=2) and mixed breeds (n=7). Furthermore, one dog of each of the following breeds: Dutch Shepherd Dog, Scottish Shepherd Dog, Small Münsterlander, German hunt terrier, Airedale terrier, Rhodesian Ridgebacks, Dobermans, Spinone Italiano, Portuguese water dog, Staffordshire Bullterrier, Afghan hound and a Dachshund. All dogs were mesocephalic or doliocephalic breeds. Twenty-four dogs were intact males, 12 castrated males, 7 intact females and 7 neutered females. Dogs referred at the University clinic with clinical signs of SNM were aged 6 months to 13 years. The mean age was 6 years.

Treatments

In total 51 dogs were treated with one of the described treatment techniques. Twenty four (47.1%) of these dogs had recurrence of SNM over the entire follow-up period after one treatment. Treatment success was 52.9% after one treatment. After two treatments 11 of these 24 dogs had again recurrence of SNM. This is 21.6% (11 of 51) of all the treated patients in total and 45.8% (11 of 24) of the patients which had recurrence of SNM after one treatment. In conclusion, 78.4% of patients receiving two treatments were successful. Patients requiring multiple treatments to cure often received treatment techniques different from the initial protocol.

Treatment option 1

Five dogs were treated with treatment option 1, local abnormalities e.g. corpus alienum and mycotic plaques were removed after which clotrimazole cream (10 mg/ml) was installed. Recurrence after one treatment was seen in one (20%) of these dogs. Success after one treatment was 80%.

Treatment option 2

A one hour long noninvasive flushing with clotrimazole (1%) without trepanation or administration of local clotrimazole cream was performed in 32 dogs (treatment 2). Seventeen (53.1%) of these dogs had recurrence; 40.6% within 12 weeks and 34.4% within 6 weeks. Overall success rate of treatment 2 was 46.9%.

Of the 32 dogs initially treated with treatment 2, nine dogs with recurrence were retreated with treatment 2. Three 33.3% (3 of 9) of these dogs again had recurrence after the second treatment. After two treatments of treatment 2, 9.4% (3 of 32) of patients still had recurrence. Treatment success after treated twice with treatment 2 is 90.6%.

Treatment option 3
Trepanation of the sinus frontalis/nasalis was performed on 2 patients with placement of temporary catheters (treatment 3). Recurrence was seen within 6 weeks in one (50%) of these patients. The same percentage was found for recurrence over the entire follow-up.

Treatment option 4
Nine patients were treated with treatment option 4: trepanation, removal of mycotic plaques and placement of tubes to administer enilconazole 2dd for 10-14 days. Three (33.3%) of these dogs had recurrence over the entire follow-up, one (11.1%) within 12 weeks. First treatment success over the entire follow-up was 66.7%.
Of the nine dogs initially treated with treatment 4, one dog with recurrence was treated with the same protocol again. This dog showed recurrence of SNM again for the second time after treatment option 4.

Treatment option 5
Three dogs were treated with a one hour flushing with clotrimazole solution without trepanation, followed by administration of clotrimazole-cream (treatment 5). Of these dogs, two (66.7%) had recurrence within 6 weeks. The same percentage was seen for recurrence over the entire follow-up. First treatment success of treatment 5 is 33.3%. Of these two dogs with recurrence, one was treated with the same treatment procedure again. Even after undergoing twice this treatment protocol, SNM reoccurred.

Treatment 2 and 5 simultaneously
When combining treatment 2 and 5 to enlarge the sample size (the only difference between these two treatments is that in treatment 5 the one hour flush with clotrimazole solution is followed by administration of clotrimazole cream (n= 35)), 19 (54.3%) dogs had recurrence. Of these dogs 42.9% (15 of 35) had recurrence within 12 weeks and 37.1% (13 of 35) within 6 weeks.

Treatment option	Number of dogs	Recurrence after 1st treatment %	Treatment success after 1st treatment %
1: Removal of local abnormalities (e.g. corpus alienum); removal of mycotic plaques by rhinoscopy, followed by the local application of clotrimazole cream	5	20.0	80.0
2: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation	32	53.1	46.9
3: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%)	2	50.0	50.0
4: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus with placement of tubes to administer enilconazole (1%) twice daily for 10-14 days	9	33.3	66.7
5: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation, removal of mycotic plaques followed by local application of clotrimazole cream (1%)	3	66.7	33.3

Table 3: Recurrence of SNM over the entire follow-up seen after 1st treatment in treated dogs at the Faculty of Veterinary Science, Medicine of Companion Animals at Utrecht University (March 2005 to March 2013).

No significant difference was found in treatment outcome (recurrence) over the entire follow-up between the different treatment groups 1 to 5. No association was seen between treatment option and recurrence over entire follow-up (Table 4).

There were only two statistically significant results found when looked at recurrence between different subdivisions (within 6 weeks, within 12 weeks and over the entire follow-up). One significant result (P= 0.049) was seen by patients treated with treatment 4 with recurrence within 6 weeks. This indicates an association between treatment 4 and recurrence of SNM within 6 weeks after this treatment. However none of the treated dogs in this group had recurrence within 6 weeks. This would indicate that there is a significant difference in recurrence within 6 weeks after treatment with treatment option 4 compared to other treatment

options. This indicates that there is 0% chance of recurrence within 6 weeks after treatment option 4.

The other significant result was seen by recurrence within 6 weeks, when treatment groups 2 and 5 were joined together (the only difference between these two treatments is that in treatment 5 the one hour flush with clotrimazole solution is followed by administration of clotrimazole cream) (p= 0.039). This indicates that there is an association between recurrence of SNM within 6 weeks after treatment with treatment option 2 or 5.

Relative risks and odds ratios with their 95% confidence intervals were calculated for each treatment option. Results are listed in table 4. Note that there is no control group that did not receive any treatment, thus patients who had recurrence after one treatment option are compared with all patients with recurrence that did not have the same treatment option. Confidence intervals of these odds ratios and relative risks were all relatively wide and almost every interval (except the confidence interval of the relative risk of treatment 4) crosses the value 1. This indicates that the relationship observed between the two variables, in this case treatment option and the chance of recurrence of SNM, is not reliable. There is a chance that in the population the relationship is opposite to what we have observed in our study population. Calculated confidence intervals are wide, meaning that the sample mean is a poor estimate of the population, and that the sample mean could be very different from the true mean of the population (Field, 2009; Petrie & Watson, 2006). Moreover, p-values calculated by Fishers exact test for each treatment were not significant. (Table 4). This means there is no association between treatment option and recurrence over the entire follow-up.

Treatment	O.R.	Sig.	95% C. I		R.R.	95% C.I	
			Lower	Upper	on recurrence	Lower	Upper
Treatment 1	0.250	0.354	0.026	2.411	2.500	0.423	14.776
Treatment 2	1.943	0.385	0.608	6.212	0.693	0.354	1.359
Treatment 3	1.130	1.00	0.067	19.118	0.939	0.227	3.847
Treatment 4	0.500	0.473	0.110	2.268	1.500	0.567	3.966
Treatment 5	2.364	0.595	0.201	27.852	0.688	0.292	1.620
Treatment 2 and 5	2.613	0.145	0.749	9.109	0.576	0.262	1.266

Table 4. Odds ratio's (O.R.), significance (Sig.), relative risks of recurrence (R.R.) and 95% confidence intervals (C.I.) are calculated for recurrence over the entire follow-up after first treatment.

Relative risks were also calculated to compare one individual treatment technique with another individual treatment technique (% of recurrence after treatment 1 / % of recurrence after treatment 2 etc.) (Table 5). For example, the chance of recurrence after treatment 1 is 0.376 times greater than after treatment 2 (this means that treatment 1 has a lower chance of recurrence).Confidence intervals for these relative risks were also calculated. Some of them are wide and they cross the value 1. This indicates that the relationship observed between the two variables is not reliable. There is a possibility that in the population the relationship is opposite to what we have observed in our study population (Field, 2009; Petrie & Watson, 2006).

	Absolute risk on recurrence %	**Relative risk**					
Treatment		**Treatment 1**	**Treatment 2**	**Treatment 3**	**Treatment 4**	**Treatment 5**	**Treatment 2 and 5**
Treatment 1	20.0	-	0.376 *0.063-2.239*	0.400 *0.043-3.738*	0.600 *0.083-4.353*	0.300 *0.044-2.061*	0.368 *0.062-2.183*
Treatment 2	53.1	2.655 *0.447-15.799*	-	1.063 *0.256-4.412*	1.594 *0.598-4.245*	0.797 *0.336-1.890*	0.979 *0.627-1.528*
Treatment 3	50.0	2.500 *0.268-23.360*	0.941 *0.227-3.908*	-	1.500 *0.284-7.934*	0.750 *0.151-3.716*	0.921 *0.223-3.806*
Treatment 4	33.3	1.667 *0.230-12.091*	0.627 *0.263-1.923*	0.667 *0.126-3.526*	-	0.500 *0,147-1.679*	0.614 *0.232-1.624*
Treatment 5	66.7	3.333 *0.485-22.898*	1.255 *0.529-2.977*	1.333 *0.269-6.606*	2.000 *0.589-6.790*	-	1.228 *0.522-2.890*
Treatment 2 and 5	54.3	2.714 *0.458-16.083*	1.022 *0.655-1.595*	1.086 *0.263-4.487*	1.629 *0.616-4.308*	0.814 *0.346-1.917*	-

Table 5. Relative risks calculated for recurrence over the entire follow-up after first treatment; comparing individual treatment options with each other (e.g. % of recurrence after treatment 1 / % of recurrence after treatment 2) 95% Confidence interval is *italic.*

Influence of different variables on recurrence of sinonasal mycosis

The Chi-squared test or the Fischer exact test calculate an association between different variables and recurrence after treatment. None of the calculated p values were smaller than 0.05. This means that treatment failure was not associated with age (0-5 years versus > 5 years; p=0.585), gender (p=0.759, affection of the frontal sinus (p=0.575), ipsilateral versus bilateral affection (p=1.00), severity of the infection (p=0.707) or enlargement of the ipsilateral mandibular lymph node (p=0.431). Odds ratios were calculated for each variable to determine an association between the variable and recurrence of SNM. None of these were significant, meaning that none of the variables were associated with recurrence over the entire follow-up. All 95% confidence intervals calculated cross the value 1, meaning that they are not accurate estimates. See table 6. Results of each variable will be discussed below.

Age

Fifty percent (16 of 32) of the dogs older than > 5 years of age had recurrence over the entire follow-up compared to 42.1% (8 of 19) of the dogs 0-5 years of age. Within 12 weeks after treatment recurrence was seen in 40.6% (13 of 32) of the animals > 5 years of age and in 21.1% (4 of 19) of the animals < 5 years of age. Within 6 weeks this was respectively 21.1% (4 of 19) in young and 31.3% (10 of 32) in older dogs. Odds ratios were all greater than one, but none of them were significant. See table 6.

Gender

The recurrence rate was higher in female dogs than in male dogs (53.3% versus 44.4% after treatment over the entire follow-up). This was also the case when calculating the percentages within the different subdivisions (male 12 weeks: 30.6% (11 of 36); male 6 weeks: 22.2% (8 of 36); female 12 weeks: 40.0% (6 of 15); females within 6 weeks: 40% (6 of 15)). The calculated odds ratios indicate that female dogs are 1.4 times more likely to have recurrence after first treatment than male dogs. However this difference is not significant (p= 0.563).

Involvement of frontal sinus

In total the frontal sinus was affected in 37.3% (19 of 51) of cases. Ten of these dogs (52.6%) had recurrence over the entire follow-up. Forty-three point eight percent43.8% (14 of 32) of the dogs without diseased frontal sinuses had recurrence. Recurrence percentages seen within 6 weeks are 26.3% (5 of 19) with an affected frontal sinus versus 28.1% (9 of 32) with only an affected sinus nasalis. Odds ratios calculated for recurrence over the entire follow-up and within 12 weeks were greater than one, but not significant. (Table 6).

Ipsilateral versus bilateral affection of the sinus nasalis/frontalis

Over the entire follow-up period, 50% (6 of 12) of the dogs that had bilaterally affected nasal/frontal sinusses had recurrence while 46.2% (18 of 39) of the dogs that only had an ipsilaterally affected sinus nasal/frontal sinusses had recurrence.

However, only 16.7% (2 of 12) of dogs with a bilaterally affected nasal/frontal sinusses showed recurrence within 6 weeks compared to 30.8% (12 of 39) of the dogs with an

ipsilaterally affected sinus nasalis/frontalis. No significant odds ratios for recurrence of SNM was found for ipsilateral versus unilateral affection of the sinus nasalis/frontalis. (Table 6). There was no statistical difference between the odds ratios for the sinus nasalis and the sinus frontalis.

Enlargement of the mandibular lymph node

Lymph node size was not always included in patient files. Mandibular lymph node enlargement ipsilateral to the diseased nasal/frontal sinus was seen in 75.8% (25 of 33) of the dogs with SNM. In 44% of these cases with enlarged mandibular lymph nodes recurrence was seen over the entire follow-up. When lymph node was not enlarged 25% (2 of 8) had recurrence over the entire follow-up. Twenty-eight percent (6 of 25) of the patients with enlarged mandibular lymph nodes had recurrence within 6 weeks versus 12.5% in animals with no enlarged mandibular lymph nodes. Calculated odds ratios were all greater than one but none of them were significant. (Table 6).

It was hypothesized that the size of the mandibular lymph nodes could possibly predict and affect the course of the disease. However, this assumption is not supported as results were not significant (p= 0.431) and the exact size of each mandibular lymph node was not measured.

Severity of the infection

Using the severity score (see table 1 and 2), 11.8% (6 of 51) of the total number of dogs treated had a very severe infection, 37.3% (19 of 51) had a severe infection; 37.3 % (19 of 51) had a moderate infection and 13.7% (7 of 51) had an mild infection.

Of the group with very severe infection 33.3% had recurrence over the entire follow-up and 16.7% (1 of 6) had recurrence within 12 and 6 weeks.

In the group with severe infection 57.9% (11 of 19) had recurrence over the entire follow-up and 42.1 (8 of 19) and 36.8% (7 of 19) had recurrence within 12 and 6 weeks respectively.

The group with moderate infection had recurrence over the entire follow-up in 42.1% (8 of 19) of cases. 26.3% (5 of 19) had recurrence within 12 weeks and 15.8% (3 of 19) within 6 weeks.

Because lack of a control group, the severity group 1 (mild infection), was used as a control group to calculate the odds ratios with. None of the odds ratios were significant. See table 6.

Depigmentation of the nasal planum was seen in 18 dogs (35.3%), hyperkeratosis was seen in 6 dogs (11.8%) and ulceration of the nasal planum was observed in 8 of the total 51 dogs (15.7%). Eleven dogs (21.7%) had local destruction of the nasal septum and on CT 13 dogs (25.4%) showed bone degeneration.

Variable	Odds ratio	Significance	95% C.I.	
			Lower	Upper
Age: young vs. old				
Entire follow-up	1.375	0.585	0.438	4.318
Within 12 weeks	2.566	0.158	0.693	9.502
Within 6 weeks	1.705	0.433	0.450	6.460
Gender: female vs. male				
Entire follow-up	1.429	0.563	0.427	4.785
Within 12 weeks	1.515	0.516	0.433	5.304
Within 6 weeks	2.333	0.201	0.637	8.543
Involvement of frontal sinus				
Entire follow-up	1.429	0.540	0.457	4.465
Within 12 weeks	1.283	0.682	0.389	4.239
Within 6 weeks	0.913	0.889	0.254	3.280
Ipsilateral vs. bilateral affection sinus nasalis/frontalis				
Entire follow-up	1.167	0.816	0.320	4.259
Within 12 weeks	0.595	0.487	0.138	2.566
Within 6 weeks	0.450	0.347	0.085	2.375
Enlargement of the mandibular lymph node				
Entire follow-up	2.357	0.346	0.396	14.041
Within 12 weeks	1.167	0.868	0.188	7.222
Within 6 weeks	2.772	0.387	0.281	26.347
Severity of the infection: group 1 vs. group 2, 3 and 4				
Group 2 Entire follow-up	0.970	0.973	0.168	5.593
Within 12 weeks	0.476	0.422	0.078	2.916
Within 6 weeks	0.250	0.161	0.036	1.739
Group 3 Entire follow-up	1.833	0.498	0.318	10.573
Within 12 weeks	0.970	0.973	0.168	5.593
Within 6 weeks	0.778	0.780	0.133	4.536
Group 4 Entire follow-up	0.667	0.725	0.069	6.409
Within 12 weeks	0.267	0.322	0.019	3.653
Within 6 weeks	0.267	0.322	0.019	3.653

Table 6. Different variables, odds ratio's, their significance and 95% confidence interval (C.I.). Group 1: mild; group 2: moderately severe, group 3: severe; group 4: very severe infection.

Research goals: Prospective case control study

Another part of this study is a prospective case control study of new patients with SNM. The goal is to define risk factors for the development of SNM in dogs and to define if there are specific abnormalities in these dogs that can be observed by performing blood analyses. Blood analysis on each patient sample was performed: hematocrit, leucocyte differentiation, albumin, total protein and protein spectrum (only measured if total protein was not within reference values). If the patient has been abroad (particularly in the south of Europe) and is suspected of Leishmania or other foreign diseases, blood analysis was performed to rule this out.
New patients participating in the study were used to investigate possible abnormalities of blood values described above, which can indicate an underlying disease and determine if there are specific blood values that are abnormal in patients with SNM. Extra blood for DNA analysis was taken from the patients (with informed consent) and stored for later research to investigate similarities between them. Specific similar DNA sequences in these patients may explain their sensitivity to Aspergillus spp.
In cooperation with Microbiology, Department of Biology, Faculty of Science, Utrecht University we did not only focus on the characteristics of the patients, but also on the characteristics of the fungus itself. Of the new patients with SNM that are examined and treated at the Faculty of Veterinary Science in Utrecht, the type of fungus that causes the infection is determined, its DNA is isolated and typed and its resistance profile and acidity is determined. Histologic evaluation of biopsies of the affected tissue is required to determine how the fungus is growing on the tissue and how deep it is capable of penetrating the tissue. These results will allow us to get a better idea of the pathogen and how to treat it.

Inclusion criteria of the study are the same as described in the retrospective part. It was decided that there should be a minimum of six patients cooperating in this part of the research. However, within the timeframe available for my research project, less than six patients with proven diagnosis of SNM contributed to the study and not all data were available yet. Not all fungal DNA was typed and the complete resistance profile was not determined. Therefore, this study will mainly focus on the retrospective aspect of it.

Material and methods: Prospective case control study

In the time period of April 25, 2013 to September 1, 2013 new patients suspected of SNM were followed. Clinical examination, CT and rhinoscopy was performed of all new patients suspected of SNM at the Faculty of Veterinary Science, Medicine of Companion Animals at Utrecht University. Dogs were under anesthesia during CT, rhinoscopy and treatment. Anesthetics used depended on the anesthetist and American Society of Anesthesiologists (ASA) scale of physical status of the dog.
All patients were scheduled for control rhinoscopy six weeks after treatment. Every two weeks the owners were contacted for follow-up.

Computed tomography:
CT images were evaluated by the Radiology Department of Clinical Science of Companion Animals, Faculty of Veterinary Medicine Utrecht University.

Rhinoscopy:
During the rhinoscopy, mycotic plaques and biopsies from affected tissue were collected.

- Pieces of fungus for culturing and species typing. (The samples were sent to Microbiology, Department of Biology, Faculty of Science, Utrecht University. This department cultures the fungus and sends samples to Centraalbureau voor Schimmelculturen (CBS) at Utrecht for typing of DNA of the fungus.).
- Pieces of fungus were transferred to a sterile tube and frozen in liquid nitrogen, these tubes were stored at -70^0C and saved for later DNA isolation. (The samples will be sent to Microbiology, Department of Biology, Faculty of Science, Utrecht University for RNA isolation).
- 1-2 biopsies from affected tissue were sent to the Veterinary Pathology Diagnostic Centre in Utrecht for histologic evaluation to determine fungal invasion.

After removal of the fungal tissue, an anti-mycotic treatment was performed.

pH measurement:
The nasal pH at the place where the fungus grows was determined. The goal is to examine if the fungus adapts to the hosts environment and how it migrates to the frontal sinus and whether or not the pH is more favorable for fungal growth. pH- measurement was performed directly (within 30 minutes) after the collection of the fungal plaques by Microbiology, Department of Biology by the means of pH paper.

Blood analyses:
Blood samples were taken while the patient was under general anesthesia and samples were analyzed by the University Veterinary Diagnostic Laboratory (UVDL). Blood parameters that were analyzed are the: hematocrit, leucocyte differentiation, albumin, total protein. Protein spectrum was only analyzed if values of total protein were high. With informed consent of the client (dog owner) extra blood (4cc EDTA) was taken for storage and later DNA research.

Culturing:

- Pieces of fungus were collected and transferred directly to Microbiology.
- Small pieces of the plaque were removed by cutting and then placed onto a PDA plate and cultured at 37^0C for two days. By this time sufficiently large colonies had formed that started to sporulate. From those plates sub culturing was performed until a single type of the fungus was defined.
- Isolates were evaluated by light microscopy, especially spore head structure to see if it resembles with *Aspergillus fumigatus*.
- Of each isolate a culture was prepared to isolate spores that were stored at -80^0C (at Microbiology, Utrecht University).

Resistance profile:

- Of all isolates, a resistance profile will be determined with a relative set of antifungals (Clotrimazol, Itraconazole, Fluconazole, Voriconazole, Amphotericin and Flucytosine).
  The resistance profile will be estimated by two tests: the broth dilution method and the epsilometer test (E-test method). (The E-test utilizes a rectangular strip that has been impregnated with the drug to be studied. A fungus is spread and grown on an plate, and the E-test strip is laid on top; the drug diffuses, producing an exponential gradient of the drug to be tested.) However, at the time of writing only an E-test with Itroconazol was performed.

DNA isolation and typing:

DNA of the collected fungal strains were isolated and typed at CBS (Centraalbureau voor Schimmelculturen) by sequencing relevant genes (Balajee et al., 2007).
DNA was isolated of each isolate, tagged with a barcode to identify the isolate (see appendix 1) and sequenced via the next generation sequencing methods.

- DNA isolation is performed by the TRIzol method.
  Ultra Clean® Microbial DNA Isolation Kit of Mo Bio Laboratories, Inc. was used to isolate DNA.
  The genome of A. fumigatus 293 (clinical isolate from humans) is used to identify expressed genes. Control for expression profiling is AF293 grown *in vitro* according to protocol used by Eric Bathoorn (who also performed a gene expression profiling, Manuscript in preparation).
- Different primer pairs were used to sequence DNA fragments, see appendix 2.

Results: Prospective case control study

In the time period of April 25, 2013 to September 1, 2013, nine patients suspected of sinonasal mycosis were presented at the Faculty of Veterinary Science, Medicine of Companion Animals at Utrecht University. These patients presented with clinical signs that are normally seen in cases of SNM (see introduction). However, other differential diagnoses or primary causes (e.g. nasal tumor, lymphoplasmacytic rhinitis, tooth root inflammation, corpus alienum and nasal fistula) were not yet excluded.
Clinical examination, CT and rhinoscopy were performed on all new patients suspected of SNM.

Ultimately four dogs were diagnosed with SNM. Breed, gender, age and severity of the infection are listed in Table 7 and 8.
With informed consent blood was collected for DNA research.
Every two weeks owners were contacted for follow-up.

Patient 4, had a previous history of sinonasal Aspergillosis. Its first diagnosis of sinonasal mycosis was established in 2011 and the right sinus nasalis was affected. In July 2013 the left side of the frontal sinus was affected. No clinical symptoms matching SNM were seen in the interim period.
Patient 2 showed recurrence of nasal discharge within two weeks after treatment, because of severe clinical deterioration probably caused by recurrence of SNM or a systemic form of Aspergillosis. The owners decided to euthanize the dog.

Treatment and recurrence of SNM
As is listened in table 9, one patient was treated with treatment option 5 (patient 1) and the other three patients were treated with treatment option 3.
Recurrence of clinical signs of SNM were seen in two of total dogs (50%) (patient 3 and 4). However, recurrence of SNM in patient 2 was not confirmed after he was euthanized.

Patient	Breed	Gender	Age (years)	Severity group
Patient 1	Labrador Retriever	Male	2	3: severe (9 points)
Patient 2	Mixed breed	Male	6	4: very severe (12 points)
Patient 3	Alaska Malamute	Male	4	4: very severe (12 points)
Patient 4	Golden Retriever	Neutered female	4	3: very severe (11 points)

Table 7. Data of new patients diagnosed with SNM at the Faculty of Veterinary Medicine, Utrecht University (time period April 25, 2013 to September 1, 2013).

Severity score					
		patient			
		1	2	3	4
Signs of painful nose or muzzle	0 to 2	1	1	1	0
Depigmentation of the nasal planum/alar fold	0 to 1	1	1	1	0
Hyperkeratosis of the nasal planum	0 to 1	0	0	0	0
Ulceration of the nasal planum	0 to 1	0	0	0	0
Episodes of epistaxis	0 to 2	1	0	1	0
Computed tomography turbinate destruction	0 to 2 (score for both left and right side)	1	3	2	3
Rhinoscopic turbinate destruction	0 to 2 (score for both left and right side)	1	2	3	3
Destruction of nasal septum	0 or 2	2	0	0	0
Involvement of the frontal sinus	0 or 2 (score for both left and right side)	0	2	2	2
Severity of intranasal or frontal sinus fungal plaques	0 to 2	2	2	2	2
Destruction/alteration of frontal bones	0 to 2	0	1	0	1
Destruction of cribriform plate	0 or 2	0	0	0	0
Total		9	12	12	11

Table 8. Severity score of new patients with SNM.

Severity of the infection, severity score and group

Using the severity score, all patients had severe or very severe mycotic infection. Painful nose or muzzle and depigmentation of the nasal planum or alar fold were seen in 3 out of 4 patients (75%). One dog had destruction of the nasal septum and in two dogs alteration of frontal bones was seen on CT. In three patients the frontal sinus was involved, in two of these three dogs with affected frontal sinus (66.6%), recurrence was seen.

Patient	1st Treatment	2nd Treatment	3rd Treatment
1	Treatment 5: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation, removal of mycotic plaques followed by local application of clotrimazole cream (1%)	No 2nd Treatment	No 3rd Treatment
2	Treatment 3: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%)	No 2nd Treatment †	No 3rd Treatment †
3	Treatment 3: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%)	Treatment 1: Removal of mycotic plaques by rhinoscopy, followed by the local application of clotrimazole cream (1%)	Treatment 3: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%)
4	Treatment 3: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%)	No 2nd Treatment	No 3rd Treatment

Table 9. Treatment option of every new dog with SNM.
† dog was euthanized

Mandibular lymph node

In three patients (patients 1, 3 and 4) the mandibular lymph node was enlarged on the affected side. Patient 3 had enlarged mandibular lymph nodes on the right and left side. In this dog both the right and left nasal sinus and the left frontal sinus were affected.

Foreign diseases
None of the four patients had been to the south of Europe. Two had been in Germany and the others had never been abroad. None of these patients were suspected of Leishmania or other foreign diseases.

Histology
Biopsies were sent to the Veterinary Pathology Diagnostic Centre (VPDC) in Utrecht for histologic evaluation. In the biopsies of three patients, the fungus grows only superficially on the mucosa, the lamina propria was not infiltrated. No vital tissue was found in biopsies of patient 2 and for that reason infiltration of the fungus could not be examined. In all patients biopsies were classified as lymphoplasmacellulair rhinitis or sinusitis, some with neutrophil infiltration.

Blood analyses
Blood parameters analyzed were: hematocrit, leucocyte differentiation, albumin, total protein and protein spectrum. The values of each patient are displayed in table 10. There was no clear similarity between blood values of the patients. The only similarity seen was that albumin was low in three of four patients. Values found were slightly lower than references values. Patients did not show any signs of hepatic disease, renal losses or immune mediated diseases that can explain hypoalbuminemia. Patient 2 showed high rod like neutrophils and low segmented neutrophils. This is seen as a left shift of the blood cells, which can possibly be explained by severe acute infection by the fungus. Patient 1 had high segmented neutrophils, that are normally seen during infection. Patient 4 had a slightly higher value of basophils which may indicate an infection and possibly an allergic component. There was no evidence for immunologic disease in any of these dogs.

pH of the mycotic plaques
After removal of fungus from the nose or frontal sinus, the pH of the fungal plaque was determined by pH-paper. All pH measurements were between 7.9 and 8.2 (patient 1: 7.9 – 8.1, patient 2: 8.0 – 8.4 and patient 3: 8.0 – 8.2, the pH of patient 4 is unknown).

DNA isolation and typing of the fungus
Isolation and typing of fungal DNA was performed on fungus collected from patients 1, 2 and 3. Although there were some small differences in nucleotide sequence, all isolates were identified as *Aspergillus fumigatus.* Isolation and typing of fungal DNA collected from patient 4 will be examined at CBS when more samples are collected.

Blood analyses											
Patient	**Ht** L/L	**Leucocytes**								**Alb.** g/L	**T. P.** g/L
		Lymph. 10^9/L	Mono. 10^9/L	Blasts 10^9/L	Rodl. 10^9/L	Seg. 10^9/L	Eo. 10^9/L	Baso. 10^9/L	Norm. 10^9/L		
1	0.42	2.2	0.8	0	0	12.1	0.9	0	0	21	57
2	0.47	0.3	0.1	0	1.0	1.4	0.2	0	0	25	59
3	0.46	1.4	0.6	0	0	7.8	1.4	0	0	22	68
4	0.48	1.2	0.3	0	0	6.3	0.8	0.1	0	29	61

Table 10. Blood values analyzed and their references: Hematocrit(Ht): 0.42-0.61 L/L, Lymphocytes (Lymph.): 0.8-4.7 x10^9/L, Monocytes (Mono.): 0.0-0.9 x10^9/L, Blasts: < 0.0 x10^9/L, Rod like (Rodl.): 0.0-0.3 x10^9/L, Segmented (Seg.): 2.9-11.0 x10^9/L, Eosinophils (Eo.): 0.0-1.6 x10^9/L, Basophils (Baso.): 0.0-0.1 x10^9/L, Albumine (Alb): 26-37 g/L, Total Protein (T.P.): 55-72 g/L, Protein Spectrum (P.S.): red = value above reference value, blue = value below reference value.

Resistance profile

Investigation of the resistance profile of the fungus was not yet completed. Resistance to the antifungal Itraconazol was determined by the E-test method on samples collected from patients 1, 2 and 3. However, MIC values are unknown at the time of writing. More tests with different concentrations should be performed in order to determine the resistance profile.

Discussion

All patients with SNM reviewed in this study showed clinical signs as described in the literature and had mesocephalic or doliocephalic skulls (Nelson et al., 2009; Peeters & Clercx, 2007; Quinn et al., 2002; M. J. Sharman & Mansfield, 2012; N. Sharp et al., 1992).
Lack of treatment success is likely to be multifactorial. It is determined by severity of the infection, the treatment option, failure to completely debride of fungal plaques, distribution of antifungal agent and the experience of the treating veterinarian (Friend, Williams, & White, 2002; Mathews et al., 1998; Schuller & Clercx, 2007; M. Sharman et al., 2010; Zonderland et al., 2002).

The goal of this retrospective study is to determine which topical treatment option is most successful. In other words, which treatment has the least chance of recurrence of sinonasal mycosis in dogs? Treatment techniques investigated were:

Treatment 1: Removal of local abnormalities (e.g. corpus alienum); removal mycotic plaques by rhinoscopy, followed by the local application of clotrimazole cream (1%).
Treatment 2: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation.
Treatment 3: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus and one hour flushing with clotrimazole solution (1%), followed by administration of local clotrimazole cream (1%).
Treatment 4: Removal of mycotic plaques by rhinoscopy and trepanation of the frontal sinus with placement of tubes to administer enilconazole (1%) twice daily for 10-14 days.
Treatment 5: Removal of mycotic plaques by rhinoscopy and one hour flushing with clotrimazole solution (1%) without trepanation, removal of mycotic plaques followed by local application of clotrimazole cream (1%).

Furthermore, the influence of different variables on treatment failure or success were determined. Variables taken into account were: age, gender, ipsilateral versus bilateral affection, affection of the frontal sinus, severity of the infection and enlargement of the mandibular lymph node.

Of the total 51 dogs treated with one of the therapies described above, 24 (47.1%) had a recurrent infection of SNM after their first treatment over the entire follow-up period. In other words, first treatment success was 52.9%. Some dogs still had some clear nasal discharge after treatment, however this is probably due to conchal destruction and is not seen as recurrence. After two treatments, the chance of success was 78.4%. This is higher than described by Sharman et al. (2010) (45.8% first treatment success) in his retrospective study (M. Sharman et al., 2010). Sharman et al. (2010) reviewed medical records of patients of six veterinary referral centers which might give a more realistic indication of clinical outcome (M. Sharman et al., 2010). In this study only the medical records of the Faculty of Veterinary Medicine at Utrecht University were reviewed. A difference in treatment protocol between the

different hospitals Sharp et al. (2010) examined may explain the difference in percentages between Sharp et al. (2010) and this study.
When only taking treatment 2 (one hour clotrimazole infusion through non-surgically placed catheters) into consideration 46.9% of the dogs were cured after one treatment. This percentage is higher than described by Sharman et al. (2010) (40%), but is less than described by Mathews et al. (1998) (65%) (Mathews et al., 1998; M. Sharman et al., 2010). First treatment success of surgically placed tubes into the frontal sinus to administer enilconazole (1%) twice daily for 10-14 days (treatment 4) was 66.7%. This is less than described by Sharp et al. (1992, 1993) (90%) (N. Sharp et al., 1992; N. J. Sharp et al., 1993). It is not clear if all patients treated by Sharp et al. (1993) had frontal sinus involvement. However, at Utrecht University, patients were only treated with treatment option 4 if the frontal sinus was severely affected. If infection was more extensive in treated animals at the Faculty of Veterinary Science compared with treated animals by Sharp et al. (1993), this might contribute to smaller treatment success.
The results from patients treated with treatment options 1, 3 and 5 had a small sample size, which makes calculated results less reliable. Treatment successes seen in this group were 80%, 50% and 33.3%.

This study did not find any significant difference in first treatment outcome (recurrence) over the entire follow-up between the different treatment groups 1 to 5. Nor was treatment failure associated with age (0-5 years versus > 5 years; p=0.585), gender (p=0.759, affection of the frontal sinus (p=0.575), ipsilateral versus bilateral affection (p=1.00), severity of the infection (p=0.707) or enlargement of the mandibular lymph nodes (p=0,431).
However, an association was seen between patients treated with treatment option 4: trepanation of the frontal sinus with placement of tubes to administer enilconazole (1%) twice daily for 10-14 days, and recurrence within 6 weeks (p=0.049). None of the patients treated with treatment 4 had recurrence within six weeks after treatment, indicating that there is significant proof that no recurrence of SNM will occur within 6 weeks after being treated with treatment option 4. Although this knowledge can be useful, sample size of treatment 4 was small (n=9) and we prefer to know what the chance of recurrence is seen over a longer period of time after treatment.
Recurrence rates of a combined group consisting of treatment 2 and 5 (in order to enlarge the sample size) showed significant (p=0.039) reduction in recurrence rates within 6 weeks when compared with other treatments. Groups were combined in order to achieve a higher sample size to improve the validity for statistic calculation. Nevertheless, administration of clotrimazole cream after one hour flush with clotrimazole solution can prolong drug contact time which may be favorable for treatment success (Burrow et al., 2013; Hayes & Demetriou, 2012; M. J. Sharman & Mansfield, 2012; Sissener et al., 2006). In all new patients with SNM, treatment was always followed by administration of local clotrimazole cream (1%).

There was no control group of dogs with SNM who did not receive any treatment as this would be ethically unsound. Patients who had recurrence after one treatment option are compared with all patients with recurrence that did not receive the same treatment option.

Treatment options are also compared individually (Table 4 and 5). As a result, however, it is not clear how much greater the risk of recurrence after treatment with a therapy is compared to animals that are not treated.
In this study different treatment options are compared with each other. Outcomes of relative risks can be used to compare each treatment technique with each other treatment technique to determine which treatment is best. However it is known that therapy choice is determined by the severity of infection. This calculation does not consider the severity of the infection. Thus, it is not quite valid to compare treatment outcomes of different treatment options. For instance, a dog with SNM with involvement of the frontal sinus probably has a higher chance at successful treatment when antifungals are inserted into the frontal sinus through surgically placed catheters compared to only administration of clotrimazole cream in the nasal sinus.
Influence of the severity of the infection was determined by using a severity score. If infection is more severe it would be more likely that higher percentages of recurrence will occur. However, no relation was found between recurrence in total and severity score ($p=0.707$).
Most recurrences occur within 6 or 12 weeks after treatment, indicating that a control rhinoscopy after 6 weeks would be beneficial to detect recurrence early. Because in most cases, recurrence was seen within 6 or 12 weeks after treatment, it is more likely that in these cases first treatment was not 100% successful. It is likely that some fungus survived the treatment and that it continues to grow.

It was expected that the chance of recurrence was higher when the frontal sinus was affected. Yet only small differences between treatment outcomes were seen (recurrence over the entire follow-up with frontal sinus involvement: 52.6% vs. 43.8% where the frontal sinus was not affected). Odds ratios calculated for the effect of frontal sinus involvement over the entire follow-up and within 12 weeks were greater than one but were not significant, and their confidence intervals cross 1.
Also, no large differences in treatment outcome calculated over the entire follow-up period were seen between ipsilateral and bilateral affected nasal and frontal sinuses (50.0% vs. 46.2%). It was remarkable that the percentage of recurrence within 6 weeks was less in bilaterally affected dogs compared to unilateral affected dogs (16.7% vs. 30.8%). This was also seen in the odds ratios but these were not significant and confidence intervals cross the value 1, which makes those results unreliable.

Age and gender seemed to play a role in treatment outcome after first treatment. Females were more likely to show recurrence than males (53.3% vs. 44.4%, seen over the entire follow-up period; OR: 1.4). However, this difference was not significant ($p=0.563$).

It is thought that an immunomodulated disease plays a part in the pathogenesis of SNM. Blood analyses can be done to detect underlying disease. Unfortunately, no blood analyses were performed in the past of patients in the retrospective study. In order to determine any underlying disease, blood analyses were performed on the 4 new patients with SNM (see prospective study). Nevertheless, no clear similarities between blood values of the patients were found. The only similarity seen was that albumin was low in three out of four patients.

Values found were slightly lower than references values. In the future, more blood analyses should be performed on more patients with SNM to find similarities and determine if specific values can indicate SNM.
Often, it was unknown whether or not the patients had visited abroad, in particular countries in the south of Europe. Therefore, association with SNM could not be evaluated.
The same applies for the notation of mandibular lymph node size. In 18 of the 51 dogs (35.3%) it is unknown if the lymph node was enlarged at the time of referral. Lymph node size evaluation is a clinical investigation that is not very specific or sensitive; individual variation exists between researchers and the mandibular lymph node is easy to confuse with the salivary gland. CT evaluation of mandibular lymph nodes has a much higher sensitivity and specificity. For this reason, statistic outcomes could be slightly different in the real population. In this study no association between treatment outcome and enlarged mandibular lymph node was seen.

Relative risks and odds ratios were calculated to compare treatment outcomes. Confidence intervals of these odds ratios and relative risks were all relatively wide and almost every one, crosses the value 1. This indicates that the sample mean is a poor estimate of the population and that the relationship observed between the two variables is not reliable. The width of the confidence interval depends on the degree of confidence required, the sample size (a larger sample provides a more precise estimate and therefore a narrower confidence interval) and the variability of the characteristic under investigation (a more variable set of observations provides a less precise estimate and a wider confidence interval) (Field, 2009; Petrie & Watson, 2006). The number of patients treated with treatment options 1, 3 and 5 are too small for reliable statistic evaluation. These groups are not a good estimate of the population. The sample size should be enlarged by reviewing more patient files, or follow up on more new patients.

The prospective study of new patients with SNM was not completed within the time available for this research project. Not enough data was obtained to draw conclusions.

Conclusion

Sinonasal mycosis is an uncommon disease which causes chronic rhinosinusitis in dogs. SNM is mostly caused by the fungus *Aspergillus fumigatus*.
The overall success rate of topical treatments performed at the Faculty of Veterinary Science, Medicine of Companion Animals at Utrecht University is 52.9% after one treatment and 78.4% after two treatments.
No statistical difference is found in treatment outcome (recurrence) over the entire follow-up between the different treatment groups 1 to 5. Nor is treatment failure associated with age, gender, disease of the frontal sinus, ipsilateral versus bilateral disease, severity of the infection or enlargement of the ipsilateral mandibular lymph node.
There is significant proof that recurrence of SNM will not occur within 6 weeks after treatment with treatment option 4 ($p=0.049$).
Recurrence rates of a combined group consisting of treatment 2 and 5 (to enlarge the sample size) shows a significant ($p=0.039$) reduction in recurrence rates within 6 weeks when compared with other treatments.
Relative risks and odds ratios calculated in this study to compare treatment outcomes and the influence of different variables (age, gender, involvement of frontal sinus, ipsilateral vs. bilateral affection, enlargement of mandibular lymph nodes and severity score) are not reliable, nothing can be concluded from these values (these results are not significant, the 95% confidence interval is wide and crosses the value 1).
The sample size per treatment option in this study is low, the highest sample size is 32 and the lowest is 2. This is most probably the reason that only a few results are significant and odds ratios and relative risks are unreliable. The sample size should be enlarged by reviewing more patient files and following up more patients diagnosed with SNM.
More research is needed into the treatment of SNM to determine which treatment is most successful.

The prospective study of new patients with SNM was not finished within the time available for this research project. Not enough results are obtained to draw conclusions.
However, mycotic samples collected from the 4 patients are all typed as *Aspergillus fumigatus*. The pH of collected mycotic plaques lies between 7.9 and 8.2. Histological evaluation of biopsies of 3 different patients shows that the fungus grows only superficially on the mucosa, the lamina propria is not infiltrated Slight hypoalbuminemia is seen in three out of four patients (75%). Values found are slightly lower than references values. More blood analyses of new patients with SNM should be done in the future to determine specific differences in parameters and/or values and to screen for parameters and/or values that can indicate an Aspergillus fumigatus infection, or a predisposing condition. No risk factors for SNM are found as of yet.

Acknowledgements

I would like to thank drs. A.N. Haagsman, dr. D. Klinkenberg, dr. J.J.P.A. de Cock and N. Escobar Salazar MSc for their assistance in writing this research paper.

Appendix 1

Protocol DNA isolation using Ultra Clean® Microbial DNA Isolation Kit of Mo Bio Laboratories, Inc.:

1. Add 300 µl Microbead Bead solution to the Microbead tube
2. Add 50 µl solution MD1 to the Microbead tube (if MD1 is cloudy warm it up with some warm water)
3. Scrape some spores and mycelium from an agar plate and add to the Microbead tube (don't add peaces of agar)
4. Vortex the Microbead tube briefly
5. **Optional:** Heat treat the sample for 10 minutes at 65°C
6. Vortex the Microbead tube for 10 min with the Mo Bio-adapter
7. Centrifuge the Microbead tube at room temperature, time=30 sec, RPM=9600
8. Transfer the supernatant in a new 2 ml collection tube (don't take any debris from the pellet)
9. Centrifuge the tube with the supernatant, time=30 sec, RPM=9600
10. Transfer the supernatant in a new 2 ml collection tube (don't take any of the pellet)
11. Centrifuge the supernatant (time=30 sec, RPM=9600)
12. Transfer the supernatant in a new 2 ml collection tube (don't take any of the pellet)
13. Add 100 µl solution MD2
14. Vortex the 2 ml collection tube for 5 sec
15. Incubate the 2 ml collection tube for 5 min at 4˚C (refrigerator)
16. Centrifuge the 2 ml collection tube, time=60 sec, RPM=9600
17. Transfer the supernatant in a new 2 ml collection tube (without pellet)
18. Add 900 µl solution MD3 to the 2 ml collection tube with supernatant
19. Vortex the 2 ml collection tube for 5 sec
20. Transfer approximately 700 µl out the 2 ml collection tube with supernatant on a spin filter
21. Centrifuge spin filter, time=30 sec, RPM=9600
22. Take the spin filter (gently) out of the collection tube
23. Discard the flow through
24. Place the spin filter back in the collection tube
25. Transfer the remaining supernatant of step 13 on to the spin filter
26. Centrifuge the spin filter, time=30 sec, RPM=9600
27. Take the spin filter (gently) out of the collection tube
28. Discard the flow through
29. Place the spin filter back in the collection tube
30. Ad 300 µl solution MD4 on the spin filter
31. Centrifuge the spin filter, time=30 sec, RPM=9600
32. Take the spin filter (gently) out of the collection tube
33. Discard the flow through
34. Place the spin filter back in the collection tube
35. Centrifuge the spin filter, time=60 sec, RPM=9600
36. Take the spin filter out of the collection tube and putt this in a new 2 ml collection tube
37. Add 50 µl solution MD5 to the middle of the spin filter
38. Let the tube stand for a few minutes
39. Centrifuge the collection tube with spin filter, time=30 sec, RPM=9600
40. Discard spin filter and store the collection tube with DNA at -20˚C

Appendix 2

Primer pairs used for PCR and sequencing are:

BT |N2|:

BT2a: 5’ – GGT AAC CAA ATC GGT GCT GCT TTC – 3’

BT2b: 5’ – ACC CTC AGT GTA GTG ACC CTT GGC – 3’
RC…. GCC AAG GGT CAC TAC ACT GAG GGT ….

ITS |N9|:

ITS1: 5’ – TCC GTA GGT GAA CCT GCG G – 3’

ITS4: 5’ – TCC TCC GCT TAT TGA TAT GC – 3’
RC…. GCA TAT CAA TAA GCG GAG GA ….

Or

ITS2: 5’ – GCT GCG TTC TTC ATC GAT GC – 3’
RC…. GCA TCG ATG AAG AAC GCA GC ….

ITS3: 5’ – GCA TCG ATG AAG AAC GCA GC – 3’

ITS4: 5’ – TCC TCC GCT TAT TGA TAT GC – 3’
RC…. GCA TAT CAA TAA GCG GAG GA ….

Camodulin |N7|:

CMD5: 5’ – CCG AGT ACA AGG ARG CCT TC – 3’

CMD6: 5’ – CCG ATR GAG GTC ATR ACG TGG – 3’
RC…. CCA CGT RAT GAC CTC RAT CGG ….

Actin |N10|:

ACT-512F: 5’ – ATG TGC AAG GCC GGT TTC GC – 3’

ACT-783R: 5’ – TAC GAG TCC TTC TGG CCC AT – 3’
RC…. ATG GGC CAG AAG GAC TCG TA….

References

Benitah, N. (2006). Canine nasal aspergillosis. *Clinical Techniques in Small Animal Practice, 21*(2), 82-88.

Burbidge, H. M., Clark, W. T., Read, R., Labuc, R., Davey, T., & Broome, C. (1997). Canine nasal aspergillosis: Results of treatment using clotrimazole as a topical agent. *Australian Veterinary Practitioner, 27*(2), 79-83.

Burrow, R., Baker, M., White, L., & McConnell, J. F. (2013). Trephination of the frontal sinuses and instillation of clotrimazole cream: A computed tomographic study in canine cadavers. *Veterinary Surgery, 42*(3), 322-328.

Claeys, S., Lefebvre, J. B., Schuller, S., Hamaide, A., & Clercx, C. (2006). Surgical treatment of canine nasal aspergillosis by rhinotomy combined with enilconazole infusion and oral itraconazole. *The Journal of Small Animal Practice, 47*(6), 320-324. doi:10.1111/j.1748-5827.2006.00154.x

Clercx, C. (2006). Surgical treatment of canine nasal aspergillosis. *The Journal of Small Animal Practice, 47*(7), 416. doi:10.1111/j.1748-5827.2006.00201.x

Field, A. (2009). *Discovering statistics using spss* [Discovering statistics using spss] (third ed.). Oriental Press, Dubai: SAGE Publications.

Friend, E. J., Williams, J. M., & White, R. A. (2002). Invasive treatment of canine nasal aspergillosis with topical clotrimazole. *The Veterinary Record, 151*(10), 298-299.

Hayes, G. M., & Demetriou, J. L. (2012). Distribution and persistence of topical clotrimazole after sinus infusion in normal canine cadavers. *The Journal of Small Animal Practice, 53*(2), 95-100. doi:10.1111/j.1748-5827.2011.01164.x; 10.1111/j.1748-5827.2011.01164.x

Mathews, K. G., Davidson, A. P., Koblik, P. D., Richardson, E. F., Komtebedde, J., Pappagianis, D., . . . Kass, P. H. (1998). Comparison of topical administration of clotrimazole through surgically placed versus nonsurgically placed catheters for treatment of nasal aspergillosis in dogs: 60 cases (1990-1996). *Journal of the American Veterinary Medical Association, 213*(4), 501-506.

Nelson, R. W., Couto, C. G., Grauwer, G. F., Hawkins, E. C., Jhonson, C. A., Lappin, M. R., . . . Willard, M. D. (2009). Nasal mycoses. In R. W. Nelson, & C. G. Couto (Eds.), *Small animal internal medicine* [Small Animal Internal Medicine] (pp. 226-229)

Peeters, D., & Clercx, C. (2007). Update on canine sinonasal aspergillosis. *The Veterinary Clinics of North America.Small Animal Practice, 37*(5), 901-16, vi. doi:10.1016/j.cvsm.2007.05.005

Petrie, A., & Watson, P. (2006). *Statistics for veterinary and animal sience* [Statistics for Veterinary and Animal Sience] (second ed.). UK: Blackwell Publishing.

Pomrantz, J. S., & Johnson, L. R. (2010). Repeated rhinoscopic and serologic assessment of the effectiveness of intranasally administered clotrimazole for the treatment of nasal aspergillosis in dogs. *Journal of the American Veterinary Medical Association, 236*(7), 757-762. doi:10.2460/javma.236.7.757; 10.2460/javma.236.7.757

Quinn, P. J., Markey, B. K., Carter, M. E., Donnelly, W. J., & Leonard, F. C. (2002). Aspergillus species. *Veterinary microbiology and microbial disease* [Veterinary Microbiology and Microbial Disease] (pp. 229-232)

Schuller, S., & Clercx, C. (2007). Long-term outcomes in dogs with sinonasal aspergillosis treated with intranasal infusions of enilconazole. *Journal of the American Animal Hospital Association, 43*(1), 33-38.

Sharman, M., Lenard, Z., Hosgood, G., & Mansfield, C. (2012). Clotrimazole and enilconazole distribution within the frontal sinuses and nasal cavity of nine dogs with sinonasal aspergillosis. *The Journal of Small Animal Practice, 53*(3), 161-167. doi:10.1111/j.1748-5827.2011.01181.x; 10.1111/j.1748-5827.2011.01181.x

Sharman, M., Paul, A., Davies, D., MacKay, B., Swinney, G., Barrs, V., . . . Mansfield, C. (2010). Multi-centre assessment of mycotic rhinosinusitis in dogs: A retrospective study of initial treatment success (1998 to 2008). *Journal of Small Animal Practice, 51*(8), 423-427.

Sharman, M. J., & Mansfield, C. S. (2012). Sinonasal aspergillosis in dogs: A review. *The Journal of Small Animal Practice, 53*(8), 434-444. doi:10.1111/j.1748-5827.2012.01245.x; 10.1111/j.1748-5827.2012.01245.x

Sharp, N., Sullivan, M., & Harvey, C. (1992). Treatment of canine nasal aspergillosis. *In Practice, 14*(1), 27-28.

Sharp, N. J., Sullivan, M., Harvey, C. E., & Webb, T. (1993). Treatment of canine nasal aspergillosis with enilconazole. *Journal of Veterinary Internal Medicine / American College of Veterinary Internal Medicine, 7*(1), 40-43.

Sissener, T. R., Bacon, N. J., Friend, E., Anderson, D. M., & White, R. A. S. (2006). Combined clotrimazole irrigation and depot therapy for canine nasal aspergillosis. *Journal of Small Animal Practice, 47*(6), 312-315.

Zonderland, J. L., Stork, C. K., Saunders, J. H., Hamaide, A. J., Balligand, M. H., & Clercx, C. M. (2002). Intranasal infusion of enilconazole for treatment of sinonasal aspergillosis in dogs. *Journal of the American Veterinary Medical Association, 221*(10), 1421-1425.