Host-pathogen interactions at the sino-nasal surface of dogs

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Host-pathogen interactions at the sino-nasal surface of dogs

Gastheer-pathogeen interacties in het sino-nasale gebied van honden
(met een samenvatting in het Nederlands)

Proefschrift

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LIST OF ABBREVIATIONS

ABPA	Allergic bronchopulmonary aspergillosis
CCPA	Chronic cavitary pulmonary aspergillosis
CNA	Chronic necrotizing aspergillosis
CPA	Chronic pulmonary aspergillosis
CT	Computed tomography
DHN-MELANIN	1,8-dihidroxynaphthalene melanin
ECM	Extracellular matrix
GAG	Galactoaminogalactan
GO	Gene ontology
IA	Invasive Aspergillosis
ICU	Intensive care unit
IL	Interleukin
INF-γ	Interferon gamma
IOE	Invasive otitis media
IPA	Invasive pulmonary aspergillosis
MEA	Malt extract agar
MPO	Myeloperoxidase
NETS	Neutrophil extracellular traps
NIA	Non invasive aspergillosis
NRPS	Non ribosomal peptide synthase
PAMP	Pathogen associated molecular pattern
PDA	Potato dextrose agar
PKS	Polyketide synthase
PRR	Pathogen recognition receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SM	Secondary metabolite
SNA	Sino-nasal aspergillosis
SNP	Single nucleotide polymorphism
SOA	Sino-orbital aspergillosis
TNF	Tumor necrosis factor
TPM	Transcripts per million
URTA	Upper respiratory tract aspergillosis
WGS	Whole genome sequencing

CHAPTER 1

General introduction

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Aspergillus fumigatus

The genus *Aspergillus* belongs to the phylum Ascomycota and consists of at least 350 species [1]. *Aspergillus fumigatus* is one of its representatives. It colonizes a large variety of substrates including soil and compost heaps [2]. Although a sexual state has been described [3] it is not clear how frequent this happens in nature. However, it is well known to reproduce asexually via the production of conidia. These spores disperse through the air over long distances and resist a broad spectrum of stresses [4]. Estimates from air samples indicate that about 10^4 conidia m^{-2} are released per second from a compost pile at a wind speed of 1 m s^{-1} [5], suggesting efficient dispersal due to minimum agitation [6]. As a consequence, between 100-1000 conidia of this fungus are inhaled by humans on a daily basis [7]. In general, they are efficiently cleared by the body but in the case of individuals with a reduced immune system they can cause invasive or non-invasive infections [8].

Biology of *A. fumigatus*

Conidia

Conidia are the main reproductive structures of *A. fumigatus.* These spores are produced on conidiophores and their surface hydrophobicity enable them to be easily dispersed by air [5, 9], water droplets or vectors [10]. Conidia are lowly metabolically active, if at all, and are resistant to drought, high temperature, UV and osmotic stress due to production of protective compounds like DHN-melanin and threhalose [11]. Due to their small size (< 5 μm), they are able to reach the alveoli in the lungs [6], where they may initiate an infection.

The conidial surface is composed of an outer rodlet layer consisting of hydrophobins with RodA being the most important [12-14] (Figure 1A). The rodlet layer confers hydrophobicity to the conidia but also acts as an immunological shield [15] by covering pathogen associated molecular patterns (PAMPs) [16, 17]. The amphipathic conidial protein CcpA also has a role as a conidial stealth protein by shielding PAMPs [18]. Dihydroxynapthalene (DHN)-melanin [19] is found underneath the RodA-CcpA layer and also has a structural role in the conidial cell wall [20]. In addition, it increases survival inside phagocytic cells by inhibition of the acidification of the phagosome [21] and assembly of the NADPH oxidase complex [22]. The rodlet and melanin layers cover an amorphous layer of α-(1,3)-glucan [14] and a complex of β-1,3 glucan, galactomannan and chitin [7].

The first step in conidial germination is swelling (Figure 1B). Swelling is the result of an increase in the osmotic pressure inside the conidium due to uptake of water [14]. When the conidium has doubled its size [13], the rodlet-melanin layer is shedding off followed by the formation of a germ tube [7, 13, 14] (Figure 1C).

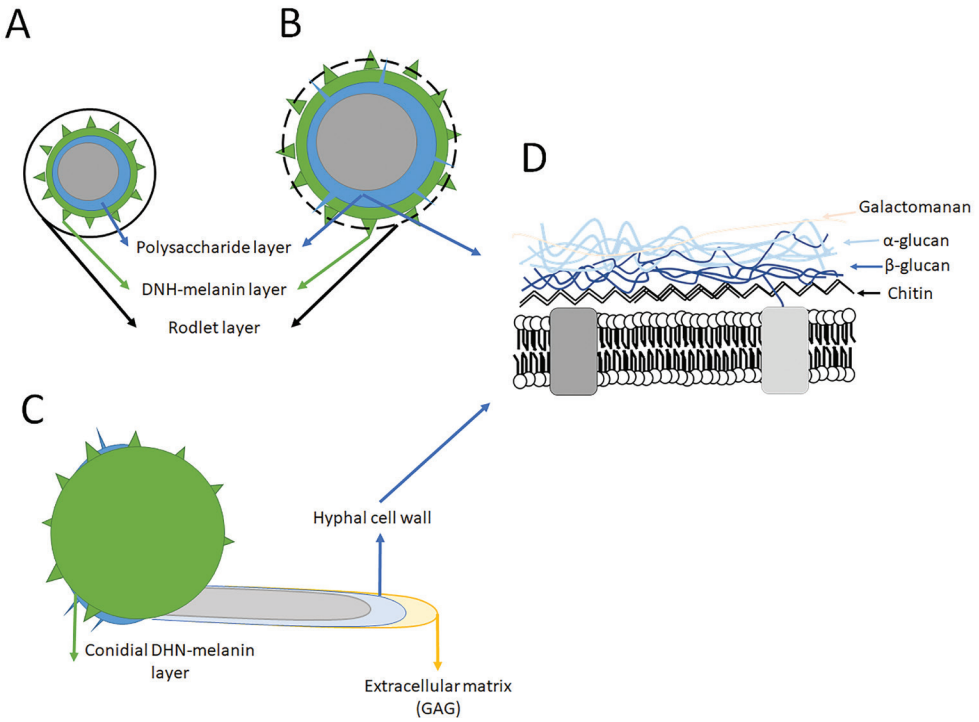

Figure 1. Schematic representation of the composition of the cell wall of *A. fumigatus* conidia and hyphae. A) Resting conidia are covered by outer layers of rodlets and DHN-melanin and an inner layer of polysaccharides (α-(1,3)-glucan, β-(1,3)-glucan, galactomannan and chitin). B) Swelling of the conidium is accompanied by rupture of the rodlet-melanin layer. C) In the case of the emerging germ tube, these outer layer are lost but the cell wall does consist of the same polysaccharides. In addition, the hyphae start to produce an extracellular polysaccharide galactosaminogalactan (GAG). D) Basic scheme of the composition of *A. fumigatus* cell wall.

Mycelium and biofilm

Germination of a conidium results in a network of branching hyphae [7]. The main components of the hyphal cell wall consist of α-(1,3)-glucan, β-(1,3)-glucan, galactomannan, and chitin. Yet, the composition is dynamic [7, 13] depending on the environmental conditions. For instance, hypoxic condition increases the content of β-1,3 glucan and chitin [23]. As the fungus grows, hyphae form a structured mycelial network that is surrounded by an extracellular matrix (ECM) [24] (Figure 1C). ECM of in vitro formed biofilms of *A. fumigatus* grown in RPMI1640 is mainly composed of polysaccharides, proteins, lipids and aromatic compounds. These compounds represent 43%, 40%, 14% and 3%, respectively, of the ECM. Galactosaminogalactan (GAG) and α-1,3 glucan are the main ECM polysaccharides, while catalase CatB and the allergen Aspf2 are the most abundant proteins [25]. In addition, DNA has been identified as a component of the biofilm [24, 26, 27]. Like the cell wall, composition

of the biofilm is affected by environmental conditions. For example, the biofilm produced in invasive aspergillosis (IA) is relatively thin and lacks α-glucan. In contrast, biofilms produced in non-invasive aspergillomas (NIA) do contain this polysaccharide [28]. Biofilm composition has important implications in the development of infection. For instance, GAG provides a protective coat against neutrophil attack [29], hides PAMPs like β-1,3 glucan [30], and can dampen the immune inflammatory response [31].

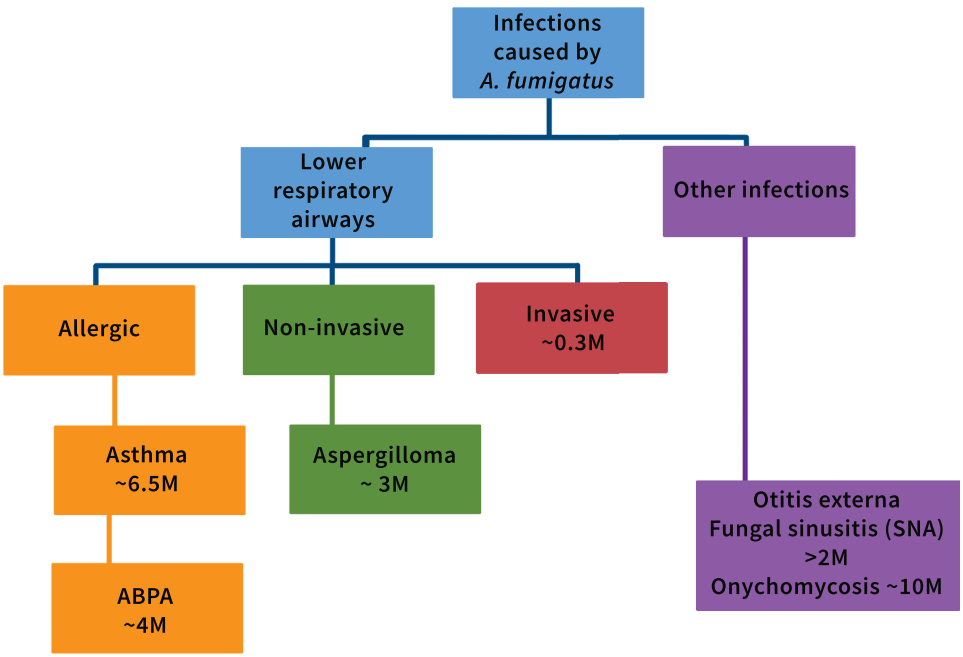

Figure 2. Type of infections caused by *A. fumigatus* with the number of million (M) cases annually [32].

Infections caused by *A. fumigatus*

Aspergillus fumigatus can cause various types of infections. They mainly occur in the lower part of the respiratory system (lungs) but can also take place in other parts of the body such as sinus, ear, eye and skin [6]. The classification in allergic aspergillosis, invasive aspergillosis (IA), and non-invasive aspergillosis (NIA) is based on the type of fungal colonization and the immune response of the host (Figure 2). Recent studies suggest that transmission *of A . fumigatus* via aerosols from cystic fibrosis patients is possible [33].

Allergic aspergillosis

Exposure to and colonization by *A. fumigatus* can elicit an exaggerated allergic reaction. This is characterized by the production of immunoglobulin E (IgE) and degranulation of eosinophils and mast cells [31, 41]. Allergic bronchopulmonary aspergillosis (ABPA) occurs in patients suffering from asthma with bronchospasm, fatigue, pneumonia and production of mucus plugs as presenting symptoms [42]. Management of the underling disease (i.e. asthma) plus antifungals is the standard therapy.

Invasive aspergillosis

IA occurs mostly in individuals that are immunocompromised [32, 34]. It is characterized by a high degree of fungal invasion of host tissue, inflammation and dispersal of the pathogen through the vascular system. IA has a high rate of mortality (58-94%) [32]. Management of disease includes the treatment with triazoles (itraconazole, voriconazole and posaconazole) that inhibit the ergosterol biosynthesis enzyme 14-α-demethylase (CYP51A). Use of azoles in the indoor environment and in agriculture has been proposed to result in resistance to these compounds [35, 36]. Tandem repeats in the promoter of the gene encoding CYP51A, mutations in the coding sequence of this gene and overexpression of efflux pumps (Cdr1B) are the most frequent examples of reported mechanisms of azole resistance in *A. fumigatus* [37]. Amphotericin B or echinocandins are used in case an azole resistant strain has infected a patient. These antifungal compounds affect cell membrane permeability and cell wall synthesis by binding to ergosterol and inhibiting β-1,3 synthase, respectively [38].

Non-invasive infections

Aspergilloma

Aspergillomas or fungal balls are a mixture of fungal hyphae with host-derived products like mucus [39]. This type of infection typically develops within lung cavities without invasion of the host tissue [40]. However, under certain conditions like neutropenia, aspergilloma can develop into IA [41]. Administration of itraconazole or voriconazole and surgical removal in the case of hemoptysis are typical treatments of aspergilloma [42].

Chronic pulmonary aspergillosis

Like aspergillomas, chronic pulmonary aspergillosis (CPA) is characterized by slow progression of the disease. In contrast to aspergilloma, it can develop in multiples cavities and is usually accompanied by inflammation, fibrosis, pleural thickening and infiltration [43, 44]. Host risk factors like mild immunosuppression or old

lesions in the lungs caused by prior pathologies like tuberculosis can worsen CPA into chronic cavitary pulmonary aspergillosis (CCPA), or chronic necrotizing aspergillosis (CNA) also known as semi-invasive aspergillosis. Oral administration of voriconazole is the usual treatment of choice for CPA [42].

Fungal sinusitis and sino-nasal aspergillosis

Fungal sinusitis occurs in the sino-nasal area including frontal sinus. It presents itself as an invasive or non-invasive form [45], both of which are mainly found in immunosuppressed patients [45]. Like IA, acute fungal sinusitis is characterized by vascular invasion of the fungus. In some cases the infection can spread to cheek and orbit of the eyes causing proptosis [46]. Noninvasive fungal sinusitis can be accompanied by inflammation of the surrounding tissue and erosion of the accompanying bone [46, 47]. A combination of antifungal treatment similar to pulmonary infection, removal of the fungal plaque and topical steroids to control inflammation are the choice of therapy [47].

Other aspergillus infections

Although rare, ear infections such as invasive otitis externa (IOE) can be caused by *A. fumigatus* [48]. IOE spreads from the auditory canal to adjacent tissue such as bone and cartilage causing loss of hearing, ear discharge, and tissue necrosis [48]. Usual treatment includes removal of the affected tissue and antifungal therapy [49]. *A. fumigatus* can also cause onychomycosis. Typical symptoms are discoloring and thickening of the nail [50]. Terbinafine and pulsed itraconazole are effective treatments for this type of infections [51].

Immune response to *A. fumigatus*

Several lines of defense have evolved to prevent infection of the host by fungi like *A. fumigatus*. Physical barriers like skin and epithelial layers are the first line of defense. Epithelial cells also produce antimicrobials and mucus and in the lung cilia facilitates muco-ciliary clearance of the upper respiratory area. Natural microbiota provide additional protection at these host surfaces [52]. The defense at epithelial layers is further strengthened by the innate and adaptive immune systems. These systems work together with the epithelial cells to form the mucosal immune system [52]. The innate system comes into action as soon as pathogens are detected. Activation and swelling of *A. fumigatus* conidia results in exposure of PAMPs like β-glucan and chitin. These molecules are recognized by pathogen recognition receptors (PPR) like TLR2 and TLR4 [7, 12], C-type-lectin Dectin-1 [53], and MelLec [54]. These receptors are mainly expressed by 'professional' tissue-resident phagocytic cells as well as epithelial cells [55, 56]. Macrophages, neutrophils and dendritic cells are the most prevalent innate immunity cells responding to *A. fumigatus* infections in

mammals [55]. TLR2 and TLR4 have been described to recognize β-glucan of several species [57]. However, in the case of *A. fumigatus* their probable ligands are chitin and α-glucan, respectively [55]. β-glucan present on swollen and germination conidia are generally recognized by the C-type-lectin PRR Dectin-1[53], which is required for optimal early defense in the lungs [58, 59]. However, Mac-1 (CD11b/CD18, complement receptor 3) and not Dectin-1 was shown to be involved in conidial recognition by human neutrophils [60]. Recently, MelLec has been identified as the PRR that recognizes DHN-melanin [54]. Activation of PRRs by PAMPs results in cytokine and chemokine production and recruitment of other types of immune cells [61, 62]. Following recognition, conidia are internalized by phagocytic cells into compartments called phagosomes. Oxidative and non-oxidative mechanisms result in intracellular killing [63, 64]. Phagosomal acidification is an important step in this process. This is inhibited by DHN-melanin, thus preventing the activation of the hydrolytic enzymes in the phagosome [55].

Neutrophils are recruited to the site of infection in response to chemokines like IL-8 [65, 66]. They also play an important role in killing of the pathogen. Besides phagocytosis of conidia, neutrophils inhibit growth of hyphal structures that are too large to ingest. To this end, they undergo a special type of programmed cell death. As a result, they form neutrophil extracellular traps (NETs) composed of their nuclear and granular constituents, such as calprotectin and lactoferrin as well as myeloperoxidase (MPO) [67, 68]. Calprotectin has zinc and manganese sequestering properties and therefore contributes to the growth inhibiting properties of NETs that are in contact with hyphae [67, 69]. MPO is required to convert peroxide to hypochlorous acid, which also inhibits growth [60]. Moreover, neutrophils secrete cytokines and chemokines upon activation thereby also potentiating the immune response for instance by attracting more immune cells to the site of infection [70, 71].

The adaptative response acts slower as the innate response but is more specific and creates immunological memory [72]. The link between these two responses are dendritic cells who survey the environment for pathogens. When exposed to fungi or fungal antigens, dendritic cells will present antigens to naïve T cells in lymphoid tissue [73]. Antigen presentation in concert with co-stimulatory cytokines are required to induce the development of naïve T-cells into different lineages (i.e. Th1, Th2, Th17,Treg). Each of them play a role in *A. fumigatus* infection [74]. The outcome is the result of an interplay of these T-cell responses. Murine models of chronic airway colonization [75] suggest that *A. fumigatus* interferes with this interplay resulting in pathogenesis [76]. Th1 cells [77] are characterized by the production of INF-γ, IL-12 and IL-18 [78]. These molecules function in pathogen killing [79] via several mechanisms. For instance, they increase the recruitment and activity of professional phagocytes [80]. Th1 response is required for clearance of *A. fumigatus* infections [74]. Allergy is mostly related with a Th2 response and participate in fungal persistence [74] by suppression of the antifungal Th1 response via secretion of IL-4 [81]. Th17 cell differentiation requires amongst others interleukins IL-1, IL-23 and IL-6

[74, 76]. In turn, these T cells secrete IL-17A, IL-17F and IL22 [76] that recruit neutrophils and induce the production of antimicrobial peptides [82]. The Th17 response has a role in fungal immune defense but can also negatively impact the host due to a detrimental inflammatory response [74]. The Th17 response has been shown to be necessary for immunity in *Candida* models of infections [83]. Yet, its role in *A. fumigatus* infections is not yet clear. This is illustrated by the fact that different results have been obtained in mouse and human infections concerning the protective role of IL-17 [56, 84]. Finally, Treg (or T- regulatory cells) has a role in controlling an over-reactive inflammatory response and immune pathology by the release of anti-inflammatory cytokines such as IL-10 and TGF-β [85]. This is important to reduce allergy and inflammation caused by the fungal allergens and the strong initial Th1 response [74]. For example, activation of the Treg response in a healthy human patient, is crucial to avoid immune dysregulation due to *A. fumigatus* [86, 87].

Defense mechanisms of *A. fumigatus*

Conidia and hyphae of *A. fumigatus* contain a variety of components that have a role in the defense against the immune system (Table 1). As mentioned above, DHN-melanin blocks acidification of phagosomes thereby avoiding intracellular killing due to pH stress [21, 88, 89]. On the other hand, fungal catalases (CatA, Cat1, Cat2) and superoxide dismutases (SOD1, SOD2, SOD3) [6] withstand ROS stress imposed by macrophages and neutrophils. Changes in the concentration of metals results in nutrition stress in the pathogen [90]. As a response to limiting iron concentration, the siderophores fusarinine C (FsC) and triacetylfusarinine C (TAFC) are secreted [91]. Similarly, pH dependent transporters are formed by the fungus to capture zinc from the host [89]. Metal concentration may also be increased by the host. A high concentration of the transition metal copper is used by macrophages to generate hydroxyl radicals [92, 93]. *A. fumigatus* uses a copper exporter (CrpA) to pump out the excess of copper from the fungal cell to counteract this killing mechanism [93].

Apart from DHN-melanin and siderophores also other secondary metabolites (SMs) contribute to survival and pathogenesis. Generally, these metabolites are formed by polymerization and modifying primary metabolites like acetyl-CoA (PKS systems) or amino acids (NRPS systems) [94]. Gliotoxin belongs to the epipolythiodioxopiperazine (ETP) family [95]. Many of its effects are due to a disulfide bond that can undergo cycles of reduction and oxidation [96, 97]. This redox activity makes that gliotoxin can inactivate proteins by conjugation to thiol groups, can deplete the glutathione pool and cause cell death [96]. Gliotoxin is immunosuppressive due to killing of cells such as macrophages [98] and T cells [99], interference with NF-κβ activation [56] and inhibition of H_2O_2 production in neutrophils via inhibition of NADPH complex assembly [100].

Table 1. Examples of survival mechanisms of *A. fumigatus*.

Class	Type	Description	Effect	References
Biophysical	Thermal response	Ability to survive and react to high temperatures	Quick colonization into mammalian host	[6, 101]
	Conidial rodlet layer	Hydrophobic proteins layer that covers the conidia	Makes the conidial immunological inert	[17]
	Galactosaminogalactan (GAG)	Hyphal polysaccharide that covers hyphae and biofilm	Dampens the immune response via indication of the interleukin receptor agonist IL-1RA	[31]
Interfering agents	DHN-melanin	Secondary metabolite that gives the conidia its characteristic green-greyish color	Avoid acidification of the phagosome, affects endocytosis and ROS activation	[21, 88]
	Gliotoxin	Secondary metabolite	Affect the glutathione pool in the host cell, kill immune cells, inhibition of the NF-κβ pathway, inhibition of H_2O_2 in the immune cells	[96]
	Fumagillin	Secondary metabolite	Reduction of phagocytic activity of immune cells, damage of epithelial cells	[131, 132]
Enzymatic	Catalases	Enzyme that converts hydron peroxide into water and oxygen	Protection from H_2O_2 produced by immune cells	[6]
	Superoxide dismutases	Enzyme that converts superoxide radical into hydrogen peroxide	Protection from superoxide radicals produced by immune cells	[6]
Nutritional	Siderophores	Secondary metabolites that sequestrate the available iron in the environment	Captures iron that can be uses for other metabolic process (i.e cofactor of catalases)	[91]
	Metal transporters	Transporters that pump in or out scare or toxic metals into the fungal cell	Captures metals that can be uses for other metabolic process (i.e zinc), or detoxify the cell from toxic concentrations (i.e copper)	[93, 102]

Infections caused by *A. fumigatus* in the veterinary context

Infections with *A. fumigatus* occurs in a wide variety of animals including birds, cows, horses, cats and dogs [103, 104]. Like in humans, the development and outcome of *A. fumigatus* infections depends on the immune status of the animal [103, 104]. Most infections reported in warm-blooded animals are related with IA. In dogs, sino-nasal aspergillosis (SNA) is the most common *Aspergillus* infection [105]. Mesocephalic, dolichocephalic and immunocompromised dogs are more prone to develop the disease [106]. The fungus can reside in the host for an extended period of time (e.g. months) before manifesting symptoms. Symptoms include sneezing, mucoid nasal discharge, nasal depigmentation, and turbinate destruction [107, 108]. This relative long build-up time facilitates the fungus to form a fungal biofilm covering the sino-nasal area up to the frontal sinus [109] before the disease is diagnosed [108]. Proper diagnosis of SNA requires a combination of computed tomography (CT), rhinoscopy, histopathology, cytology, fungal cultures and serology [108]. Topical administration of azole remains the most widely used and most successful treatment for SNA in dogs [105, 108]. This technique allows for direct administration and therefore immediate effect on fungal plaques. Yet, additional treatments like chirurgical removal of the plaque and trephination of the frontal sinuses in combination with antifungal are frequently required [105, 110].

SNA has been associated with up-regulation of Th1 cytokines like IL-8 and TNF-α [111-113]. This may indicate a localized response to the fungus to restrain the dispersal and development of an invasive infection. Yet a high level of inflammation and incapability to clear the infection might be due to the up-regulation of anti-inflammatory molecules such as TGF-β and IL-10 [111, 113]. Additional research is required to uncover additional host and pathogen factors related with the development of this chronic disease.

Scope of this thesis

In contrast to IA, much less is known of non-invasive *A. fumigatus* infections, especially chronic non-invasive sino-nasal infections. The aim of this Thesis was to understand host-pathogen interactions during sino-nasal aspergillosis in dogs. This knowledge is expected to contribute to the understanding of similar infections in human (i.e. non-invasive chronic fungal sinusitis) [106] and the biology of the fungus and host response, in general.

Chapter 2 shows that dog are colonized by a single genotype, while different genotypes are found in human individuals infected by *A. fumigatus.* Phenotypic characterization demonstrated that the fungal isolates from individual dogs have a high degree of phenotypical heterogeneity indicating in-host adaption of the fungus during infection.

Fungal gene expression was studied to understand the biology of *A. fumigatus* in the sino-nasal area (**Chapter 3)**. Transcriptomic profiles were obtained of four fungal plaques derived from three different dogs. These transcriptomes shared a set of genes including those of the SMs gliotoxin, neosartoricin and hexadehydroastechrome. In addition, genes were found to be expressed that counteract oxidative stress and nutritional immunity and that encode immunomodulatory molecules such as prostaglandin. Together, these data indicate that fungal plaques have several strategies to survive and counteract the immune response of the host.

Chapter 4 describes the expression profile of the host tissue impacted by SNA. This revealed a dominant proinflammatory response. Ranking of expressed genes showed high expression of interleukin 8 (IL-8) followed by proteins of the S100 family and iron metabolism. These results indicate activation of inflammatory responses and nutritional immunity by changing the concentrations of metals like iron, zinc, and copper at the host-pathogen interface. Notably, results showed the absence of the Th17 response since IL-17 and IL-23 were found not to be expressed. The results indicate a dysregulation of the antifungal Th17 response possibly due to formation of immunomodulatory compounds like kynurenine by expression of IDO1.

Culturing fungal isolates under various stress conditions showed large phenotypical variability between fungal isolates from a single dog patient (**Chapter 5**). This was associated with genomic changes (SNPs). Some of the fungal isolates from dogs contained 2.5 times more SNPs than other strains from dogs or from the indoor environment. This increase in SNPs correlated with mutations in genes encoding components of the DNA repair system. Moreover, non-synonymous SNPs were found to impact genes related with secondary metabolism, copper detoxification and oxidative stress. Together, data indicate that the sinus environment of the dog triggers in host adaptation of *A. fumigatus*.

Results are summarized and discussed in **Chapter 6**.

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Chapter 2

Comparative genotyping and phenotyping of *Aspergillus fumigatus* isolates from humans, dogs and the environment

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ABSTRACT

Aspergillus fumigatus is an ubiquitous saprotrophic fungus and an opportunistic pathogen of humans and animals. Humans and animals can inhale hundreds of *A. fumigatus* spores on a daily basis. Normally this is harmless for humans, but in case of immunodeficiency, invasive pulmonary aspergillosis (IPA) can develop with a high mortality rate. *A. fumigatus* also causes non-invasive mycoses like sino-nasal aspergillosis (SNA) in dogs. In this study we compared *A. fumigatus* isolates from humans with suspected IPA, dogs with SNA, and a set of environmental isolates. Phylogenetic inference based on calmodulin (*CaM*) and beta-tubulin (*benA*) sequences did not reveal *A. fumigatus* sub-groups linked to the origin of the isolates. Genotyping and microsatellite analysis showed that each dog was infected by one *A. fumigatus* genotype, whereas human patients had mixed infections. Azole resistance was determined by antifungal susceptibility testing and sequencing of the *cyp51A* gene. A total of 12 out of 29 human isolates and 1 out of 27 environmental isolates were azole resistant. Of the azole resistant strains, 11 human isolates showed TR_{34}/L98H n=6; TR46/Y121F/T289A n=5 Phenotypically, isolates from dogs were more variable in growth speed and morphology when compared to those isolated from human and the environment. The relationship between the observed differences and evolution during the infection process are discussed.

INTRODUCTION

Aspergillus fumigatus is a filamentous saprotrophic ascomycete that occurs worldwide [1]. This fungus produces large numbers of conidia that are released into the air [2-5]. Estimates indicate that humans can inhale a few hundred of these asexual spores of *A. fumigatus* a day [2, 6]. Their small diameter of 2-3 µm and hydrophobic surface enables them to reach the alveoli [7]. Yet, most of them are trapped and removed by the mucociliary escalator or by immune cells present in the lung tissue. Upon germination, they can cause invasive or non-invasive forms of aspergillosis. The former is characterised by the penetration and infection of tissues [8]. *A. fumigatus* has been shown to secrete mycotoxins like gliotoxin, verruculogen, fumagillin and helvolic acid, which can affect ciliary beat frequency and promote colonization of lung epithelium [9, 10]. Invasive aspergillosis most often occurs in individuals with impaired cell-mediated immunity [8] Additionally this fungus is able to cause mycosis in animals such as cats and dogs [11]. For instance, it is responsible for most cases of sino-nasal aspergillosis in dogs (SNA) [12]. Canine SNA is clinicopathologically similar to chronic erosive non-invasive fungal sinusitis in humans [13]. It is characterized by the formation of fungal plaques (i.e. fungal biofilms) in the nasal cavity and frontal sinus. Other than in some invasive forms of aspergillosis in humans [14], no evidence of impaired innate or adaptive immune responses have been described for dogs suffering from SNA [15, 16]. Notably, mesocephalic and dolichocephalic dogs seem to be affected with a higher frequency as compared to brachycephalic dogs for reasons that remain elusive but which could be related to a smaller sino-nasal surface area of the latter [13]. In cats *A. fumigatus* is one of the causative agents of upper respiratory aspergillosis (URTA). URTA in cats can be divided into sino-nasal aspergillosis (SNA) and sino-orbital aspergillosis (SOA) of which SOA is the most common condition. SNA in cats resembles SNA in dogs and is caused primarily by *A. fumigatus* but also by *Aspergillus niger*. SOA, on the other hand, is most commonly caused by *Aspergillus felis* sensu lato [17]. Still, these infections in humans, dogs and cats are, like most fungal infection in mammals, relatively uncommon, especially considering the ubiquity of *A. fumigatus* conidia in the environment.

Fungal infections are treated with a range of antimycotic compounds. The antifungal activity of azoles is based on the binding to ergosterol synthase. Both *cyp51A* and *cyp51B* of *A. fumigatus* encode for this lanosterol 14α-demethylase. Azole resistance induced mutations have been described mainly in *cyp51A* [18-20]. These mutations can involve tandem repeats in the promotor area in combination with point mutations in the cyp51A gene. Of these, the TR_{34}/L98H [21, 22] and TR_{46}/Y121F/T289A [23, 24] are most frequently reported with an incidence of up to 20% % in the Netherlands depending on the site of isolation of the resistant strain [25]. Additionally it has been suggested that about 10% of azole resistant strains that does not possess a mutation of the *cyp51A* gene can be explained by increased activity in ABC transporters that helps to detoxificate the fungal cell from azoles, or mutation in other genes like *hapE* [20, 26, 27].

Here we performed a comparative phenotypic and genetic analysis of *A. fumigatus* isolates from dogs with SNA, human patients suspected of having invasive *A. fumigatus* infection from a well-defined group of high-risk patients from the haematology ward and the intensive care unit (ICU), and isolates from indoor and outdoor substrates. Phylogenetic inference based on calmodulin (*CaM*) and beta-tubulin (*benA*) sequences did not reveal *A. fumigatus* sub-groups but dog isolates were more variable in growth speed and morphology. Possibly, *A. fumigatus* evolves within the dog during the infection process by mutations and / or epigenetic variations.

MATERIALS AND METHODS

Fungal isolates and culturing

A total of 87 isolates of *A. fumigatus* were used (Table 1; Table S1) that were derived from the environment (n=27), infected dogs (n=27), and humans (n=29). *A. fumigatus* var. *ellipticus* type-strain [28], *A. fumigatus* Af293, and *A. fumigatus* Af1163 were included as reference strains. *A. fumigatus* isolates were originated from 9 dogs (Canine patient CP1-8.2, Supplementary table S1) suffering from SNA presented at the Faculty of Veterinary Medicine in Utrecht between 2010-2012. One dog (CP8) was presented twice at the hospital due to a reinfection after 2 years (CP8.2 with isolates DTO 303-F1 and -F2, Table S1). Hospitalized canine patients suspected to suffer from SNA were subjected to computer tomography (CT), and endoscopic/rhinoscopic, and haematological analysis. Clinical signs specific of SNA are unilateral or bilateral mucopurulent nasal discharge, depigmentation of the nasal planum, an increased airflow through the nostrils, and an enlarged ipsilateral mandibular lymph node. In the case a fungal plaque was detected (Supplementary table S1), tissue and blood samples were sent to the Veterinary Pathology Diagnostic Center and the Veterinary Diagnostic Laboratory of Utrecht University. Haematocrit, leucocytes, blood cell differentiation, total protein, albumin and the protein spectrum was measured in the blood samples.

Fungal plaques were obtained via trephination of the frontal sinus and/or rhinoscopic removal in the nasal sinus with a 30 degrees 2.7 mm rigid endoscope and using a combination of suction and a metal hook. Trephination was performed if mycotic plaques in the frontal sinus were diagnosed by CT. After removal of the fungal plaques the nasal sinus was flushed in most cases with clotrimazol for 15 min each in ventral, left and right lateral, and dorsal recumbency, followed by flushing for 15 min in ventral recumbency, after which the clotrimazol was allowed to leave the nasal sinus by gravity (head down). Part of the fungal plaque was immediately frozen in liquid nitrogen and stored at -80°C. Remaining material was placed on ice and used for microbial culturing and histological analysis. Culturing was started at the same day on potato dextrose agar (PDA, Becton Dickinson, Le-Pont-De-Claix, France) and care was taken to inoculate plates with different pieces of fungal plaques of the same dog. Colonies incubated for 2 days at 37 °C presenting macro-

scopical characteristics of *A. fumigatus* were sub-cultured on PDA plates using vegetative mycelium from the outer part of the colony as an inoculum. Conidial suspensions were made as described [29].

Table 1. Overview of *A. fumigatus* isolates.

	Number of isolates	Provided by	Country of origin	Reference/Year
Environmental	23	Westerdijk Institute Utrecht	The Netherlands and Germany	2005-2008
	4	Westerdijk Institute Utrecht	Ireland	[4], 2005
Canine isolates	27	Clinical Sciences of Companion Animals, Veterinary Medicine, Utrecht University	The Netherlands	2010-2012
Human isolates	29	University Medical Centre Utrecht	The Netherlands	[30], 2011-2013
***Aspergillius fumigatus* Af293 (CBS 126847)**	1	Westerdijk Institute Utrecht	UK	[51], 1993
***Aspergillius fumigatus* A1163**	1	Westerdijk institute Utrecht	France	[52], 2008
Aspergillius fumigatus* var. *ellipticus	1	Westerdijk Institute Utrecht	USA	[28], 1965

Isolates from *A. fumigatus* were obtained from 9 high-risk patients from the intensive care and haematology units of the Utrecht University Medical Center (UMCU) who were suspected to have developed invasive aspergillosis (patients HP1-9; Supplementary table S1) but none of them had received prophylactic antifungal therapy. These isolates were obtained between 2011 and 2013 from sputum, bronchoalveolar lavage, or pleural fluid (Supplementary table S1; [30]). The human isolates were provided as colonies on Malt Extract agar (MEA) plates. In order to eliminate the possibility of having more than one isolate per plate, colonies were sub-cultured on PDA plates using vegetative mycelium from the edge and the centre of the colonies as inoculum. Plates were inoculated by tracing a double zigzag pattern with a plastic inoculating loop. After incubation at 37°C for 3 days, plates were inspected for the presence of colonies with different morphology. Clearly distinct colonies were treated as different isolates. Conidial suspensions were prepared as described [29].

Phenotypic characterization of fungal isolates

Phenotypic characterization was performed on creatine sucrose agar (CREA), Czapek yeast agar (CYA), CYA with 5% NaCl (CYAS), dichloran 18% glycerol agar (DG18), malt extract agar (MEA), yeast extract sucrose agar (YES) as recommended [29]. A 3-point inoculation was used and plates were incubated lid-side up at 25°C for a period of 7 days in the dark. Diameter of colonies was analysed by ImageJ [31] after taken photographs from a fixed height with a Canon camera with a telephoto lens. They were subjected to PCA and k-means clustering with k=3 corresponding to the initial number of groups (i.e. indoor and outdoor substrates, dog, and human). In addition, colour of the medium underneath the colony, spatial distribution of conidia formation, and morphology of the conidiophores was analysed. Pictures of conidiophores and conidia were made as described [29]. Mycelium was scraped of colonies that had been grown on MEA for 7 days. This mycelium was mounted on an object glass in a drop of lactic acid and a very small drop of ethanol (70%) was used to flush the excess conidia outward. Samples were studied using Olympus BH2 and Zeiss Axioskop microscopes.

DNA isolation, sequencing, genetic and phylogenetic analysis

Genomic DNA was isolated with the UltraClean Microbial DNA Isolation kit (MO BIO Laboratories, Solana Beach CA) and used to PCR amplify fragments of calmodulin (*CaM*), β-tubulin (*benA*), and the mating type loci *MAT1-1* and *MAT1-2* (Table 2; [29]). Sequencing of the reverse and forward strands was performed using the Big Dye® Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). Products of the sequencing reactions were purified using Sephadex G-50 gel filtration (GE Healthcare, Little Chalfont, Buckinghamshire, UK), equilibrated in double-distilled water, and analyzed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The *CaM* and *benA* sequences of the isolates and *A. niger* NRRL326, *A. clavatus* NRRL1, *A. felis* CBS130245, *A. lentulus* NRRL35552, *A. fumigatus* NRRL5109, *A. fumigatus* NRRL164, *A. fumigatus* NRRL163, and *N. fischeri* NRRL181 were used to construct a phylogenetic inference. Phylogenetic trees were constructed using the ETE Toolkit pipeline [32] and visualized with Figtree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Microsatellite analysis

Microsatellite genotyping was performed using the STR*Af* assay as described [33]. All nine markers data were imported into R package poppr [34] to construct a minimum spanning tree. Bruvo distance was used to determine genetic diversity between isolates [35].

TR profiling and microdilution assay

TR_{34} and TR_{46} repeat analysis within *cyp51A* was performed using qPCR as described [36], additional information about the sequence of the cyp51A was available for human isolates (Supplementary table S1). Microdilution assays to determine Minimum inhibitory concentrations (MIC) of itraconazole (Santa Cruz Biotech, Dallas, USA), posaconazole (MSD, Kenilworth, USA) and voriconazole (Pfizer, New York, USA) were performed according to EUCAST (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9_3_Mould_testing_definitive.pdf).

Table 2. Primers and temperature conditions used for PCR [29].

Locus	Primer	Annealing temp (°C)	Cycles	Direction	Sequence (5'-3')	Reference
CaM	CMD5	55	35	Forward	CCGAGTACAAG GARGCCTTC	[53]
	CMD6	55	35	Reverse	CCGATRGAGGT CATRACG TGG	
BenA	Bt_2a	55	35	Forward	GGTAACCAAAT CGGTGCTGTTC	[54]
	Bt_2b	55	35	Reverse	ACCCTCAGTGT AGTGACCCTTG GC	
MAT1-1	AFM1_F65655 MAT1_R6215	48 48	30 30	Forward Reverse	CCTYGACGMGA TGGGITGG TGTCAAAGARTC CAAAAGGAGG	this study
MAT1-2	MAT2_F6086 MAT2_R6580	48 48	30 30	Forward Reverse	TCGACAAGATCA AAWCYCGTC CTTYTTGARCTC TTCYGCTAGG	this study

RESULTS

Histology and haematological analysis

CT scan and rhinoscopy confirmed sinonasal mycosis in 9 dogs presented at the Veterinary hospital at Utrecht University. Histopathological biopsies showed non-invading hyphae on the mucosal surface. The lamina propria was not invaded (Supplementary figure. S1). Conidiophores were rarely observed, which is consistent with the observation that fungal plaques in the nasal cavities were white (Supplementary figure S1B). The lamina propria was infiltrated by large amounts of lymphocytes, plasma cells, and neutrophil granulocytes. Haematological analysis showed differences in the levels of blood albumin in all dogs. 4 dogs showed signs of mild blood loss in the weeks before the treatment, which could explain the hypoalbuminemia. Together, all 9 dogs suffered from a chronic lymphoplasmacellular rhinitis with fungal hyphae and ulcerations.

Genotyping and phylogeny

A phylogenetic tree of the *A. fumigatus* isolates was constructed using the *benA* and *CaM* genes (Supplementary figure S2). In 4 out of 87 isolates only one of these genes was amplified successfully (Supplementary table S1). The tree based on *CaM* (Supplementary figure. S2) showed no subgroups of isolates. They all grouped with *A. fumigatus* Af293 and NRRL 163 (Supplementary figure S2, left) but not with the emerging fungal pathogen *A. felis* [17] (Supplementary figure S2). The same result was obtained when the tree was constructed with *benA* sequences (Supplementary figure S2, right), indicating that these markers are unable to distinguish between the set of isolates used in this study.

Fungal isolates from an individual dog were of the same mating type, while both mating types were present in individual human patients (4 out of 9). The 9 infected dogs had either isolates with *MAT1.1* (4) or *MAT1.2* (5). The fungal isolates from humans (15 isolates having *MAT1.1* and 12 isolates having *MAT1.2*) and indoor and outdoor substrates (12 isolates having *MAT1.1* and 13 isolates having *MAT1.2*) also showed a ratio close to 1. Because of the inability of the previous markers to differentiate between isolates, genetic heterogeneity was further analyzed by microsatellite analysis using the STR*Af* assay. A total of 59 *A. fumigatus* genotypes were found within the 87 isolates with a genetic distance between the isolates ranging between 0.056 and 0.496 (Figure 1). Most of the isolates were found to be of a unique distant genotype. In the case of indoor and outdoor substrates only two genotypes were represented by more than one isolate. Similarly, most of the isolates from a human individual were of a different genotype (Figure 2). Moreover, genotypes were different between human individuals except for genotype 10 (Table S1) which was found in two human patients. In contrast, the 27 dog isolates from the 9 dogs grouped only in six clusters. The isolates derived from an individual dog all had the same genotype (Figure 2, Table S1).

Phenotyping of *A. fumigatus* isolates

Most isolates showed a colony and conidiophore morphology typical for *A. fumigatus*(Figure 3). However, the isolates from dogs were phenotypically more diverse as compared to those from the environment or humans (Tables 3 and 4). Variation was also observed between isolates from a single dog. This was the case for dogs CP1, CP2, CP4, CP7 and CP8.2. Conidiophore morphology was atypical in 12 out of 27 isolates from dogs. Furthermore, whitish colonies with concentric green rings of conidia forming conidiophores were observed in 12 out of the 27 fungal isolates (Figure 3). This phenotype was associated with reduced production of conidia as compared to the fungal isolates from human patients and outdoor en indoor substrates.
Acidification of CREA medium was not observed in the case of human isolates and the reference strains but was detected in 7 out of 27 fungal isolates from dogs and 12 out of 27 environmental isolates (Table 3 and Table S1).

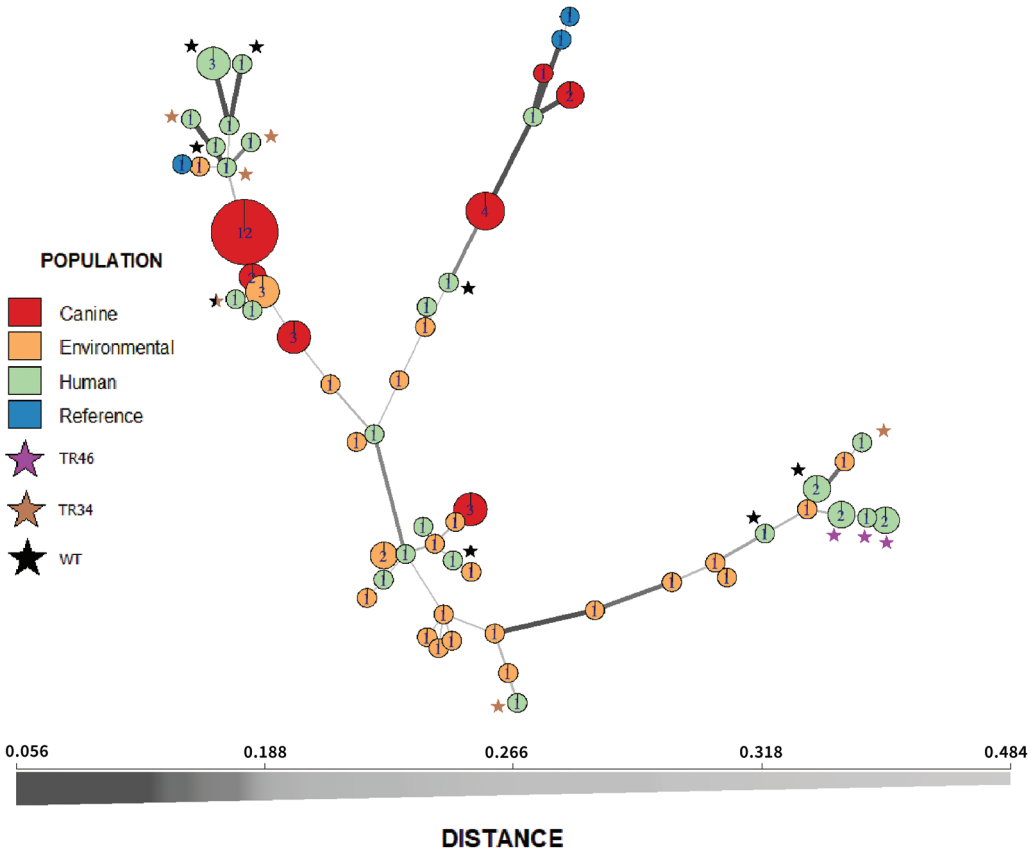

Figure 1. Minimum spanning tree of *A. fumigatus* isolates. Circles indicate different genotypes, numbers inside the circles indicate the number of isolates belonging to that particular genotype, thickness of the line indicates the relatedness of the connected isolates determined by Bruvo distance; values close to zero indicate identical isolates, while values close to 1 indicate unrelated isolates [35]. Additionally, TR mutations present in the isolates are depicted as color-coded stars. The black star (WT) refers to azole sensitive phenotype.

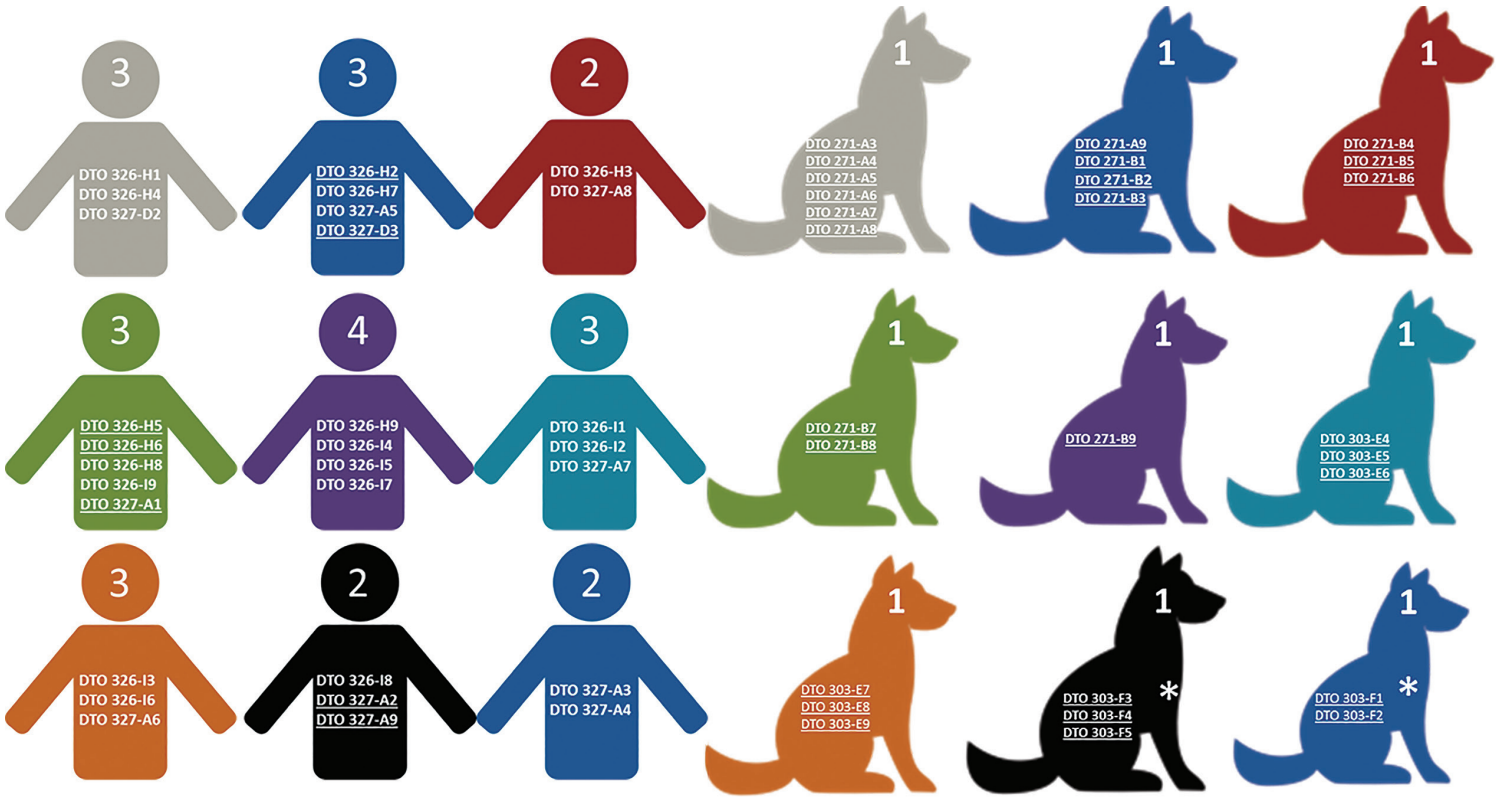

Figure 2. Schematic overview of the nine human and canine patients. The total number of genotypes and the codes of the A. fumigatus isolates from each patient is indicated. Isolates that are underlined belong to the same genotype in the case of canine patients, an asterisk indicates the same patient (CP8 – CP8.2) being re-infected with a different A. fumigatus isolate.

Figure 3. Morphology of colonies (A) and conidiophores and conidia (B-D) of A. fumigatus isolates from dogs, humans and the environment. Top row of panel A shows the obverse side of the colony grown on MEA of isolates DTO 028-D6, DTO 271-A5, and DTO 303-E8, while the bottom row shows the reverse side of CYA-grown colonies of DTO 028-D6, DTO 326-I9, DTO 326-H2. A conidiophore of DTO 327-D3 with flask-shaped phialides and abundant production of conidia is shown in (B), while (C) shows similar phialides of isolate DTO 326-I9 with sparse production of conidia. Reduced, cylindrical shaped phialides of DTO 303-F2 and abundant production of conidia are shown in (D).

Table 3. Number of isolates from human, dog and indoor and outdoor substrates per phenotypic trait. Total number of isolates per source is indicated between brackets.

	Phenotype	Canine (27)	Environmental (27)	Human (29)	Reference (3)
Degree of sporulation and phialide shape in MEA	Strong sporulation and cylindrical phialides	1			
	Strong sporulation and flask-shaped phialides	15	27	29	3
	Sparse sporulation and flask-shaped phialides	11			
Sporulation in MEA	Dark greenish colony	14	27	29	2
	Mostly whitish colony, with the center of the colony green followed by concentric rings of pale yellowish green	12			1
	Whitish colony with star-like green patches	1			
Acid production on CREA	Acid production absent	20	15	29	3
	Acid production present	7	12		
Reverse color on CYA	Brownish	1		3	
	Green with brown center and white margins		1	2	1
	White	26	26	24	2

Additionally, high variation was observed in the average diameter of colonies of isolates from dogs grown on CREA, MEA, YES, DG18, CYAS (Table 5). PCA and k-means clustering using colony diameter as a proxy for growth showed that one cluster contained only 5 isolates from dogs (DTO 303-E6, DTO 303-F5, DTO 303-E5, DTO 271-B3, DTO 271-B2) as well as *A. fumigatus* var. *ellipticus* (cluster 1 in Figure 4) The isolates from indoor and outdoor substrates and human patients were distributed in clusters 2 and 3. 14 and 8 isolates from dogs clustered in clusters 2 and 3, respectively (Figure 4).

Table 4. Distribution of phenotypes of *A. fumigatus* isolated from 9 dogs suffering from SNA. The total number of isolates per dog is between brackets.

	Phenotype	CP1 (6)	CP2 (4)	CP3 (3)	CP4 (2)	CP5 (1)	CP6 (3)	CP7 (3)	CP8 (3)	CP8.2 (2)
Degree of sporulation and phialide shape in MEA	Strong sporulation and cylindrical phialides									1
	Strong sporulation and flask-shaped phialides	1	1	3	2	1	3	3		1
	Sparse sporulation and flask-shaped phialides	5	3						3	
Sporulation in MEA	Dark greenish colony	1	1	3	1	1	3	2		2
	Mostly whitish colony, with the centre of the colony green followed by concentric rings of pale yellowish green	5	3		1				3	
	Whitish colony with star-like green patches							1		
Pigmentation in CREA	No pigment	5	4		2	1		3	3	2
	Yellow pigment	1		3			3			
Pigmentation in CYA	Brownish reverse		1							
	White reverse	6	3	3	2	1	3	3	3	2

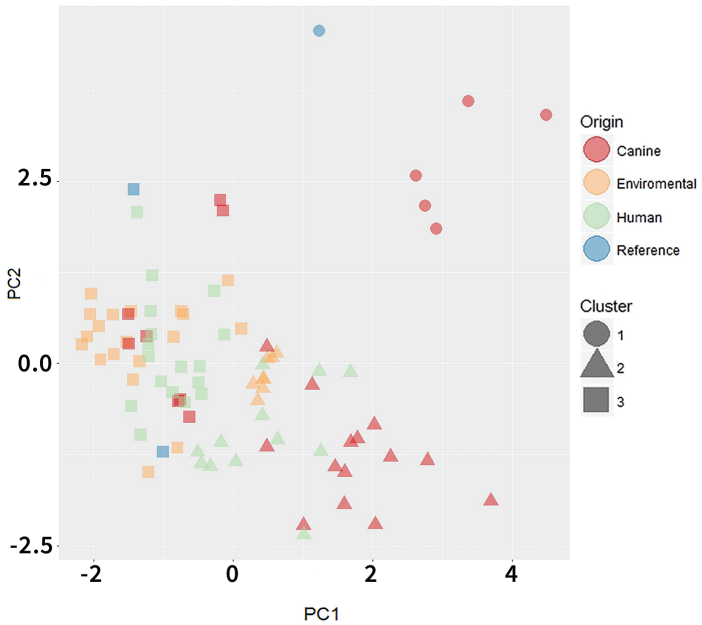

Figure 4. PCA and K-means clustering of A. fumigatus isolates based on colony grown on several media. The arrow indicates the position of *A. fumigatus var. ellipticus*.

Table 5. Mean diameter with standard deviation of colonies (mm) on each medium for each investigated isolate set.

Medium	Dog isolates	Environmental isolates	Human isolates	References
CREA	31.81±10.52	26.73±6.90	29.55±7.98	25.31±12.02
CYA	36.90±11.25	44.57±4.62	42.44±4.32	39.98±10.96
CYAS	28.66±10.76	25.54±3.53	24.83±6.81	14.74±11.30
DG18	28.65±9.99	19.92±2.85	28.67±8.76	11.56±11.86
MEA	36.37±11.04	44.55±5.96	45.63±3.87	39.06±11.22
YES	38.98±11.90	49.02±3.20	47.36±5.72	41.78±13.26

TR profiling and resistance to azoles

Microdilution assays showed that 12 out of the 29 human isolates were resistant to itraconazole, posaconazole and/or voriconazole. Of these isolates, 6 showed a TR_{34} promotor duplication in *cyp51A* and 5 a TR_{46} duplication in this gene. One resistant (DTO 060-G9) and 3 intermediate resistant (DTO 028-D6, DTO 086-C1, DTO 089-G1) posaconazole strains were identified in the environmental isolates from the Netherlands. Dog isolates were all sensitive to the azoles tested, and both groups of strains (Dog and environmental) did not show TR_{34} and TR_{46} mutations. The Af293 strain was sensitive to itraconazole and posaconazole and resistant to voriconazole like the results presented by Mowat *et al* 2008 [37](Supplementary table S1) and the EUCAST MIC distribution for Af293 (Supplementary figure S3) Af293 is however generally considered susceptible for this azole.

DISCUSSION

We assessed phenotypic and genetic differences between strains of *A. fumigatus* isolated from indoor and outdoor substrates and from infected humans and dogs. Genetic analyses did not indicate the existence of sub-groups within *A. fumigatus* specialized in infecting human or dog. A notable variation in phialide morphology, sporulation, colony size, and colony color was observed between strains isolated from dogs suffering from SNA; even between isolates from the same infected individual. This phenotypic variability was not associated with genetic differences in the *benA, CaM, MAT1-1, MAT1-2, cyp51A* loci and microsatellites. This indicates that the dogs were infected with a single strain. It is tempting to speculate that the phenotypic differences evolve within the dog during the infection process due to epigenetic changes or mutations. The fact that phenotypic variability was hardly observed in isolates from human and from indoor and outdoor substrates suggests that the dog sinus induces phenotypic evolution. SNA in dogs is a chronic infection starting with an inoculum in the front of the sinus [13]. This may be the cause of the observed variation in the dog isolates. A long exposure to a stressful environment

and for example host immune response, nutrient, and gas (oxygen) availability may act as a selection pressure [36}. Reduced asexual reproduction, as observed in 11 out of 27 dog isolates can be an adaptation to increase fitness in the host [38, 39]. Similarly, higher growth rate at 37°C have been correlated with increased virulence [40]. Notably, in our study isolates from dog and humans had a higher growth rate on DG18 media as compared to the environmental isolates. The real meaning of this variation of growth for virulence is unclear and requires further investigation.

The observation that a single genotype persist in a dog could suggest that the spread of the fungus in the sinus proceeds gradually associated with a non-sporulating biofilm but not invading the epithelium of the sinus [41]. The infection within human patients is also different from dogs because human patients can carry multiple *A. fumigatus* strains [42-44]. This was confirmed in our study by the presence of *MAT1-1* and *MAT1-2* strains isolated from an individual patient that had different microsatellite profiles. It should be noted that it is difficult to associate the human isolates with a proven infection since they were obtained via bronchoalveolar lavage or sputum samples and not from deep tissue samples.

Clinical azole resistance in the Netherlands is 5-20% for *A. fumigatus* isolated from human patients [25, 30, 45]. The majority (89%) contain TR_{34}/L98H or TR_{46}/Y121F/T289A mutations, while 11% of the resistant isolates carry wild type *cyp51A* [46]. In our study, tandem repeats TR_{34} or TR_{46} were only detected in human clinical isolates. A single posaconazole resistant isolate from environmental isolates was found which was not TR associated and future genome sequencing should illuminate the resistance mechanism involved. Jensen and colleagues [47] found few azole resistant environmental isolates (4/133) in the National Mycology Reference Laboratory of Denmark. In contrast, Klaassen et al. [48] reported that 16 out of the 213 clinical isolates and 9 of the 42 environmental isolates contained the TR_{34}/L98H allele associated with multi-triazole-resistance. Clearly, extensive azole fungicide use in the environment can result in selection of azole resistant strains and complicate clinical management[49]. A survey including whole genome sequencing (WGS) between environmental and clinical isolates can give clues about the evolution of resistance in both niches.[50].

Taken together our results showed that phenotypic and genetic heterogenicity is a key factor to understand several aspects of infections caused by *A.fumigatus*. Dog SNA isolates were phenotypically more diverse than human and environmental isolates. Additionally, using the STRAf assay we observed that fungal plaques from dog suffering SNA harbour isolates genetically identical (one genotype), while humans with suspected IPA are infected with multiple genotypes. Phenotypic variation in dog isolates might be due to genomic differences or epigenetic variations and due to host adaption during the chronic infection process in dogs. New experiments aiming to understand the genomic, epigenetic and physiological adaptations of the isolates studied can provide clues host-driven evolution and its implication in virulence and control of the disease caused by this pathogen in humans.

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SUPPLEMENTARY MATERIAL

Supplementary table S1. Excel file with detailed phenotypic and genetic data .n.d, not determined (no PCR product or sequence obtained) https://static-content.springer.com/esm/art%3A10.1186%2Fs12866-018-1244 2/MediaObjects/12866_2018_1244_MOESM1_ESM.xlsx

Supplementary figure S1. Histology section of the mucosa of a dog with SNA (A) and rhinoscopic image of the fungal plaque within the sinus nasalis (B). Arrow in (A) indicates mucosa tissue with high infiltration of immune cells. Note that the hyphae indicated with a star in (B) do not penetrate the epithelial tissue https://static-content.springer.com/esm/art%3A10.1186%2Fs12866-018-1244-2/MediaObjects/12866_2018_1244_MOESM2_ESM.tif

Supplementary figure S2. Phylogenetic inference constructed using *CaM* (left) and *benA* (right) sequences of human, dog, and indoor and outdoor isolates as well as reference strains. Green represents indoor and outdoor isolates, blue and red represent isolates from dogs and human, respectively, and black represents reference strains. *A.felis* indicated with *. https://static-content.springer.com/esm/art%3A10.1186%2Fs12866-018-1244-2/MediaObjects/12866_2018_1244_MOESM3_ESM.tif

Supplementary figure S3. Voriconazole MIC distribution for Microdilution assay from EUCAST (consulted 22/06/2018), note that MIC of 4 mg/L are reported. https://static-content.springer.com/esm/art%3A10.1186%2Fs12866-018-1244-2/MediaObjects/12866_2018_1244_MOESM4_ESM.jpg

Chapter 3

Transcriptomic analysis of *A. fumigatus* during natural non-invasive sino-nasal infections in dogs

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ABSTRACT

Aspergillus fumigatus can cause life-threatening systemic of superficial infections in human and animals. For instance, sino-nasal aspergillosis (SNA) is a non-invasive infection that is mainly found in dolichocephalic and mesocephalic dogs but also occurs in human. Severe cases in dogs result in facial deformation, destruction of the turbinates and bones surrounding the nasal cavity and cribriform plate, and in lethal infection of the brain. So far, only *A. fumigatus* transcriptomes have been described from murine infection models but these expression studies may not mimic a natural infection. Here, the first transcriptomic profiling is described of natural *A. fumigatus* infections. Transcriptomes were studied of two fungal plaques with different genotypes from the sinus of the same dog (re-infected after approximately two years) and from two fungal plaques with the same genotype but from two different dogs. Close to 93% of all genes were expressed in at least one plaque. The expression profiles were most similar for *A. fumigatus* growing in the same dog, despite the fact that they had different genotypes. Yet, the four expression profiles shared 87% of the expressed genes (8029), 6% of which showed high variability in RNA levels. This subset included secondary metabolite and defence genes and genes encoding resistance proteins like MFS and ABC transporters. The expression profiles explain the virtual absence of asexual reproduction and the observed high pH in the fungal plaques.

INTRODUCTION

Sino-nasal aspergillosis (SNA) is a non-invasive infection that affects mostly dolichocephalic and mesocephalic dogs [1], probably because of their enlarged nasal surface area. The symptoms of the disease are nasal discharge, ulceration and depigmentation of the nasal planum [2, 3]. In severe cases, facial deformation and destruction of the turbinates and bones surrounding the nasal cavity and cibriform plate is observed, which results in a lethal infection of the brain [2]. The infection process starts when *Aspergillus fumigatus* conidia enter the frontal nasal cavity via inhalation or a foreign body. Subsequently, conidia germinate and if the growing fungus is not removed by the immune system a fungal plaque is formed in the sinus nasalis which can ultimately reach the frontal sinus. To alleviate the clinical signs that can be presented up to years [4], removal of the fungal plaques is needed by trephination of the frontal sinus and/or rhinoscopic removal from the nasal sinus, followed by flushes with antifungals [3].

Histological preparation of SNA plaques showed that hyphae do not enter the epithelium of the sinus, hence the non-invasive character of this infection [3-5]. Notorious infiltration of the lamina propria by lymphocytes, plasma cells, and neutrophil granulocytes shows that the immune system is strongly activated [2, 5]. However, the infection is not cleared implying that a balance between fungal growth and immune defence is established. Destruction of tissue and breaching of the sinonasal-brain barrier can be due to an overactive immune system and, in addition, to the production of fungal cytotoxic compounds and tissue-damaging factors [1, 3].

We previously described the phenotypic- and genotypic characterization of fungal isolates derived from sino-nasal plaques of dogs suffering from SNA [5]. Fungal plaques were shown to be rather basic with a pH of about 8 [6]. The rare presence of conidiophores with conidia and the white appearance of fungal plaques *in situ* indicates the repression of the asexual reproduction program in the sinus. In contrast to human clinical isolates from lungs, each canine fungal plaque was composed of one genotype of *A. fumigatus*. Interestingly, high variability in phenotypic differences of isolates of the same genotype and derived from a single canine patient was observed. Amongst others, part of the isolates were characterized by a reduced sporulation phenotype [5]. This stable variability occurred at a much higher frequency when compared to growth on agar plates. This suggests that *A. fumigatus* adapts in the sinus by an increased mutation rate, possibly due to nutritional limitation and/or stress imposed by the immunity of the host.

Transcriptomic analysis is a powerful technique to understand host-pathogen interactions [7]. To date, only in vivo *A. fumigatus* transcriptomes are available from murine infection models [8, 9]. These micro-array studies revealed new insights in sub-telomere directed gene expression during initiation of invasive aspergillosis and an important role of the *A. fumigatus* pH-responsive transcription factor PacC during epithelial entry and tissue invasion. Both studies made use of mice that were immunocompromised by administration of cyclophosphamide, required to

mimic the immunocompromised status of human patients that develop invasive aspergillosis. Clearly, these studies provide valuable information but may not be representative of natural infections. Here, we describe for the first time transcriptomic profiling of natural *A. fumigatus* infections in the sinus of canine hosts using RNA sequencing. Transcriptomes of two fungal plaques with different genotypes from the same dog (re-infected after approximately two years) were compared and from two fungal plaques with the same genotype but from two different dogs. This study is challenging for several reasons. Differential gene expression can be not be assessed since we are dealing with a variable set of natural infections in dogs of different breeds. In addition, sino-nasal fungal plaques represent large three-dimensional structures that differ in location in the nasal cavity, being either in the sinus nasalis and/or frontal sinus. The time of infection and their micro-environments also differ. Nevertheless, in all cases, the fungus had formed a mature biofilm. Results show that expression profiles were most similar for *A. fumigatus* growing in the same canine patient, despite the fact that these fungi had different genotypes. Notably, 87% of the expressed genes were active in all four plaques, only 6% of these genes showed high variability. This set included genes involved in secondary metabolite production and defense and encoded resistance proteins like MFS and ABC transporters. Expression profiles explain virtual absence of asexual reproduction during infection and the relatively high pH in the fungal plaques.

MATERIALS AND METHODS

SNA samples

Fungal plaques from nine dogs with SNA were obtained by rhinoscopic removal (sinus nasalis) or trephination (frontal sinus) at the faculty of Veterinary Sciences at Utrecht University [5]. After extraction, part of the fungal plaques were immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation and sequencing.

RNA isolation and sequencing

Frozen fungal plaques (100 – 200 mg) were homogenized in 1 ml of TRIzol (Thermo Fisher Scientific, USA) with 2 metal balls (4.76 mm in diameter) using a Tissuelyzer for 5 min at 20 Hz min^{-1} (Qiagen, Venlo, The Netherlands). After homogenization, the mixture was incubated for 5 min at RT, followed by mixing with 200 µl chloroform and subsequent incubation for 3 min at RT. Samples were centrifuged for 10 min at 4 °C and 10.000 g. RNA was purified from the aqueous phase using the RNeasy NucleoSpin® RNA kit. RNA sequencing was performed at GenomeScan (Leiden, The Netherlands) using Illumina NextSeq 500 according to manufacturer protocols. Briefly, RNA quality was checked using the Fragment Analyzer. Libraries were made using the MEBNext Ultra Directional RNA Library PrepKit for Illumina. The size of cDNA fragments was checked to be between 300-500 bp and a concentration of 1.6 pM

of cDNA was used for sequencing. The RNA quality of four out of nine isolates was found sufficient to proceed with RNA sequencing.

RNA sequencing analysis

The quality of raw reads was checked using fastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Cleaning and trimming were performed using Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). *A.fumigatus* Af293 transcripts from AspGD (http://www.aspergillusgenome.org/) were used as reference to quantify expression using Kallisto [10]. Transcripts per million (TPM) was used as unit of expression. Transcripts that had a TPM >1 in all four samples were considered expressed.

Functional categorization and enrichment of expressed transcripts

Gene ontology (GO) enrichment analysis was performed using the R package gProfileR [11], with best term per parent group (strong filtering). REVIGO (http://revigo.irb.hr/) was used for removing redundancy in GO terms. FunCat ontology was determined using FungiFun2 [12]. Additionally, published lists of *A. fumigatus* genes involved in different processes were used to query the transcriptomes (Table 1).

Table 1. Sets of *Aspergillus fumigatus* genes involved in biological processes.

Category	Reference
Transcription factors involved in virulence and pathogenesis	Bultman et al. 2017[13]
Reproduction and Stress response	de Vries et al. 2017[14]
Aspergilli Stress response	Miskei et al. 2009[15]
Secondary metabolism	Khadi et al. 2010[16]
Genes involved in host-pathogen interaction	Urban et al. 2017[17]

RESULTS

RNA-Seq was used to study *A.fumigatus* gene expression in dogs with SNA (Figure 1A). Two fungal plaques were derived from either a Labrador Retriever (CP6; plaque isolated half way sinus nasalis) or Sint Bernard (CP7; plaque isolated from both sinus nasalis and frontal sinus). Fungal isolates derived from the fungal plaque CP6 (isolates DTO303 E4/5/6) and CP7 (DTO 303 E7/8/9) differed in their genotype with a Bruvo genetic distance of 0.572 as determined via STR*Af* analysis (Valdes *et al*. 2018) (Figure 1B). The other two fungal plaques, CP8 from sinus nasalis and frontal sinus and CP8.2 from sinus nasalis, were derived from the same Golden Retriever. Individual isolates from fungal plaque CP8.2 (DTO303-F1/F2) were isolated in 2010 and the isolates from fungal plaque CP8 (DTO303-F3/F4/F5) were isolated in 2012, respectively (Fig. 1A) showing different genotypes with a Bruvo genetic distance of

0.46 (Fig. 2B). A total of 9213 genes, representing 92.8% of the genes of the published Af293 genome[18], were found to be expressed in the SNA samples (TPM >1). Of these genes, (8029 (~87%) were shared among all four fungal plaques. We observed a high variation of expression levels between these samples, in some cases > 1000-fold. Therefore, the coefficient of variation (CV) of the log10 TPM (CV=standard deviation in four samples/mean in four samples) of the 8029 expressed genes was calculated and classified as highly stable expressed (CV < 0.05), medium stable expressed (0.5 > CV > 0.05) and highly variable expressed (CV > 0.5). These groups compromised 13, 81, and 6% of the genes.

Transcriptomic profiling of *A. fumigatus* in fungal plaques of dogs with SNA

Hierarchical clustering of the transcriptomes of shared genes in each of the fungal plaques of the three dogs revealed that fungal plaques from the same dog (CP8 and CP8.2) have a more similar expression profile than those of CP6 and CP7 (Figure. 2). Notably, the expression profiles of CP6 and CP8 are most different although the fungal isolates derived from these two plaques have the same genotype.

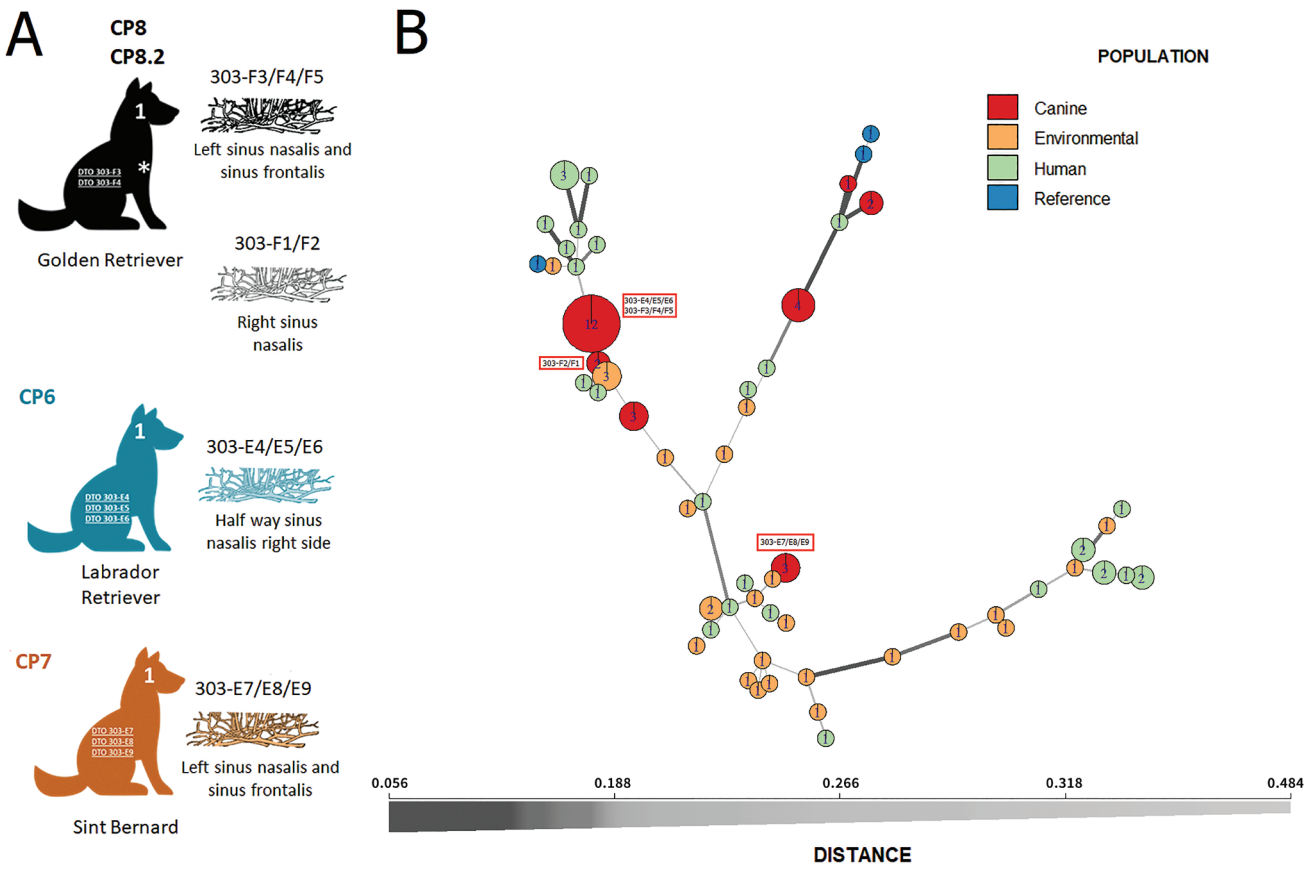

Figure 1. Overview of the source of the fungal plaques used for transcriptomic profiling. Breed and location of the fungal plaque inside the sino-nasal area of the dog are indicated. A) DTO 303 - codes of the isolates derived from the fungal plaque of each patient are indicated as well. B) Minimum spanning network and the Bruvo genetic distance of different *A. fumigatus* isolates. Position of the genotypes of the isolates used in this study are shown in red boxes (modified from [5]).

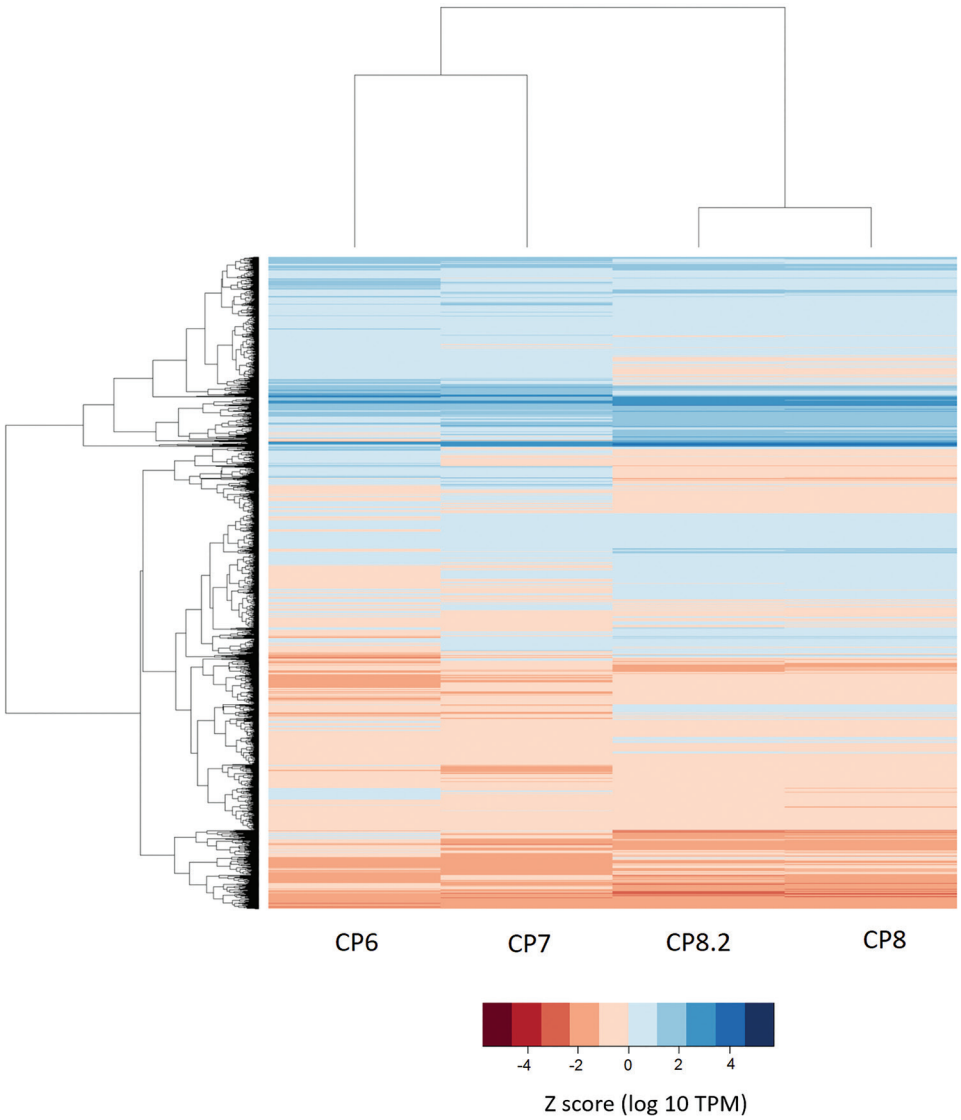

Figure 2. Heatmap clustering expression of shared genes expressed in the fungal plaques of CP6, CP7, CP8 and CP8.2.

A similar distribution of gene expression was observed when shared expressed genes were compared that are related to secondary metabolism [16] (Figure 3A), stress- response genes [15] (Figure 3B) and pathogen-host interaction [17] (Fig. 3C) and transcription factors related to virulence [13] and pathogenesis combined with reproduction and stress response [14] (Figure 3D). Again, the transcriptomes of CP6 and CP8 were most distinct although these strains have the same genotype. Apparently, the host is more directive in gene expression. The variability of gene expression differed to some extent between the four functional categories. More medium

and highly variable genes were observed in the secondary metabolism group (Figure 3A, indicated with yellow and green) as compared to genes that were low in expression variability (Figure 3A, indicated with red). The opposite pattern was observed for genes related to the other 3 categories (Figure 3B-D).

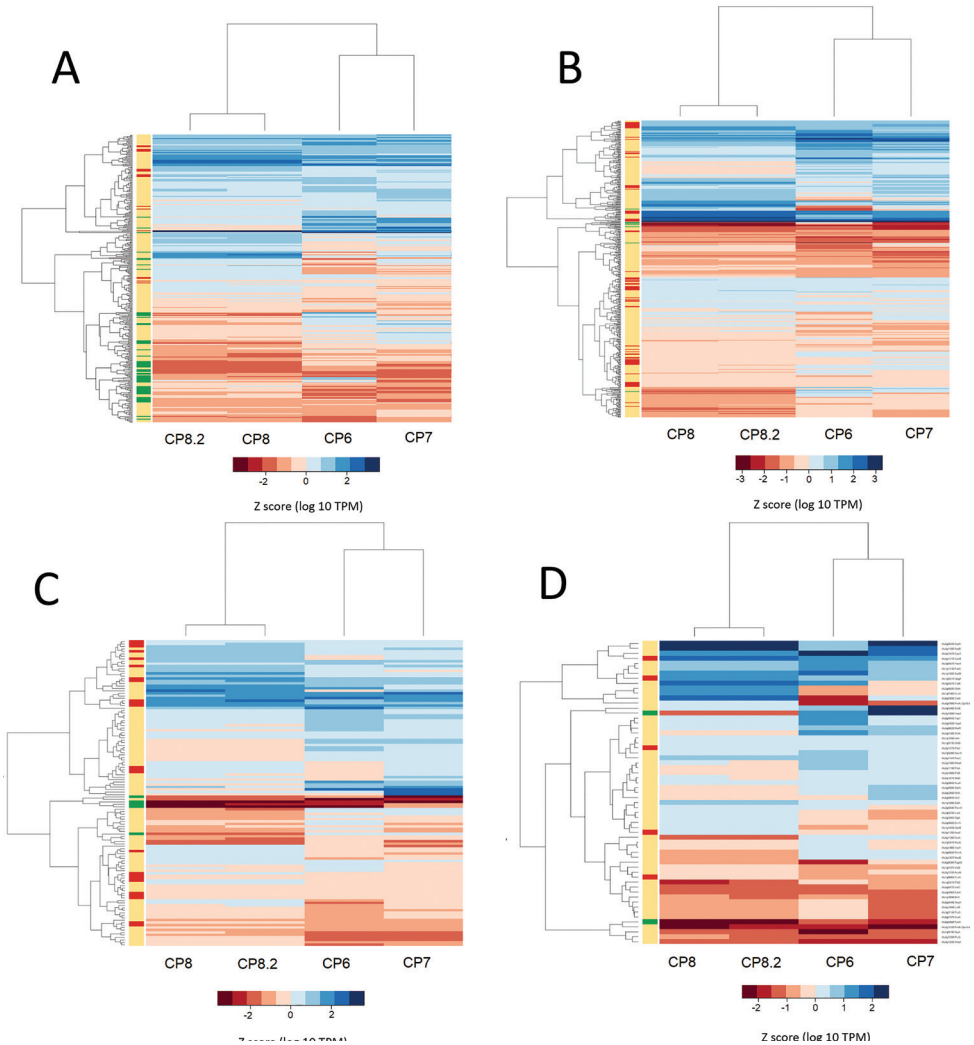

Figure 3. Heatmaps with clustering of total expression of genes implied in secondary metabolism (A), stress response (B), and pathogen-host interaction (C) and transcription factors related with virulence and reproduction and stress (D). The color code under the graphs represents Z scores of TPM. The color bar along the side of graphs A-D indicates the variability based on the CV representing low (red), medium (yellow) or highly variable (green) genes.

Enrichment

Fifteen GO terms were enriched within the 1046 expressed genes with low variability most of them belonging to general categories like mRNA metabolic process (GO:0016071), translation (GO:0006412) and proton-transporting ATP-synthase activity (GO:0046933) (Supplementary material S1). In agreement, FunCat ontology analysis also showed enrichment in categories like translation (12.04), aerobic respiration (02.13.03), ribosome biogenesis (12.01), and ribosomal proteins (12.01.01) (Figure 4). Enrichment of genes categorized as having a medium variability in expression showed general categories like cellular protein catabolic process (GO:0044257), cellular process (GO:0009987) and binding (GO:0005488). On the other hand, the 514 genes with highly variable expression were only enriched in the GO terms oxidation-reduction process (GO:0055114) and oxidoreductase activity (GO:0016491) (Supplementary material S1). In the case of FunCat analysis, this group of genes showed enrichment in secondary metabolism (01.20), defense related proteins (32.05.03), resistance proteins (32.05.01) and other terms related to virulence (Figure 4).

Reproduction and Stress response

GpgA (Afu1g05210) and CpcB (Afu4g13170), two negative regulators of asexual reproduction, [19] were found to have a low variation in expression among the four samples (Figure 5). This was also the case for FlbC (Afu2g13770), which is an activator of the master regulator of asexual reproduction BrlA [20, 21]. On the other hand, BrlA and another main regulator, WetA, showed medium variable expression among the four samples, while AbaA was not expressed (Figure 5).

418 genes (~5% of the total expressed genes) were found to be related with *Aspergillus* stress response (Table 2, Supplementary material S2). Among the genes with low variability in expression; 29 were categorized as having a role in DNA repair (29) followed by oxidative (22), osmotic (23), and temperature and UPR stress (12). This category also included the calcineurin CalA (Afu5g09360) [22], the histone deacetylase HdaA (Afu5g01980) [23] and PpoA (Afu4g10770) that is involved in oxylipin production [24, 25]. These proteins have been previously linked to virulence and stress response. The class of highly variable genes were represented by 8 genes including 2 putative α(1-3)glucan synthases being Ags2 (Afu2g11270). Ags3 (Afu1g15440), and one α(1,3)glucanase /mutase (Afu2g03980).Furthermore, the glutathione S-transferase TpcF (Afu4g14530) that is present in the trypacidin cluster [26], the ABC transporter AtrF (Afu6g04360), which is postulated as being related to azole resistance [27], and AtfB (Afu5g12960) and Ppoc (Afu3g12120) belong to this category. The former is a paralog of the AtfA transcription factor and might be involved in fine-tuning of stress response like AtfA but also plays a role in conidiogenesis [28]. PpoC is key in the production of prostaglandin production together with PpoB (Afu4g00180)and PpoA [25] (Fig 5).

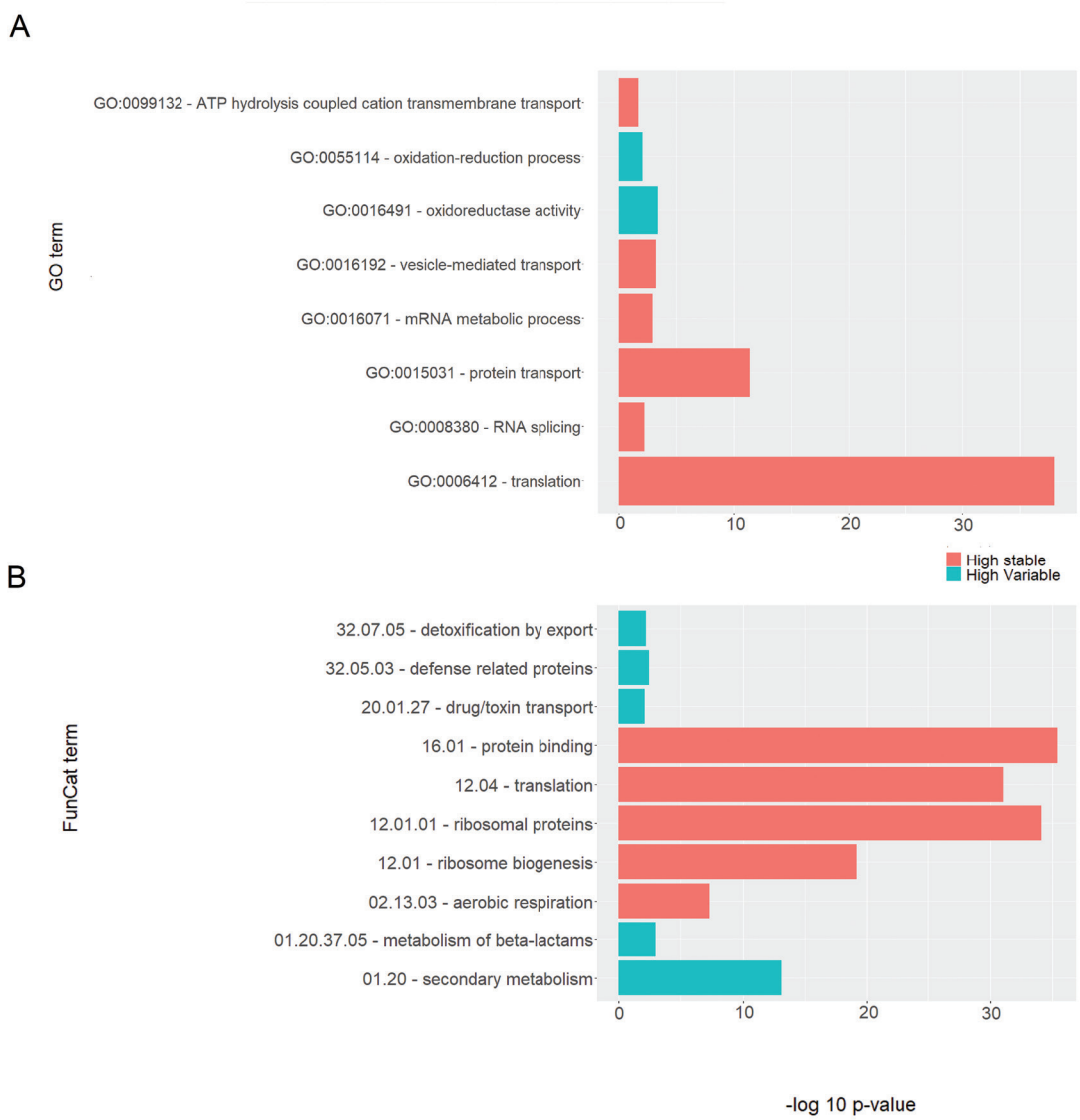

Figure 4. GO (A) and FunCat enrichment (B; representing the first 5 terms) of shared expressed genes with low and high variation in expression. See Supplementary Material S1 for the complete enrichment analysis.

Table 2. Categorization of expressed genes found in four SNA fungal plaques according to the classification made by Miskei *et al.*2009 [15].

Miskei category	Number of genes*	Genes with CV > 0.5 (highly variable)	Genes with CV < 0.05 (lowly variable)
DNA repair	155	0	29
Osmotic	110	2	23
Oxidative	88	5	22
Temperature and UPR	74	1	12
Other (xenobiotics and or heavy metals) / general response to stress	68	0	8
Starvation	34	0	7

*Expressed genes can belong to more than one category, the full list is available as Supplementary material S2.

Secondary metabolism

SMURF [16] classifies 479 genes of *A. fumigatus* as genes involved in secondary metabolism. These genes are distributed in 36 clusters. Of this group, 18 genes from 15 clusters (Table 3) were found to be lowly variable). For instance, this group includes SidH (Afu3g03410) that is involved in the production of the extracellular siderophore TAFC [29], and Afu2g05750 that is predicted to encode an agmatinase that converts agmatine into putrecine and urea. Agmatine has many physiological roles in humans, for example, modulation of NO concentrations, inhibition of NO synthase in neurons via H_2O_2 production, and inhibiting formation of advanced glycation end product (AGE) molecules [30, 31]. On the other hand, 58 secondary metabolism genes from 17 clusters showed high variably of expression (TPM:594.2429 – 1.353088, log10 CV: 1.208995 – 0.515171). This group includes the cluster of fumitremorgin (Afu8g00170-Afu8g00260), the transcription factor HasA (Afu3g12890) and its companion non-ribosomal peptide synthase HasD (Afu3g12920) (Table 3).

Notably, all genes of seven clusters were expressed in all plaques indicating that their encoded metabolites are likely produced (Table 3). For instance, this holds for gliotoxin and neosartoricin that are known to have immunosuppressive activity [32, 33]. Interestingly, only 4 out of 8 genes in the PKS cluster for DHN-melanin synthesis are expressed indicating the absence of DHN melanin formation. In contrast, all genes of the pyomelanin cluster were found to be expressed indicating that this type of melanin is produced.

Virulence and pathogen-host interaction

Three known transcription factors were found to be expressed in a highly stable pattern being Ace2 (Fig 4) (Afu3g11250), RsmA (Afu2g02540) and CrzA (Afu1g06900). Ace2 has been described to have a role in conidiogenesis and environmental sensing of germination cues [34]. Interestingly, a mutant of Ace2 is hypervirulent in a mice model of infection, caused by increased fungal burden and over induction of immune response related genes like myeloperoxidase (MPO), tumor necrosis factor (TNF) and interleukin 6 (IL-6) [34]. RsmA is involved in gliotoxin production and resistance to menadione [35], while in *Aspergillus nidulans* it was implicated in resistance against fungivory [36].

Metals like iron, zinc and copper are reported to function in host-pathogen interaction [37, 38]. In our data set the ZrfC (Afu4g09560) transporter that is responsible for zinc uptake under alkaline conditions [39] and the copper toxicity transcription factor AceA (Afu6g07780) were constantly highly expressed (Fig 4). On the other hand, the iron transcription factors HapX and SreA were classified as medium variable expressed and the copper exportin ATPase CrpA (Afu3g12740) showed highly variable expression among the four SNA fungal plaques.

In *A. fumigatus* septins are a family of GTPases that participate in fungal processes like cell wall integrity, conidiation, and hyphal morphology [40]. Their role in pathogenesis is not very clear but in the SNA sample we observed all four septins AspA-D being expressed in a stable manner as well as part of their regulatory proteins ParA (Afu5g05900) and Cla4 (Afu5g02560) that are also required for full hyphal extension and septation [40].

Table 3. Overview of expressed secondary metabolite clusters in SNA fungal plaques. Clusters indicated in red were found to express all their genes in all four plaques.

	Number of genes	Expressed in SNA	SNA CV > 0,5	SNA CV < 0,05
1 - Afu1g01010 -	5	0	0	0
2 - Afu1g10380 - Nidulanin-like*	9	9	0	1
3 - Afu1g17200 -	17	14	0	0
4 - Afu1g17740 - Fusarielin-like*	6	0	0	0
5 - Afu2g01290 -	12	9	0	2
6 - Afu2g05760 -	12	12	0	3
7 - Afu2g17600 - DHN melanin spore pigment	8	4	0	0
8 - Afu2g18040 - Fumigaclavine C (fga)	9	0	0	0
9 - Afu3g01410 -	16	13	2	0
10 - Afu3g02530 -	4	2	2	0
11 - Afu3g02570 -	7	0	0	0
12 - Afu3g02670 -	10	6	4	0
13 - Afu3g03420 - Siderophore (sid) - Fusarine C*	32	18	3	1
14 - Afu3g12930 - HAS*	18	18	5	1
15 - Afu3g13730 -	14	6	1	0
16 - Afu3g14700 -	16	4	0	0
17 - Afu3g15270 -	5	1	1	0
18 - Afu4g00210 - Endocrocin*	8	2	0	0
19 - Afu4g14560 - Trypacidin*	30	15	6	1
20 - Afu5g10120 -	27	12	4	1
21 - Afu5g12730 -	13	13	1	2
22 - Afu6g03480 - fumisoquin*	19	15	2	0
23 - Afu6g08560 -	3	3	0	0
24 - Afu6g09660 - Gliotoxin (gli)	17	17	1	0
25 - Afu6g12080 - Fumiquinozalines*	14	6	3	0
26 - Afu6g13930 - Pyripyropene A*	21	17	8	0
27 - Afu7g00170 - Neosartoricin*	5	5	1	0
28 - Afu8g00250 - Fumitremorgin B (ftm)	17	14	12	0
29 - Afu8g00540 - Pseurotin A - Fumagillin *	26	25	2	1
30 - Afu8g02350 -	10	4	0	1
31 - Afu1g13160 -	10	10	0	0
32 - N.D -	14	13	0	1
33 - N.D -	10	9	0	1
34 - Afu5g00110 -	7	0	0	0
35 - Afu5g04080 -	15	15	0	2
36 - N.D -	13	8	0	0

DISCUSSION

Most of the literature focusing on SNA in dogs involves detection, treatment, and immune response of the disease. To date, two transcriptomic studies have been performed using qPCR and microarray technology to investigate the immune response of the host [41-43]. Here, we report for the first time a transcriptomic profiling of fungal plaques isolated from dogs with natural sino-nasal infections.

The transcriptomic profiles of *A. fumigatus* present in fungal plaques of dogs with SNA revealed that the host has a stronger impact on expression than the genotype of the infecting fungus. This contrasts recent findings that *A. fumigatus* strains Af293 and CEA10 do not show a conserved transcriptional response when infecting A549 lung cells [44]. Two plaques isolated from the same dog but having different fungal genotypes showed the most similar transcriptomic profiles. Notably, these plaques were isolated either in the sinus nasalis or both in the sinus nasalis and frontal sinus, indicating that the sino-nasal location also does not have a major effect on gene expression. Nevertheless, the four transcriptomic profiles resulting from 3 different dogs, 3 fungal genotypes and 4 different plaques did share 87% of the expressed genes with rather similar expression levels. This indicates a similar transcriptional response. SNA is a chronic infection and it is very likely that the four fungal plaques were mature and present for a long time [3]. Our transcriptomic data therefor represent the gene expression profile of a late stage of infection. The absence of actual data on the time of infection of the four patients can be a disadvantage as compared to time course experiments in laboratory settings. However, since we expect that the fungal plaques are mature and are all related to chronic infections we believe comparison of the profiles is a valid approach.

The chronic SNA infections imply that the dog immune system is unable to clear the host from the pathogen [1, 42]. Fungal growth and the immune system continuously fight each other at the epithelial surface being underscored by our transcriptomic data. Genes that showed a stable expression were enriched in general processes of fungal growth (e.g. translation, morphogenetic processes and transcriptional control) but were also enriched in genes involved in stress response. The presence of GpgA transcripts and the absence of AbaA transcripts indicates that *A. fumigatus* does not sporulate in SNA fungal plaques, which agrees with the white color of plaques *in situ* and the few conidiophores that are detected by histological analysis [5].

A. fumigatus is likely to form the immunosuppressive secondary metabolites gliotoxin and neosartoricin in the plaque since all genes in their clusters are expressed. The same holds for 5 other secondary metabolite clusters, while 24 clusters are only partly or highly variable expressed. For instance, only 4 out of 8 genes of the DHN-melanin cluster are expressed, while the fumitremorgin b cluster was shown to be highly variable in expression. This toxin has been reported as a cell cycle inhibitor and to be neurotoxic [45, 46]. Possibly, partial expression of clusters may still result in secondary metabolites that can affect sino-nasal survival. These

precursors may present an activity or may be used by enzymes of another cluster resulting in another bioactive metabolite. For example, the production of endocrocin in *A.fumigatus* can be carried out by enzymes present in the trypacidin cluster [26]. Metal homeostasis is an important aspect of pathogenesis. In fact, the host immune system has developed strategies to regulate the availability of for instance iron, zinc, and copper [38]. Reducing the availability of these metals will reduce the growth of the pathogen [38, 47], while high concentrations can be toxic to the pathogen e.g. via production of hydroxyl radicals via the Fenton reaction [37]. Fungal plaques showed indeed low variability in the expression of genes related to copper detoxification regulation (AceA) and zinc transport (ZrfC). In addition, transcriptomic analysis revealed a stable expression of SidH that is vital for the production of the siderophore TAFC [29]. This result is in accordance with Crawford & Wilson [48] who propose that siderophore-mediated scavenging if preferred over the reductive pathway.

Immune response of the dog to an *A. fumigatus* infection is characterized by the Th1 response and the expression of markers like IFN-y CCL3, CCL4, and CXCL10. However, it has also been observed that the immune damping factors IL-10 and IL-16 are also upregulated in dogs [43]. Our results suggest an increase in production of fungal-derived prostaglandin E2 (PGE2). This molecule is involved in many processes including the recruitment of phagocytic cells, inflammation and allergic response. Therefore manipulation of the pathways in which these molecules are involved could be a strategy to modulate an "aggressive" host response since it has been reported that PGE2 can induce the production of IL-10 [49, 50], leading to an anti-inflammatory response.

The breakdown of agmatine into urea followed by a conversion to ammonia may also function in modulation of the host defence. Production of ammonia explains the local pH of 8 in plaques. This increased pH impacts amongst others protein-protein interactions or might even result in tissue damage [6, 51]. In addition, an increased pH is expected to induce PacC dependent gene expression which also modulates epithelial cell entry and tissue invasion. Yet, the non-invasive nature of SNA suggests that the host counteract the PacC induced gene expression by the massive presence of immune cells in the epithelium.

Together, our results reveal a "war zone" at the sino-nasal epithelium where fungal gene expression is adapted to fight the host and its defence mechanisms. *A. fumigatus* is proposed to control the host immune response by molecules like gliotoxin, neosartoricin, and oxylipin-derived prostaglandin. In addition, the fungus seems to respond to nutritional immunity by expression of genes involved in metal homeostasis and by protection against ROS and RNS that are most likely generated at the epithelial surface by the massive amount of infiltrated immune cells in this area. The analysis also suggests that *A. fumigatus* modulates the environment by increasing pH via the agmatine pathway.

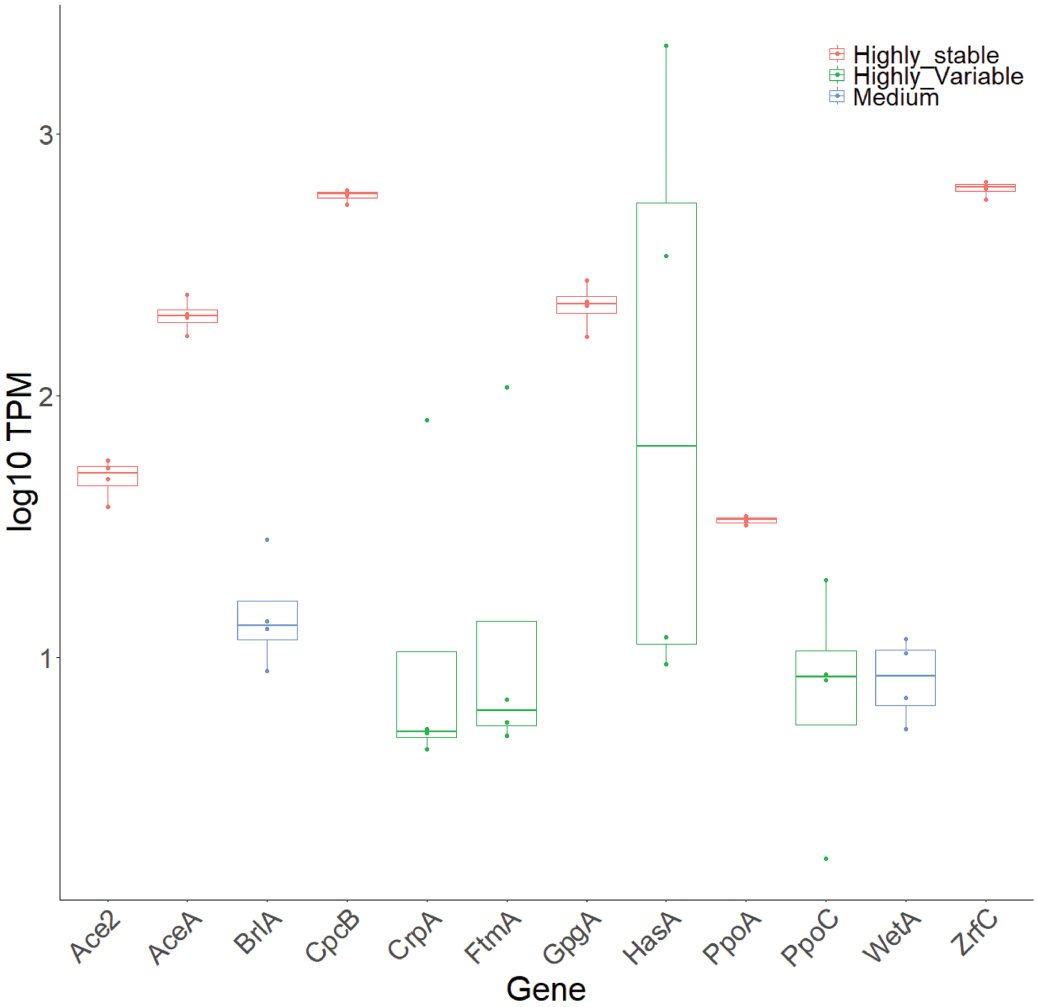

Figure 5.Box plot showing differences between expression of selected genes. Green, blue and red boxes represent highly variable, medium stable and highly stable expressed genes in the fungal plaques.

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SUPPLEMENTARY MATERIAL.

Supplementary material S1. Excel file with detailed GO en FunCat enrichment analysis of Highly variable and highly stable expressed genes. http://tinyurl.com/y4qo99ds

Supplementary material S2. Excel file with detailed expression data (log10 TPM per sample, mean and coefficient of variation), of the genes corresponding to categories explained in Table 1. http://tinyurl.com/y3hl7cd8

CHAPTER 4

Mucosal-immunity in canine patients with sino-nasal aspergillosis

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ABSTRACT

Aspergillus fumigatus is a cosmopolitan fungus and a common pathogen of humans and animals. Sinonasal aspergillosis (SNA) is a chronic non-invasive infection in dogs. QRT-PCR and microarray expression studies indicated that *A. fumigatus* induces a Th1 response in the host. Here, RNA sequencing was used to analyse gene expression of infected mucosal tissue in 4 fungal plaques derived from 3 dogs with SNA. Dog RNA comprised 1-17% of total RNA and ~ 17 % of the dog transcripts were detected. Of these transcripts, 44.2% were shared among all samples. The proinflammatory chemokine IL-8, transcripts of nutritional immunity and transcripts of S100 family alarmin genes were among the top 50 % of highest expressed genes after ranking by mean TPM. Data indicate a dysregulation of the Th17 response and confirms the activation of the Th1 response during SNA. The combination of these responses suggests a disruption of the mechanism for complete fungal clearance. While these responses were observed in all three dogs, clustering of the transcriptome data does suggest a breed specific immune response during SNA, although intra-breed variation can not be excluded. This implies that the genetic background of the patients within breeds has to be taken into account for the occurrence and management of SNA in dogs.

INTRODUCTION

Aspergillus fumigatus causes both invasive and non-invasive infections. Sino-nasal aspergillosis (SNA), chronic pulmonary aspergillosis (CPA) and allergic bronchopulmonary aspergillosis (ABPA) are types of non-invasive infections. In general, infection success depends on a combination of the immune status of the host and the fungal virulence and infection dose [1-5].

Similar to fungal rhinosinusitis in humans, dogs can be affected by SNA. Symptoms include sneezing, mucoid nasal discharge, nasal depigmentation, and turbinate and bone destruction [6]. Dolicho- and mesocephalic dogs are more prone to develop SNA, which might be due to the larger sino-nasal surface area relative to brachycephalic (short-headed) dogs. No apparent immunodeficiency has been reported in dogs but Labrador Retriever, Golden Retriever, Rottweiler and Beauceron Sheepdog appear more susceptible to SNA [7]. Single-nucleotide polymorphisms (SNPs) in Toll-like receptors 2,4 and 9 have been associated with increased risk of fungal infections in human [8]. However, no correlation was observed between SNPs in these Toll-like receptors and occurrence of SNA in dogs. Yet, other SNPs found in innate immunity genes may impact SNA in this host [7].

Histological observations showed a superficial fungal biofilm on the sino-nasal epithelium of the dog, which was associated with high infiltration of immune cells [6, 9-12] and mucosal inflammation [11]. We recently reported that fungal isolates derived from an individual canine patient with SNA have the same genotype. Yet, the phenotypes of these isolates varied more than to those isolated from human patients and the environment [12]. Based on these results it was proposed that phenotypic variation results from fungal adaptation to environmental conditions in the dog, for example by stress imposed by the mucosal immune response [12]. This response is mediated by both sino-nasal epithelial cells and immune cells present in and at the mucosal surface. The mucosal immune response of canine patients has been studied in biopsies from affected areas using qRT-PCR [13] and Affymetrix microarray analysis [13, 14]. These studies pointed to a local Th1 immune response that is characterized by upregulated expression of cytokines IL-6, IL-12, IL-18, tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ), and the transcription factors STAT1 and STAT4. No indications were found for a Th17 response that was previously described to play an important role during invasive aspergillosis [15] and mucosal candidiasis [16]. Particularly, no upregulation of IL-17 chemokine was reported in SNA. Yet, IL-6, TGF-ß and IL-23 (IL-12p19 and IL-12p40) that are implicated in Th17 differentiation of CD4+ T cells were upregulated [15]. A variety of chemokines were also upregulated in dogs suffering from SNA in dogs including IL-8, eotaxin-2 (CCL24), MCP1-4 (CCL2, CCL8, CCL7 and CCL13, respectively), IL-16, CCL3, CCL4, CCL21, CCL28 and CXCL10, and CXCL19. This response explains the high number and variety of lymphocytes, macrophages and granulocytes that infiltrate the sino-nasal mucosal area [6, 9-12, 17]. Importantly, SNA is not cleared and canine patients thus suffer from a chronic fungal infection that becomes lethal

if not treated in time. Why the infection is not cleared remains unclear but it was proposed to be related to the expression of anti-inflammatory cytokines like IL-10, TGF-ß and DNA-binding protein Ikaros (IKLZF1) [18]. The latter activates IL-10 expression and down regulates IFN-γ expression [9, 13, 14, 19].

In this study, RNA sequencing was used to expand gene expression analysis of mucosal tissue in dogs suffering from SNA. To this end, transcriptomes of four fungal plaques of three canine patients were analyzed. Results show upregulation of the Th1 response and dysregulation of the Th17 response, indicating that a defective pathogen clearance is one of the hallmarks of SNA. Furthermore, genes involved in nutritional immunity and S100 protein family alarmins were shown to be highly expressed. The latter may be key in local tissue damage. Despite these global responses, canine sino-nasal gene expression appears to be patient-specific, this in conjunction with the type of skull implies that susceptibility to SNA might have a genetic cause.

MATERIALS AND METHODS.

Fungal plaques from nine dogs with SNA (Table 1) were obtained by endoscopy or trephination at the Faculty of Veterinary Medicine at Utrecht University [12]. Part of these plaques were immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation and sequencing.

RNA isolation and sequencing.

Material of fungal plaques (100 – 200 mg) was homogenized for 5 min with 2 metal balls (4.76 mm in diameter) in a Tissuelyzer at 20 Hz min-1 (Qiagen, Venlo, The Netherlands) in the presence of 1 mL of TRIzol (Thermo Fisher Scientific, USA). After homogenization, the mixture was incubated for 5 min at RT, followed by the addition of 200 µL chloroform and subsequent incubation at RT for 3 min. Samples were centrifuged for 10 min at 4 °C at 10.000 g and the RNA in the aqueous phase was isolated and purified using the RNeasy RNA kit (Qiagen).

RNA sequencing was performed at GenomeScan (Leiden, The Netherlands) using Illumina NextSeq 500 according to manufacturer protocols. Briefly, RNA quality was checked using the Fragment Analyzer. Libraries were made using the MEB-Next Ultra Directional RNA Library PrepKit for Illumina. The size of the cDNA fragments was confirmed to be in the 300-500 bp range and 1.6 pM of DNA was used for sequencing.

RNA sequencing analysis.

Quality of raw reads was checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), while cleaning and trimming were performed using Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). CanFam3.1 transcripts

from Ensembl (https://www.ensembl.org/Canis_familiaris/) served as reference to quantify expression using Kallisto [20]. Transcripts per million (TPM) was used as unit of expression. Transcripts with a TPM > 1 in all 4 samples were considered expressed.

Functional categorization and enrichment of expressed transcripts.

Gene ontology (GO) and KEGG enrichment analysis was performed using the R package gProfileR with best term per parent group (strong filtering) [21].

Comparison with reported microarray data

Expression data [14] were extracted from the array express database using the R package Array express V1.42.0. Raw data were processed and reanalyzed using the RMA method contained in the R package affy V1.60.0. Expression values of each transcript corresponded to the mean value of expression of the probes targeting that particular transcript. Transcripts were considered differentially expressed when they showed a Log2 Fold change (control vs. affected dogs) > +/- 2 (up, down-regulation, respectively).

RESULTS

RNA of sufficient quality for sequencing was isolated from 4 out of 9 fungal plaques (i.e. from patients CP6, CP7 CP8, and CP8.2). The latter two belonged to the same dog that suffered SNA in 2010 (CP8) and 2012 (CP8.2) (Table 1).

Read mapping to the dog reference transcriptome (38019 transcripts), resulted in 8706-12578 transcripts with TPM >1 per sample (Figure 1A). About 4% of the transcripts (i.e. between 514 and 619) were unique for each fungal plaque, whereas 44% (i.e. 6536) of were shared between the 4 plaques (Figure 1A). This number corresponds to 17% of the total transcriptome of *C. lupus familiaris*. The relatively low number of expressed transcripts reflects the low amount of canine RNA (i.e. 1-17% of a total of 3-6 $*10^7$ reads) (Table 2) due to the relatively small amount of dog cells of the sino-nasal area present in the fungal plaques. The transcripts that were unique to each dog were enriched in a few unrelated terms (Supplementary material S1).

The 6536 shared transcripts were considered as the SNA dog transcriptome and were used for further analysis. Hierarchical clustering of the shared transcriptomes showed that the two samples derived from the same dog, CP8 and CP8.2, clustered together (Figure 1B). This suggests similar core transcriptome responses to the fungal pathogens having different genotypes [12]. GO and KEGG term analysis (Supplementary material S1; see Goprofiler) showed significant enrichment in the shared expressed transcripts in general terms like cellular metabolic process (GO:0044237), RNA binding (GO:0003723), membrane-bounded organelle (GO:0043227) and spliceosome (KEGG:03040). In addition, immune response terms

like Toll-like receptor binding (GO:0035325), cytokine production (GO:0001816), TNF signaling pathway (KEGG:04668),NOD-like receptor signaling pathway (KEGG:04621), Th1 and Th2 cell differentiation(KEGG:04658), NF-kappa b signaling pathway (KEGG:04064), as well as osteoclast differentiation (KEGG: 03480) were overrepresented. The latter is related to bone destruction and remodelling (Figure 2).

Table 1. Origin of fungal plaques from canine patients. Patient code, the origin of the patient and location of the fungal plaque within the sino-nasal area are indicated. Fungal plaques of which RNA was isolated of sufficient quality for sequencing are marked with an asterisk.

Patient code	Origin	Location of fungal plaque	Breed
CP1	Province Noord-Holland, The Netherlands	Left sinus nasalis and sinus frontalis	Alaska Malamute
CP2	Province Utrecht, The Netherlands	Left sinus nasalis and sinus frontalis	Cross
CP3	Province Limburg, The Netherlands	N.D	Labrador Retriever
CP4	The Netherlands	N.D	Cross
CP5	The Netherlands	Sinus nasalis caudal, before the entrance of sinus frontalis	Heidewachtel
CP6*	Province Noord-Holland, The Netherlands	Halfway sinus nasalis right site	Labrador Retriever
CP7*	Province Zuid-Holland, The Netherlands	Left sinus nasalis and sinus frontalis	Saint Bernard
CP8*	Province Noord-Holland, The Netherlands	Right sinus nasalis	Golden Retriever
CP8.2*	Province Noord-Holland, The Netherlands	Left sinus nasalis and sinus frontalis	Golden Retriever

Table 2. Read mapping statistics.

Fungal plaque	Total number of reads	Number of pseudo-aligned reads to *C. lupus familiaris*	Percentage
CP6	$6{,}04*10^7$	$2{,}75*10^6$	5%
CP8	$3{,}2*10^7$	$5{,}9*10^6$	18%
CP8.2	$2{,}49*10^7$	$4{,}6*10^6$	19%
CP7	$4{,}44*10^7$	$6{,}52*10^6$	1%

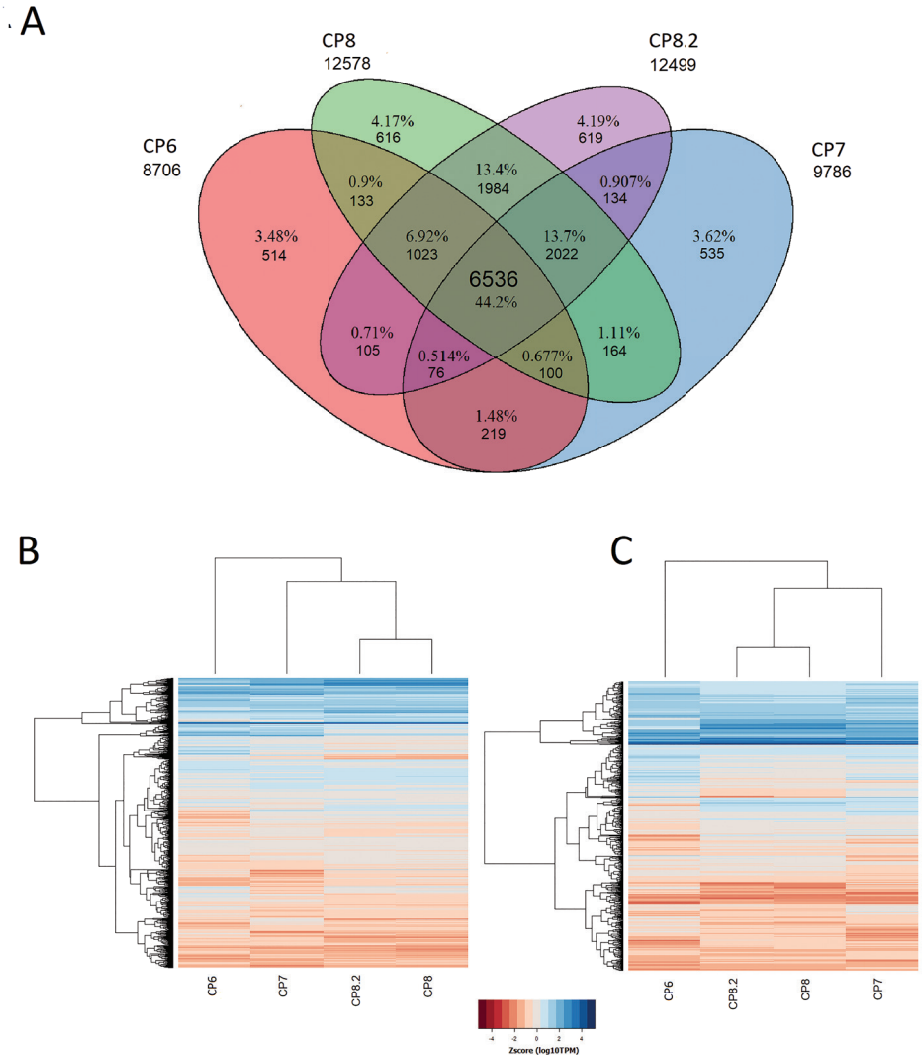

Figure 1. Transcriptome profiles of 4 fungal plaques of 3 different dogs. Venn diagram (A) showing number and percentage of shared transcripts (TPM > 1) between all four fungal plaques and heatmaps with hierarchical clustering of the 6536 shared transcripts (B) and the 660 transcripts mapped to the InnateDB database [23] (C). Number of transcripts with TPM >1 are shown under each patient code in panel A.

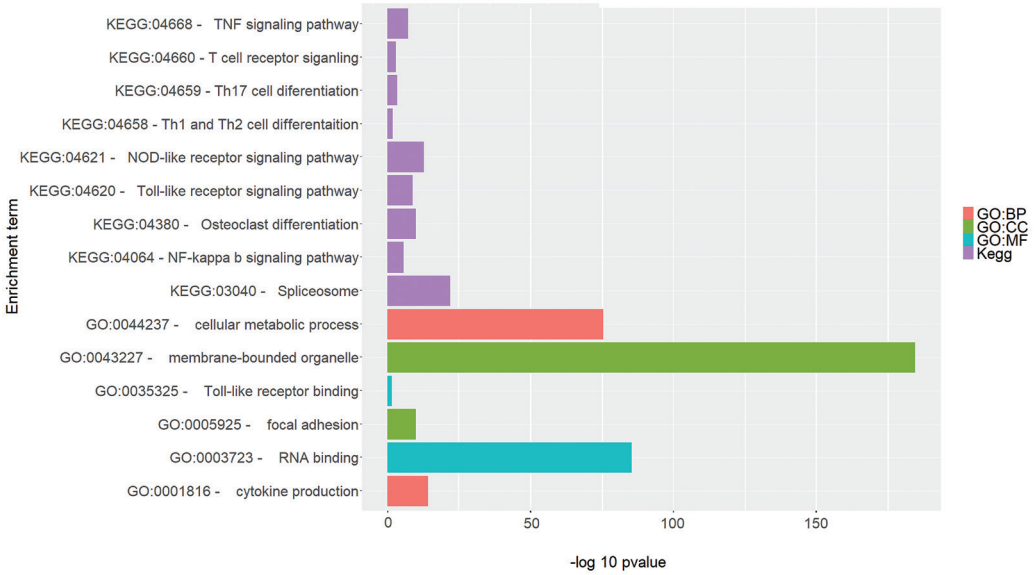

Figure 2. GO and KEGG term enrichment analysis of the 6536 shared expressed transcripts in four fungal plaques from 3 dogs with SNA. BP indicates biological process, CC cellular component and MF molecular function (for full list see Supplementary material S1).

Ranking of the transcripts by their mean TPM revealed that the top 50 had 20 transcripts related to immune response with IL-8 as the highest ranked transcript (Table 3). Also, high amounts of transcripts related to nutritional immunity (e.g. ferritin, and S100 A8, A9, and A12) were detected. These type of proteins have several functions in host defence including inducing cytokine production, acting as damage-associated molecular patterns (DAMPs) and chelating divalent ions like zinc [22]. Interestingly, cathepsin proteases were highly expressed and these lysosomal proteases are not only implicated in intracellular protein turnover but also in antigen presentation, degradation of extracellular matrix components and signalling in a large variety of biological processes [23]. In the next step, the shared SNA dog transcriptome was mapped to the immune database InnateDB [24] using the closest human ortholog (according to Ensembl). This resulted in 660 hits (i.e. 11% of the 6536 shared transcripts) (Supplementary material S1) This included six interleukins (IL-1,8,10,15,16,18,23A), twelve interleukin receptors (IL 1R2, 2RG, 4R, 10RA, 13RA1, 15RA, 17RA/B, 18R1, 22RA2, 27RA, 31RA), seven toll-like receptors (TLR-1, 2, 4, 6, 7, 8, 10), six signal transducers and activators of transcription (STAT1, 2, 3, 5A, 5B, 6), two chemokine C-X-C motif receptors (CXCR4,2), three chemokine C-C motif receptors (CCR3, 5, 7), five chemokine C-C motif ligands (CCL2, 3, 4, 5, 8), one chemokine C-X-C motif ligand (CXCL10),one nucleotide-binding oligomerization domain containing protein 2 (NOD2), and Interferon gamma (IFNγ) (Supplementary material S1).

As observed with the general SNA transcriptome (Figure 1B), the InnateDB transcripts derived from the same dog (CP8, CP8.2) also clustered (Figure 1C). This indicates that the host and not the genotype of *A. fumigatus* has the strongest impact on the immune response.

We compared our RNA-seq results with these previously published microarray data describing differential gene expression of healthy dogs versus dogs with SNA [14]. We used a log2 fold change -/+ 2 observed in the microarray data for the identification of differentially expressed genes. This resulted in 948 out of 5551 differentially expressed transcripts. Of these differentially expressed transcripts, 307 transcripts were also expressed in our RNA-seq data (Supplementary material S1). Enrichment analysis of these 307 differentially expressed genes showed that up-regulated genes (264) were enriched in immune-related terms like superoxide anion generation (GO:0042554), phagocytosis (GO:0006909) and immune system process (GO:002376). Terms related to lipid metabolism were found to be enriched in the 43 down-regulated genes (Table 4). Interestingly, 15 transcripts within the top 50 highest expressed genes detected by RNAseq analysis were differently expressed in the microarray (Table 3). Genes like S100 A12, A9, A8, and SOD2 were not differentially expressed in the microarray. In contrast, IL-8 and IDO1 were up-regulated and LTF was downregulated. A correlation between log10 mean TPM and log2FC from the microarray was not clear (Figure 3), indicating that transcripts with high TPM are not up-regulated and vice versa. In addition, it may suggest differences between the type of samples (individual samples) between our study and the one from [14] (pooled samples).

Table 3. List of Top 50 genes ranked by mean TPM. NA= not available *= related to Immunity.

Gene name emsembl	RANK	Description	Function	DEG in Microarray	Up/Down Microarray
IL-8 (CXCL8)*	1	Interleukin-8	Chemokine and neutrophil activation factor	Yes	Up
FTL*	2	Ferritin light chain	Iron storage	No	
ENSCAFG00000030286	3	NA	NA	Yes	Up
SAT1	4	Spermine N1-acetyltransferase 1	Polyamine metabolism	No	
B2M	5	Beta2 microglobulin	Component of Class I MHC (major histocompatibility complex)	No	
S100A8*	6	Calcoprotein	Zinc and transition metal sequestration	No	
FTH1*	7	Ferritin heavy chain	Iron storage	No	
FTH1*	8	Ferritin Heavy chain	Iron storage	No	
S100A12*	9	Calgranulin C	Zinc and transition metal sequestration	No	
ENSCAFG00000024443	10				
SOD2*	11	Superoxide dismutase -2	Conversion of superoxide into hydrogen peroxide and oxygen	No	
S100A9*	12	Calgranulin B	Calcium-binding protein	No	
FTL	13	Ferritin light chain	Iron storage	No	
ENSCAFG00000030265	14	NA	NA	No	
MT-CO1	15	NA	NA	No	
S100A8*	16	Calcoprotein	Zinc and transition metal sequestration	No	
PLEK	17	Pleckstrin	Substrate for protein kinase C in platelets	Yes	Up
SRGN	18	Serglycin	Hematopoietic cell granule proteoglycan	Yes	Up
CTSB*	19	Cathepsin B	C1-peptidase	No	
C15orf48	20	NA	NA	No	
MT-ATP6	21	ATP synthase membrane subunit 6	NA	No	
HLA-DRB1*	22	MHC class II DR alpha chain	Antigenic peptide presentation to T helper cells	No	
MT-CO2	23	Cytochrome oxidase II	Respiration	No	

SERPINA1*	24	alpha-1 antitrypsin	Serine protease inhibitor	Yes	Up
SPP1	25	Secreted phosphoprotein 1	Cytokine and osteoclast attachment	Yes	Up
DLA DRA*	26	MHC class II DR chain	T-cell activation	Yes	Up
RPS14	27	NA	NA	No	
COX3	28	cytochrome c oxidase subunit III	NA	No	
ENSCAFG00000032276	29				
ENSCAFG00000004916	30	NA	NA	No	
ENSCAFG00000029904	31	NA	NA	Yes	Up
ENSCAFG00000013979	33	NA	NA	No	
TPT1	34	Translationally controlled tumor protein	Several roles (cancer development, cell cycle, heat shock, among others)	No	
S100A12*	35	Calgranulin C	Zinc and transition metal sequestration	No	
TNFAIP6*	36	TNF alpha-induced protein 6	Hyaluronan-binding domain, induced by proinflammatory cytokines	Yes	Up
DLA-DQB1*	38	MHC class II DQ beta chain	Antigenic peptide presentation to T helper cells	Yes	Up
ENSCAFG00000032615	39	NA	NA	Yes	Up
RPS27	40	NA	NA	No	
PSAP	41	Prosaposin	Generation of saponins who facilitates catabolism of glycosphingolipids	No	
GGH	42	Gamma-glutamyl hydrolase	NA	Yes	Up
GAPDH	43	Glyceraldehyde-3-phosphate dehydrogenase	involved in glycolysis	No	
ENSCAFG00000004730	44	NA	NA	Yes	Up
CD74*	45	HLA class II gamma chain	T-cell activation	No	
ND4L	46	NADH dehydrogenase subunit 4L	Respiration	No	
CSTB*	47	Cathepsin B	C1-peptidase	No	
CTSS*	48	Cathepsin S	C1-peptidase	Yes	Up
ENSCAFG00000030164	49	NA	NA	No	
DLA-DQA1*	50	MHC class II DRQ alpha chain	T-cell activation	Yes	Up

Table 4. Go term enrichment for shared genes between RNA-seq and the Microarray of Vanherbergen *et al*. 2012 [14].

Term		GO space	Expression in Microarray	p-value
GO:0002376	immune system process	BP	Up	$3.66 * 10^{-42}$
GO:0044459	plasma membrane part	CC	Up	$4.38 * 10^{-9}$
GO:1903561	extracellular vesicle	CC	Down	0.00124
GO:0001906	cell killing	BP	Up	0.00676
GO:0051247	positive regulation of protein metabolic process	BP	Up	0.0075
GO:0006629	lipid metabolic process	BP	Down	0.0113
GO:0042554	superoxide anion generation	BP	Up	0.0148
GO:0006909	phagocytosis	BP	Up	0.0246
GO:0042101	T cell receptor complex	CC	Up	0.0296
GO:0050664	oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor	MF	Up	0.0296
GO:0016126	sterol biosynthetic process	BP	Down	0.0497

DISCUSSION

Whole genome transcriptome analysis of the sino-nasal mucosa in dogs affected by SNA had a "core" transcriptome profile composed of 44% of the total number of detected transcripts and were enriched in immune-related GO and Kegg terms. Comparing four transcriptomes from three dogs showed that the general and immune transcriptome of the dog that was infected twice (i.e. in 2010 and 2012) were most similar despite the fact that it was infected by a different strain. In contrast, the transcriptomes of two different dogs with the same species were most different. Apparently, the mucosal transcriptome of the host is determined by the genetic differences between the breeds of the dogs. Breeds are defined as intraspecies groups with common physical traits. This man-controlled selection has resulted in a different degree of loss of genetic variability, as indicated for instance by microsatellite analysis [25] and genome analysis [26]. Moreover, haematological parameters can be specific for breeds and vary with age and sex [27, 28]. Our observation that the mucosal transcriptomes might be breed specific impacts differential gene expression analysis. Transcriptomes of a canine patient with SNA should be compared to a control of the same breed and pooling of RNA from different breeds should not be employed. This, in fact, may have resulted in the large variation in gene expression that was previously observed in canine patients [14]. Comparing differential gene expression in different breeds or individuals within the same breed may allow the identification of genetic factors that explain susceptibility to SNA.

A total of 32% (307 out of 948) of the differentially expressed genes resulting from microarray analysis were also expressed in our RNA-Seq data. This confirmed the predominant Th1 response [13, 14]. In addition, our data imply a dysregulation of the Th17 response that was shown to function in clearance of mucosal *Candida* infections [29]. Although factors related with the Th17 response (i.e. IL-6 and TGF-ß) were shown to be upregulated in SNA [30], IL-6 and IL-22 were not detected in our study and in microarray expression analysis [14]. IL-6 was shown to regulate the balance between IL-17-producing Th17 cells and regulatory T cells [31] and its absence would explain the absence of expression of IL17. The absence of IL-22 affects not only the proinflammatory response but also eliminates its protective role in tissue damage and inflammation [32, 33]. More research is required to investigate how a dysregulated Th17 response in canine patients contributes to chronic mucosal infections and tissue damage by *A. fumigatus.* For example, the hyphal cell wall component galactoaminogalactan (GAG) has been reported to have a role in the inhibition of proinflammatory responses (Th1 and Th17 related cytokines) on a model of invasive aspergillosis via induction of IL-1 receptor antagonist (IL-1Ra) [34]. We did detect expression of the UGE3 gene required for the formation of GAG in the fungal transcriptomes (Valdes *et al.* 2019 Chapter 3) but, importantly, we did not detect expression of the IL-1 receptor antagonist (IL-1Ra) suggesting alternative ways of Th17 response regulation in SNA.

Evidence was found for a role of nutritional immunity in SNA. High expression levels were detected of transcripts encoding the iron storage protein ferritin and proteins belonging to the S100 family also called alarmins, especially A8/9 (calprotectin) and A12 (calgranulin C). These proteins bind Ca^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} or Mn^{2+} at their dimer interface and, if released at the site of infections, control growth of pathogens by imposing nutritional immunity [35]. The A8/9 and A12 proteins have also a proinflammatory role and are abundantly expressed in a variety of cells. S100 A8/A9 is expressed in immune cells like neutrophils and monocytes, while S100 A9 can also be expressed in healthy mucosal tissues [36, 37]. Besides their role in nutritional immunity, they are involved in a large variety of cellular activities including cellular differentiation and migration, metabolism, cell death and in inflammation [38, 39] If released by immune cells in a non-programmed apoptotic process they contribute to inflammatory responses by recruitment and activation of immune cells [40]. Depending on the amount released, they can create a cytokine storm. The expression of a broad variety of cytokines in this study may be related to the action of the alarmins. The nasal tissue destruction may be due to high infiltration and activation of immune cells in addition to osteoclast differentiation and the action of fungal cytotoxic metabolites (Valdes *et al.* 2019 Chapter 3). In this context, it is interesting to mention that we find relative high expression of IL-1ß, which is co-released with S100 proteins during inflammasome activation [41]. This is in agreement with a strong inflammatory response from the mucosa to the fungi.

The transcript encoding β2-microglobulin B2M was found to be highly expressed in our study. B2M associates with the major histocompatibility complex (MHC) class 1–like protein HFE, which is expressed at low levels in our analysis. HFE is required for cell surface expression and forms a stable complex with the transferrin receptor 1. Mutations in HFE affects iron homeostasis [42] via regulation of hepcidin levels, which is regarded as the master hormonal regulator of iron metabolism [43]. Moreover, local production of hepcidin by macrophages and neutrophils upon bacterial infection has been linked to limited Fe availability at the site of infection. This is regulated via PRR TLR4 [44] that also recognizes *O*-linked mannan of fungi. TLR4 polymorphisms in humans were previously correlated with aspergillosis and keratitis [45, 46]. Whether local hepcidin regulated iron homeostasis plays a role in SNA needs further investigation since the expression of the encoding gene was not observed in the current analysis. In addition, the observed down-regulation of LTF might be linked to locally limit the iron availability together with increased intracellular storage with ferritin.

Interestingly, indoleamine 2,3-dioxygenase (IDO1) was found to be upregulated in SNA. This enzyme is involved in the conversion of tryptophan into the immunomodulatory kynurenine. This compound is involved in the development of allergic reactions to *A. fumigatus* [47] and prevention of invasive aspergillosis [48]. Taken together, it is concluded that a combination of nutritional immunity, S100 protein-mediated effects on immunity and tissue damage together with the absence of a Th17 response appear to play an important role in the development of this chronic-destructive sino-nasal aspergillosis. A breed-specific defect in mucosal immunity might be an underlying predisposing factor.

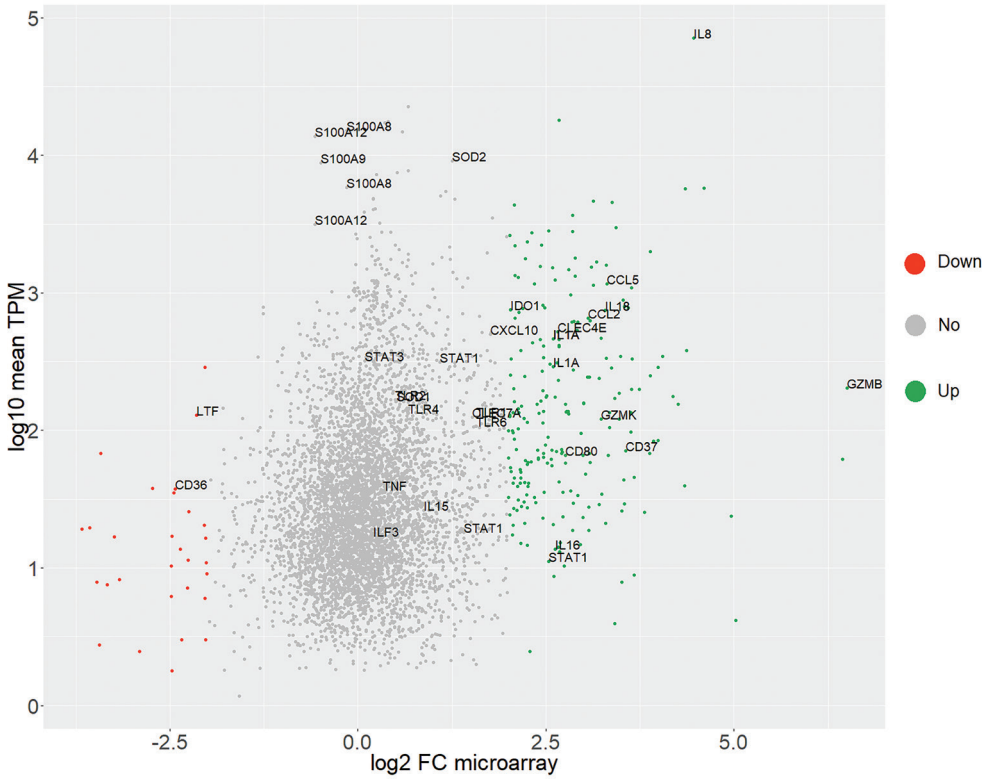

Figure 3. Comparison between the RNA-seq data of this study and the microarray from [11]. Shared transcripts between our RNA-seq data and found to be upregulated, down regulated and not differentially expressed (log 2 FC >=2) in the microarray are in green, red, and grey, respectively. Transcripts mentioned in the Results and/ or Discussion section are labelled.

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SUPPLEMENTARY MATERIAL

Supplementary material S1. Expression data, mapping of expressed transcript into InnateDB, GO-Profiler results, and comparison with Microarray of [14].

Chapter 5

Increased mutation rate and heterogeneity of *Aspergillus fumigatus* in sino-nasal biofilms in infected dogs

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ABSTRACT

Aspergillus fumigatus causes a variety of infections in humans and animals. For instance, it causes chronic sino-nasal aspergillosis (SNA) in dogs by forming a biofilm covering the mucosal surface. It was previously shown that dogs are infected by a single genotype. Here, we assessed whether *A. fumigatus* acquires mutations when exposed to the sino-nasal environment of the dog. Single-nucleotide polymorphisms (SNPs) were identified by whole genome sequencing (WGS) of 26 isolates from nine dogs suffering from SNA using the Af293 genome sequence as reference. Eight environmental *A. fumigatus* isolates were included as control. A total of 28 isolates contained on average around 45.000 non-synonymous (ns)SNPs but 6 isolates from a total of 3 dogs contained approximately 2.5-fold more mutations. The high SNP isolates formed a closely related subclade, even though they originated from 3 genotypes. This indicates that the 6 isolates had acquired similar genetic changes. NsSNP's within the 26 dog isolates were enriched in secondary metabolite gene clusters and in genes associated with stress conditions in the host. Part of these nsSNP's were shared suggesting they resulted from in host selection pressure. Strains also contained unique alleles, which would explain the variation in growth between the isolates that was observed under stress conditions (H_2O_2, pH, or high copper). Together, we show here for the first time a large genetic and phenotypic heterogeneity in a fungal biofilm of a dog. We propose that such a biofilm at the sino-nasal mucosal surface is composed of a mixture variants of *A. fumigatus* as a result of in-host adaptation.

INTRODUCTION

Aspergillus fumigatus is a cosmopolitan fungus that causes several types of infections in human, ranging from acute invasive to chronic non-invasive forms. It is most notorious for causing invasive aspergillosis (IA) in immunocompromised patients. IA is a severe acute pulmonary infection, with mortality rates ranging from 40% to 90% [1]. A chronic, non-invasive form of infection is known as aspergilloma. In this case, the fungus forms a fungal ball in pre-existing lung cavities [2]. *A. fumigatus* is also a major respiratory pathogen in poultry [3] and seemingly healthy dogs and cats [4]. In dogs with a chronic infection called sino-nasal aspergillosis (SNA), *A. fumigatus* forms a biofilm (i.e. fungal plaque) in the nasal cavity up to the frontal sinus [5-7]. Inside the host, the fungus is exposed to the sino-nasal mucosal immunity which encompasses both nutritional immunity and innate- and adaptive immune responses by epithelial and immune cells [8]. Antimicrobial effector molecules like lactoferrin, antimicrobial peptides, reactive oxygen species (ROS), and chemo- and cytokines are involved in the defense response. On the other hand, *A. fumigatus* has an arsenal of mechanisms to withstand the host response. For example, it produces superoxide dismutase and catalase [9], which neutralize ROS [10]. Furthermore, siderophores, melanin and secondary metabolites (SM) like gliotoxin promote survival of *A. fumigatus* in the mammalian host. Siderophores are used to acquire iron in the host tissue [11], while DHN-melanin, the grey-green pigment of *A. fumigatus* conidia, has multiple roles in protection against killing by immune cells [12, 13]. Gliotoxin is cytotoxic due to interference with the glutathione pool in cells [14] and due to inhibition of NADPH-oxidase thereby affecting ROS production in neutrophils which is required to induce NETosis to inhibit hyphal growth [15, 16].

Survival in the host may also be caused by in-host mutations. Hagiwara *et al.* [17] studied genome dynamics of serial isolates of *A. fumigatus*. Genome sequencing of 13 *A. fumigatus* isolates recovered from a human patient with invasive pulmonary aspergillosis (IPA) and a patient with aspergilloma over a time frame of 3 days and 1 year, respectively, revealed 22 SNPs. Interestingly, a *cyp51A* mutation, indicative of resistance to itraconazole, had been acquired in the patient with aspergilloma. Indeed, resistance to itraconazole was observed at later stages of the infection.

A remarkable phenotype of *A. fumigatus* from patients with a chronic infection is the appearance of whitish, low sporulating isolates after prolonged growth in the host [17] . However, no SNPs were described explaining this phenotype. Ballard *et al.* [18] described that 6 out of 13 isolates from a patient with chronic granulomatous disease (CGD) showed poor conidiation. Interestingly, sporulation by some of them could be restored in the presence of itraconazole. Moreover, 248 nsSNPs were found at later stages of infection that were absent in earlier ones. For instance, a nsSNP was found in the *pksP* gene (Afu2g17600) required for DHN-melanin production but the impact on functionality of PksP was not assessed. It remains to

be determined how conidiation phenotypes links to in-host adaptation and which adaptive mechanisms are involved in such clinical isolates.

We previously characterized a set of clinical *A. fumigatus* isolates from dogs with SNA and concluded that each individual dog had been infected with a single strain (i.e. a single genotype) [19]. In spite of this, isolates did display phenotypic variability e.g. in pigmentation. The fact that in our study [19] phenotypic variability was hardly observed in isolates from human and from indoor and outdoor substrates suggested that the dog sinus is a highly selective environment inducing in-host adaptations resulting in phenotypic evolution. The fungus is expected to experience nutrient stress, host immune responses, and limited oxygen availability. These types of stress may act as a selection pressure. Here, a SNP analysis was performed to assess whether genetic changes occur that are associated with in-host adaptation to survive the sino-nasal environment. Six isolates from 3 dogs contained approximately 2.5-fold more nsSNPs when compared to 20 other dog isolates and 8 environmental strains. In addition, it was found that nsSNPs of the 26 dog isolates were enriched in secondary metabolite gene clusters and genes associated with stress conditions in the host. In contrast, environmental isolates were not enriched in gene function. A part of the nsSNPs were shared between isolates from different dogs suggesting similar adaptation mechanisms. Over and above this, strains have their own SNPs, which would add to the high phenotypic heterogeneity between the isolates when grown under stress conditions (H_2O_2, different pH values or high copper). Together, results show that growth of *A. fumigatus* in the sino-nasal environment of a dog is accompanied with in-host adaptation via genetic changes resulting in genetic heterogeneity within the infecting isolate.

MATERIALS AND METHODS

Strains

A total of 26 isolates from fungal plaques of 8 canine SNA patients (CP1-8) were used in this study [19] using 8 environmental isolates as a control (Table 1).

Table 1. *A. fumigatus* isolates used in this study.

	Number of isolates	Source	Origin	Reference/Year of isolation
Environmental	8	Westerdijk Institute Utrecht	The Netherlands, Indoor air	Valdes *et al*. (2018)[19]/ 2005-2008
Dog	26	Microbiology, Utrecht University	The Netherlands	Valdes *et al*. (2018) [19]/2010-2012

Culturing

Strains were grown at 37 °C on potato dextrose agar (PDA). Spores were harvested with Milli-Q® (Millipore Corp) water and filtered through 3 layers of sterile Miracloth (Merck, Darmstadt, Germany). They were diluted to 1 x 10^8 ml^{-1} and stored at 4 °C for maximally two weeks.

Genomic DNA isolation

Genomic DNA was isolated using a modification of the protocol described previously [20]. Briefly, 200 µl conidial suspension (1 x 10^8 conidia ml^{-1}) was seeded on PDA and cultured for 7 days as described [21]. A loop of conidia was used to inoculate 20 mL of potato dextrose broth in a 9-cm-wide Petri dish. After incubation for 3-4 days, mycelium was harvested by filtration over 3 layers of sterile Miracloth (Merck, Darmstadt, Germany), washed 3 times with sterile H_2O, and lyophilized. Mycelium was homogenized using mortar and pestle and ~30 mg was used for DNA isolation using the Qiagen DNeasy PowerPlant Pro Kit following manufacturer's recommendations for problematic samples. DNA concentration and quality was checked using the Qubit® assay system.

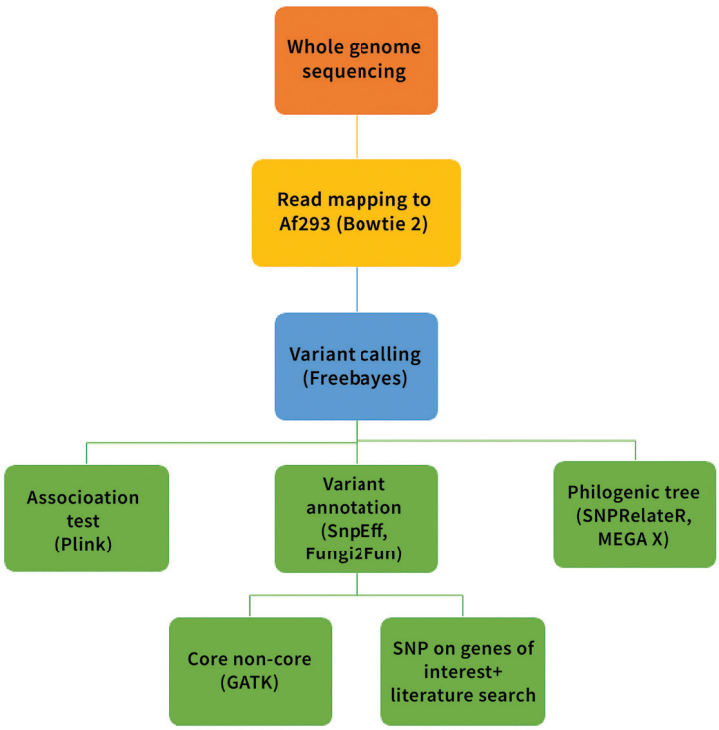

Figure 1. Scheme of the workflow of mutation analysis of *A. fumigatus* dog isolates.

Whole-genome sequencing

Whole genome sequencing was performed by the Utrecht sequencing facility. Briefly, libraries were prepared using Truseq DNA Nano library and sequenced on an Illumina NextSeq500 with150 bp pair end mid output configuration.

Sequence analysis

Quality of raw reads was checked using fastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Cleaning and trimming were performed using Fastx-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Reads were mapped to the genome of reference strain Af293 (Genome version from AspGD: s03-m05-r07 http://www.aspergillusgenome.org/) with bowtie2 v2.2.9 using options end to end and very sensitive. SAMtools v1.3 was used for further quality control. Freebayes v0.9.10-3 [22] with option ploidy 1 was used for variant calling. Post filtering of the vcf file was performed using vcfilter (" qual > 20", depth 5X).

Identification of variants related to *A. fumigatus* evolution in the host

Mutation analysis in dog isolates was performed using association tests, core / non-core SNP analysis, and variant selection and annotation per group of interest (Figure 1).

Association tests. PLINK 1.90b5.3 [22] was used to perform association tests to identify possible relations between the previously reported green/white phenotypes of the strains [19] or source of the isolates (dog / environmental). The closest-features tool in bedops [23] was used for the identification of SNPs within the upstream regions (max 1 kb) of annotated genes in Af293.

Variant annotation. The filtered vcf file was used as input for SnpEff v4.3k in order to predict the effect of the variants [24]. For downstream analysis HIGH (gain or loss of stop codon, splice region variant) or MODERATE (missense) effects were used.

Core / non-core variants. A variant that was found in all isolates of a group was considered a core variant, while variants that were different in at least one isolate were classified as non-core variant. FungiFun2 [8] was used to perform FunCat enrichment of core/non-core variants having a HIGH impact (for example loss of start or stop codon).

A targeted search of variants affecting genes of interest

SNPs present in genes of interest [25-27] were compared to whole genome sequencing data of 66 isolates of *A. fumigatus* [28]. To this end, the full annotated vcf file (environmental and dog isolates) was used and SNPs with a HIGH and MODERATE effect were taking into account.

Phylogenetic tree

A phylogenetic tree was constructed based on the filtered biallelic SNPs with no missing data using the R package SNPRelate v3.7 [29]. The SNPs were pruned using thresholds reported for *A. fumigatus* (linkage disequilibrium threshold of 0.8 and a minor allele frequency of 0.03) [28]. MEGA X [30] was used for the calculation of the phylogenetic tree. Briefly, a multi-fasta file with the SNP per strain was aligned with MUSCLE [31] and the resulting alignment was used for statistical test of best-fit model of nucleotide substitution. The Generalised Time-Reversible (GTR) model resulted in the best fit using the corrected Akaike information criterion (AICc). A total of 3826 pruned marker SNPs were used to build a maximum likelihood phylogenetic tree based on the GTR substitution model with 1000 bootstrap replicates.

Phenotyping

pH growth test. Minimal agar medium [32] with 25 mM glucose (GMMA) was titrated at pH 5.0, 6.5, 7.2 and 8.0 [33] (see Supplementary material S1). Wells of 12-well plates (Greiner Bio One International GmbH) were filled with 2 ml medium, inoculated with 10^3 conidia in the centre of each well, and incubated at 37 °C. Colony diameter was measured after 24 and 48 hours using biological duplicates.
Copper stress. Copper excess agar (GMMA-Cu containing 60 mM glucose and 0.9 mM Cu^{2+}) was prepared by dissolving 0.015 g anhydrous copper sulfate (Merck, Darmstadt, Germany) in 47 ml MM (Supplementary material S1), followed by addition of 3 ml 1 M glucose. GMMA-Cu (2 ml) was added to wells of 12-well plates, inoculated with 10^8 conidia in the centre of the wells and incubated at 37 °C up to 4 days. GMMA (containing 1.25 µM Cu^{2+}) was used as negative control. Colony size was determined in biological triplicates.

Hydrogen peroxide stress. A two-layered agar plate (60 mm Petri dish) was used containing 100 µl 500 mM H_2O_2 in a 5 mm wide hole in the centre. The bottom layer consisted of 10 ml GMMA and a top layer of 5 ml GMMA containing 5 10^7 conidia (conidia were always two weeks old). After 16 hours of incubation, diameter of inhibition zones (as biological triplicates) was determined using ImageJ 1.52a [34].

RESULTS

Variant calling

Genomic DNA of 34 isolates was sequenced with an average 23.61-fold coverage (Supplementary material S2) and compared to the Af293 reference genome [35]. The variation of the genomes of the isolates ranged between 0.11% and 0.3% relative to Af293. The SNPs were regularly distributed along the 29,388,377 bp Af293 genome Biallelic SNPs were the most prevalent variant (average number 56064), while multiallelic SNPs (average number 219) were much less frequently detected (Supplementary material S3). Interestingly, the genomes of six isolates from a total of 3 dogs contained 2.5 fold more biallelic SNPs when compared to the other dog and environmental isolates (Figure 2).This high SNP number was generally distributed all along the genome (Supplementary material S4).Three out of the 6 isolates had been isolated from dog CP2 from which also an isolate had been obtained with a low SNP incidence (Figure 2).

Phylogenetic tree

Clades A-D were identified using a SNP-based phylogeny (Figure 3). Clades B and D contain both environmental and dog isolates, while clades A and C contain canine isolates only. The isolates of clade A are derived from patients CP1, CP6 and CP8 [19] and have a very small genetic distance based on the SNP-based phylogenetic tree. This is in agreement with genotyping based on STR*Af* (Supplementary material S2). Clade B contained isolates of CP3 only, while clade C contained isolates of CP7 and 8.2 and clade D of CP2, CP4, CP5, and CP8. In general, clustering was in line with microsatellite analysis [19] with two exceptions. Isolates DTO 271-A9 (from CP2) and DTO 303-F3 (from CP8.2) are now in subclade D1 but were expected to be in subclade D2 and cluster A, respectively, based on the micro-satellite analysis. The six high SNP isolates form subclade D2 even though they have different genotypes based on STR*Af* [19]. This may be due to the selection pressure within the dog.

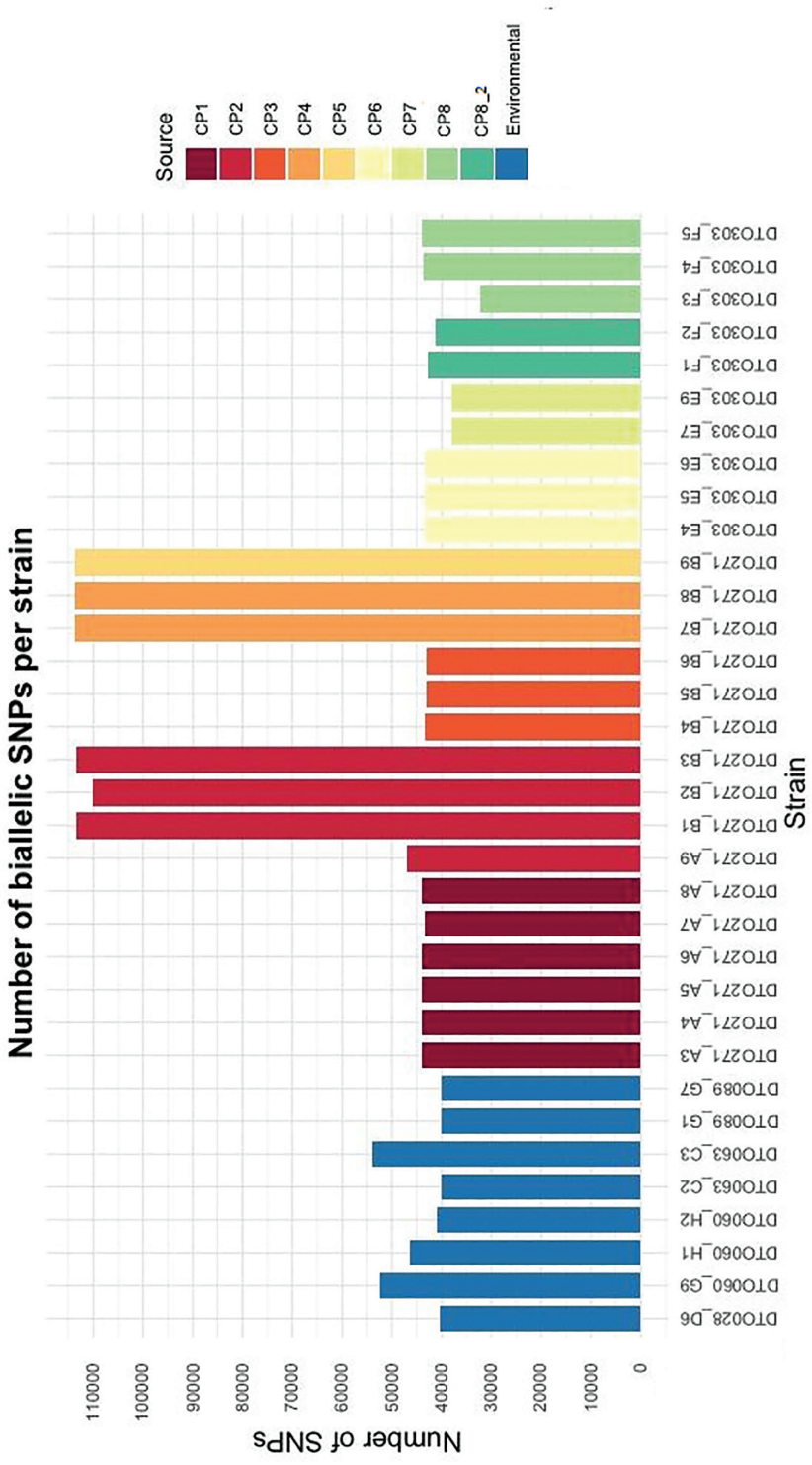

Figure 2. Number of biallelic SNPs per *A. fumigatus* isolate.

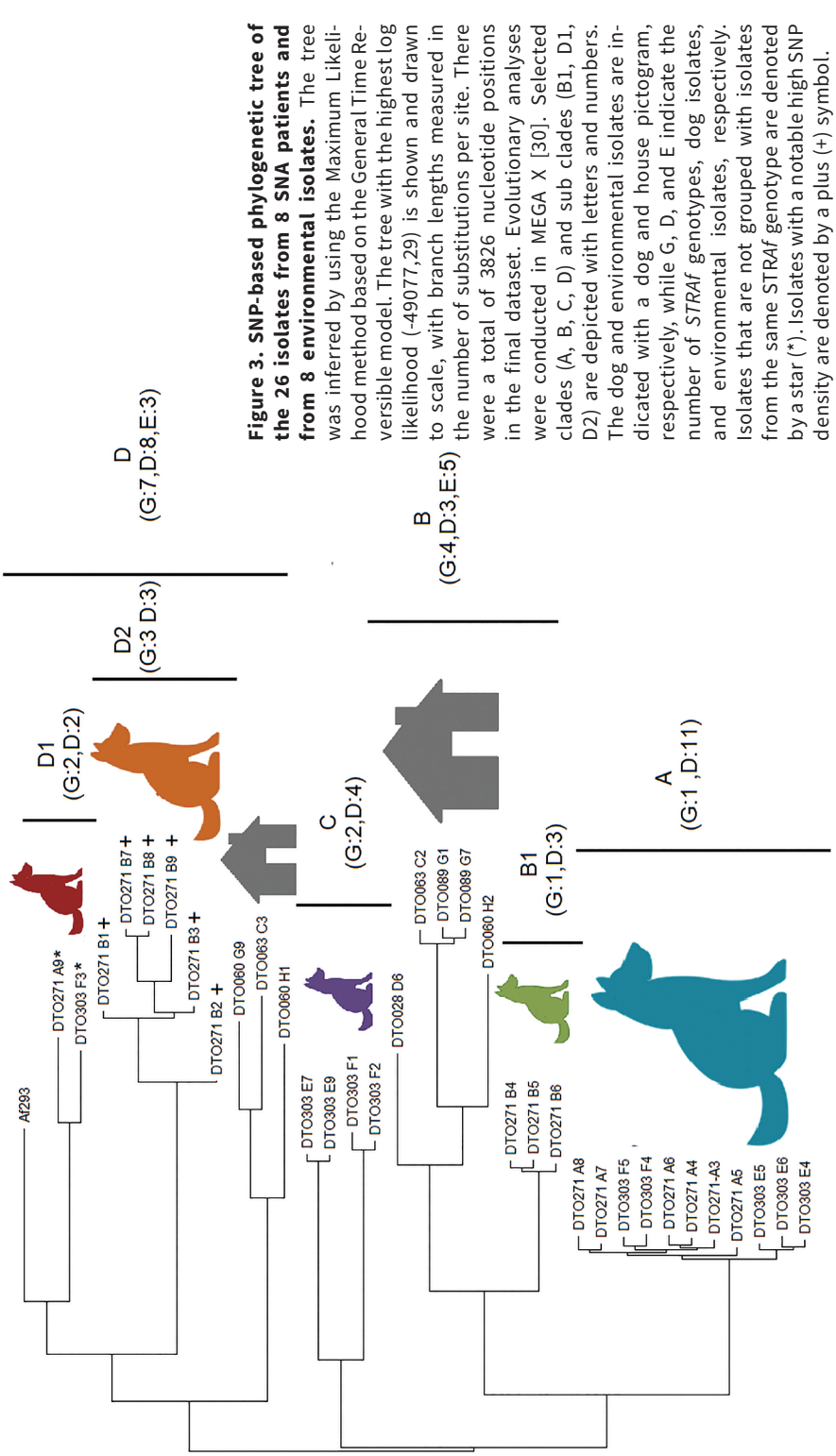

Figure 3. SNP-based phylogenetic tree of the 26 isolates from 8 SNA patients and from 8 environmental isolates. The tree was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-49077,29) is shown and drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 3826 nucleotide positions in the final dataset. Evolutionary analyses were conducted in MEGA X [30]. Selected clades (A, B, C, D) and sub clades (B1, D1, D2) are depicted with letters and numbers. The dog and environmental isolates are indicated with a dog and house pictogram, respectively, while G, D, and E indicate the number of *STRAf* genotypes, dog isolates, and environmental isolates, respectively. Isolates that are not grouped with isolates from the same STR*Af* genotype are denoted by a star (*). Isolates with a notable high SNP density are denoted by a plus (+) symbol.

Association tests

A total of 12 SNPs were related to the white phenotype (p-value ≤ 0.01) (Table 2 and Supplementary material S5) but only one was located in an upstream region and one in a coding sequence of genes of unknown function (Table 2). Therefore a functional relationship between the SNPs and the green/white phenotype is not conclusive. Next, an association test was done between the source of the isolates and the SNP data (environmental vs dog) (Table 2, Supplementary material S5). A total of 7 canine-specific SNPs were identified (p-value ≤ 1 10^{-9}). Two SNPs were located in the upstream region of genes Afu8tD(GUC)9 and Afu2g01650 that encode $tRNA^{asp}$ and is involved in folate metabolism, respectively. A synonymous variant of gene Afu611300 (c.2934G>A | p.Gly978Gly) is expected not to affect the gene product (Table 2). The rest of the associated SNP were outside upstream regions of genes or were not located in a coding sequence (Supplementary material S5). Together, no clear dog specific SNPs were detected.

Table 2. Association test between SNP data and the source of the isolate (dog vs environmental) and the white/green phenotype [19] (see also Supplementary material S5).

Chromosome	SNP location	Corrected p-value	Type of test	Distance to gene start	Gene
2	398126	3,289*10 $^{-9}$	Dog / Environment	60	Afu2g01650
8	1409099	3,289*10 $^{-9}$	Dog / Environment	371	Afu8tD(GUC)9
6	2796163	9,093*10 $^{-9}$	Dog / Environment	Afu6g11300	Afu6g11300
6	2991043	0,003435	White / Green	702	Afu6g11970
8	1473863	0,005829	White / Green	Afu8g06170	Afu8g06170

Core / non-core variant analysis

Non-core variants are the predominant type of variant in environmental and dog isolates (Table 3). The 62 core variants in dog isolates were not enriched in a functional gene group as assessed by FUNCAT enrichment analysis using SNPEff. Interestingly, the non-core variants of dog isolates with a HIGH effect in genes were enriched in the functional classes secondary metabolism and C-compound and carbohydrate metabolism. In contrast, HIGH impact variants of environmental isolates were not enriched in particular functions (Table 3). However, an effect of unequal sample size per group in this analysis cannot be discarded. Next, the number of variations between isolates of the same patient was analyzed (Table 4). Since we had only one isolate from CP5 no core/non-core was determined. A relative small amount (125-1458) non-core SNPs were detected in the isolates of each dog except for CP2 and CP8 that contained 95639 and 29163 non-core SNPs, respectively. A subset of the non-core SNPs were within coding regions with a predicted moderate or high impact. The presence of many non-core SNPs in CP2 might be expected since three

out of the four isolates are high-SNP variants. Together, these results show that significant genetic variation occurs between fungal isolates that share the same STR*Af* genotype.

Table 3. Number of total, core and non-core SNPs in environmental and dog isolates. NA indicates no significant enrichment of FUNCAT categories.

Group	N	Total SNP variants	Core/Genes with HIGH impact	FunCat High impact	Non-core / Genes with HIGH impact	FunCat High impact
Environmental	8	33525	0 / 0	NA	33525 / 269	NA
Dog	26	118873	62 / 0	NA	118751 / 654	Secondary metabolism

Table 4. presence of core and non-core SNP in dog isolates. I/P = Isolates per patient, Mod/high account for annotated moderate and high impact. Only patients with more than 1 isolate were divided into core and non-core, while patients with more than 3 isolates were subdivided into unique and common.

I/P	CP	Total SNPs	Core	Non-core	SNPs protein coding region			
					All	Mod/high	Unique	Common
1	CP5	113677	113677		/	/	/	/
	CP4	113640	113515	125	18	14	/	/
2	CP7	38014	37519	495	45	36	/	/
	CP8.2	42752	41307	1445	147	68	/	/
	CP1	43976	42518	1458	277	138	41	97
	CP2	126258	30619	95639	38314	16723	2469	14254
>2	CP3	43587	42702	885	149	81	47	34
	CP6	43660	43155	505	36	22	8	14
	CP8	52003	22840	29163	10773	5367	1883	3484

SNPs in secondary metabolite clusters

Non-core SNPs of dog isolates with high impact are enriched in secondary metabolic gene clusters. A missense mutation (Cys2335Ser) in Fma-PKS (Afu8g00370) (Table 5) encoding a polyketide synthase (PKS) of the fumagillin cluster (cluster 30) is present in 15 out of the 26 isolates present in clades B, C and D but absent in clade A (CP1, CP6 and CP8.2). This nsSNP is not present in 2 out of 8 environmental isolates (i.e. DTO 060-G9 and DTO 063-C3) (Figure 3). Fumagillin is one of the mycotoxins of *A. fumigatus* and has immunomodulatory properties like inhibition of the mucociliary-mediated fungal clearance of the respiratory tracts [36]. Whether this SNP affects the fumagillin function needs to be addressed.

Gliotoxin has various roles in *A. fumigatus* infections [15] and two missense mutations were detected in the cytochrome P450 (CYP450) monooxygenase GliC gene (Afu6g09670) (Table 5) that is involved in the gliotoxin biosynthesis pathway. Interestingly, the nsSNP 1310A>T resulting in the Asp437Val substitution in GliC was absent in 66 isolates [28] and is also absent in the eight environmental isolates described here. However, this nsSNP is present in 19 out 26 dog isolates (Figure 4). Additionally, we found many SNPs in dog isolates causing missense mutations in genes encoding GliT, GliA and GtmA that are involved in transport (GliA), detoxification of gliotoxin (GliT, GtmA), and down-regulation of the expression of the gliotoxin cluster [37-39] . Together, these results indicate that these SNPs affect production of gliotoxin (GliC) and at the same time affect the detoxification mechanisms (GliT, GliA and GtmA).

Various SNPs with HIGH impact were detected in the *pes3* gene *(*Afu5g12730) encoding a multi-modular NRP synthetase of 8,515 amino acids [40] (Table 5). A SNP resulting in a stop codon at amino acid 1590 (Gly1590*) was found in the isolates belonging to cluster D1 (Figure 3) and was absent in the environmental isolates (Figure 4). Similarly, introduction of a stop codon at position 3299 (Trp3299*) was found in isolates of clade C. These modifications could impact development of SNA given the fact that disruption of Pes3 leads to increased virulence possibly due to aberrant innate immune recognition [40].

Fumitremorgins are tremorgenic mycotoxins of *A. fumigatus* but their role in infection is not clear yet. Interestingly, the encoding FTM cluster is inactive in reference strain Af293 because of a Arg202Leu missense mutation in the FtmD gene (Afu8g00200) [41]. All fungal isolates (i.e. both environmental and dog isolates) did not have this missense mutation, indicating that these isolates do produce fumitremorgin.

Stress response and reproduction

We investigated which nsSNPs of the dog isolates are present in genes involved in reproduction and stress response (Table 5, Supplementary material S6). One of the SNPs resulted in a stop codon at Leu87* within the orthologue of the *fgaCat* gene (Afu2g00200). This is a bifunctional catalase-peroxidase involved in the oxidative stress response [25]. Remarkably, this nonsense mutation is present in none of the environmental isolates and only in the 6 high-SNP isolates, which indicate a loss of function of this catalase-peroxidase. Additionally, we found a nonsense mutation in the mycelial catalase CatB/Cat1 (Afu3g02270) present only in dog isolates from CP2 (DTO 271-A9/B1/B2/B3) (Figure 4, Table 5). Two other nonsense SNPs, Gln20* and Gln375*, are present in the orthologous gene of the *A. nidulans* blue light responsive transcription factor LreB (Afu4g12690). This protein is thought to be involved in asexual sporulation in *A. fumigatus* [25]. The first nonsense SNP is present in all isolates of clade A (Figure 2, 3, Table 5), while the latter is present in the 6 high SNP isolates only. Additionally, 132 missense SNPs were found in genes related with stress response, asexual and sexual

regulation (Supplementary material S6). Notably, 24 missense SNPs were present in the light sensing regulator FphA (Afu6g09260) [42], of which 21 are only present in the high SNP isolates. This indicates a difference in light-sensing between isolates [43].

Transcription factors with known roles in pathogenesis

A total of 70 missense mutations were found in 17 transcription factors (TFs) involved in virulence [44] (Table 5; Supplementary material S6). However, no disruptive (stop codons) HIGH impact SNPs were present. The Ser147Pro SNP in TF CpcA was only found in dog isolates of subclade A and both isolates of CP9. SrbB is a TF involved in the early response to hypoxia [45] and showed the Ala113Thr SNP in almost all dog isolates, except for the ones of CP7 and environmental isolates (Figure 3). PacC is involved in pH response and is important in the epithelial entry of conidia and invasive growth [46]. The SNP Arg529Cys was present in all dog isolates, except for the high SNP isolates. One of the other principal regulators of stress response and adaptation in *A. fumigatus* is Yap1 [47] (Afu6g09930). Knock-out strains are sensitive for H_2O_2 [47, 48], while a shorter truncated version of the protein is related with increased resistance to oxidase stress [49]. We found seven nsSNPs in Yap1 (Supplementary material S6), of which five only occurred in dog isolates. Two of them (Figure 3,Table 5) were only present in isolate DTO 271-B9 (Clade D2), suggesting that nsSNPs in this gene might have an impact on oxidative stress response (see below).

Other SNPs in genes of interest

A nonsense SNP (Tyr123*) was observed in the mitogen-activated protein kinase (MAPK) gene MpkC (Afu5g09100) (Supplementary material S6). MAPKs play a central role in signaling of nutrients and environmental stress factors. Thus, mutants defective in MAPK signalling show reduced virulence [50]. The mutation was observed in the 6 high SNP isolates only. Another high impact SNP was only present in the 6 high SNP isolates and results in the loss of the stop codon (* 1270Gln) of Gin4 (Afu6g02300). This gene is involved in conidiation, apical compartment length and virulence in an invertebrate and intranasal murine infection model of IA [51].

One mechanism of the immune system to attack pathogens is the generation of reactive oxygen intermediates via for example the Fenton reaction with transition metals like copper as a catalyst [52]. Mechanisms to transport such metals out of the cell is vital for the survival of the fungus inside a host. Gene AceA (Afu6g07780) encodes a transcription factor involved in the copper toxicity response and regulates the copper transporters CrpA (Afu3g12740) and Ctr2 (Afu3g08180) [53]. Interestingly, SNPs were found in AceA and CrpA in both environmental and dog isolates (Supplementary material S2). In the case of CrpA (Figure 3, Table 5), one SNP was found that was present in 18 dog isolates, and one which was only present in isolates DTO 303-E7 and E9 (clade C). The latter two dog isolates also contained a

MODERATE impact nsSNP in Ctr2 (Figure 4, Table 5). These mutations suggest that in-host adaption to copper stress occurs in dogs.

We did not find alterations in the *cyp51A* gene in the environmental or dog isolates which are in accordance with our previous observations that none of these dog isolates showed resistance to three tested azoles.

Micro- evolution in the host

NsSNPs previously described in 13 *A. fumigatus* isolates taken from a patient with CGD over a 2 year-time frame [18] were compared with the nsSNPs from dog and environmental isolates (Supplementary material S6). We found an amino acid change Thr214Ala in the exo-alpha-sialidase Afu4g13800, this protein has been proposed to have a role in the interaction between *A. fumigatus* and its host. This missense SNP is absent in the environmental isolates but present in the six high SNP variants and in DTO 303-F1 and F2 in clade C and D, respectively (Figure 2,3). In contrast [18], we did not detect SNPs in Afu6g14720 encoding a putative telomere-associated RecQ helicase and which in human isolates had a broad variety of nucleotide changes (Supplementary material S6).

Table 5. Selected SNPs with resulting amino acid change, its potential effect and frequency in environmental and dog isolates.

Process / molecule	Gene	Nucleotide	Protein	Environmental	Dog	Possible effect
Fumagillin	Fma-PKS (Afu8g00370)	c.7003T>A	p.Cys2335Ser	6	15	Inhibition of production of metabolite
Gliotoxin	GliC (Afu6g09670)	c.1310A>T	p.Asp437Val	0	19	Production of intermediate species changed
	GliT (Afu6g09740)	c.946C>A	p.Leu316Ile	1	19	Production of active gliotoxin changed
		c.769G>A	p.Val257.Ile	0	19	
		c.635C>G	p.Ala212Gly	0	19	
	GliA (Afu6g09710)	c.867G>T	p.Trp289Cys	1	13	Transport of gliotoxin changed
		c.1279C>T	p.Arg427Cys	1	3	
		c.1612c>T	p.Pro538Ser	0	19	
	GtmA (Afu2g11120)	c.746A>G	p.Glu249Gly	0	6	Detoxification of gliotoxin changed
		c.851G>C	p.Gly284Ala	1	17	

Process / molecule	Gene	Nucleotide	Protein	Environmental	Dog	Possible effect
NRP	Pes3 (Afu5g12730)	c.4768G>T	p.Gly1590*	0	2	Inhibition of production of metabolite
		c.3934C>T	p.Gln1312*	8	18	
		c.3719G>A	p.Trp1240*	2	0	
		c.23359C>T	p.Gln7787*	4	14	
		c.9500T>A	p.Leu3167*	2	0	
NRP	Pes3 (Afu5g12730)	c.18229G>T	p.Glu6077*	5	14	Inhibition of production of metabolite
		c.9896G>A	p.Trp3299*	0	4	
		c.9896G>A	p.Lys208*	8	18	
Fumitremorgin	FtmD (Afu8g00200)	c.605T>G	p.Leu202Arg	8	26	Production of metabolite
Exo sialidase	Afu4g13800	c.640A>G	p.Thr214Ala	0	7	Host recognition changed
pH response	PacC (Afu3g11970)	c.1585C>T	p.Arg529Cys	0	20	Changes in response to pH
		c.1418G>C	p.Gly473Ala	0	6	
		c.1933T>C	p.Ser654Pro	0	6	
Catalase	fgaCat (Afu2g00200)	c.260T>A	p.Leu87*	0	6	Protein non-functional
	Cat2 (Afu8g01670)		p.Lys67Thr	0	11	Changes in the function of the protein
	CatB (Afu3g0227)	c.361A>T	p.Ile121Phe	0	4	Changes in function of protein
Hypoxia	SrbB (Afu4g03460)	c.397G>A	p.Ala133Thr	0	24	changes in response to hypoxia
Amino acid metabolism	CpcA (Afu4g12470)	c.439T>C	p.Ser147Pro	0	4	Amino acid homeostasis changed
Light sensing	LreB (Afu4g12690)	c.58C>T	p.Gln20*	0	11	Light induced morphogenesis changed
		c.1123C>T	p.Gln375*	0	6	
Hyphal morphology	Gin4 (Afu6g02300)	c.3808T>C	p.*1270Gln	0	6	Hyphal ,conidiation and virulence in murine model altered
Stress response	Yap1 (Afu6g09930)	c.1249C>T	p.His417Tyr	0	1	Stress response altered
		c.1004C>T	p.Thr335Ile	0	1	
Copper transport	CrpA (Afu3g1274)	c.2480C>G	p.Ala827Gly	0	2	Export of copper improved
		c.585G>C	p.Gln195His	0	18	Export of copper changed
	Ctr2 (Afu3g08180)	c.539C>T	p.Ala180Val	0	2	Import of copper improved

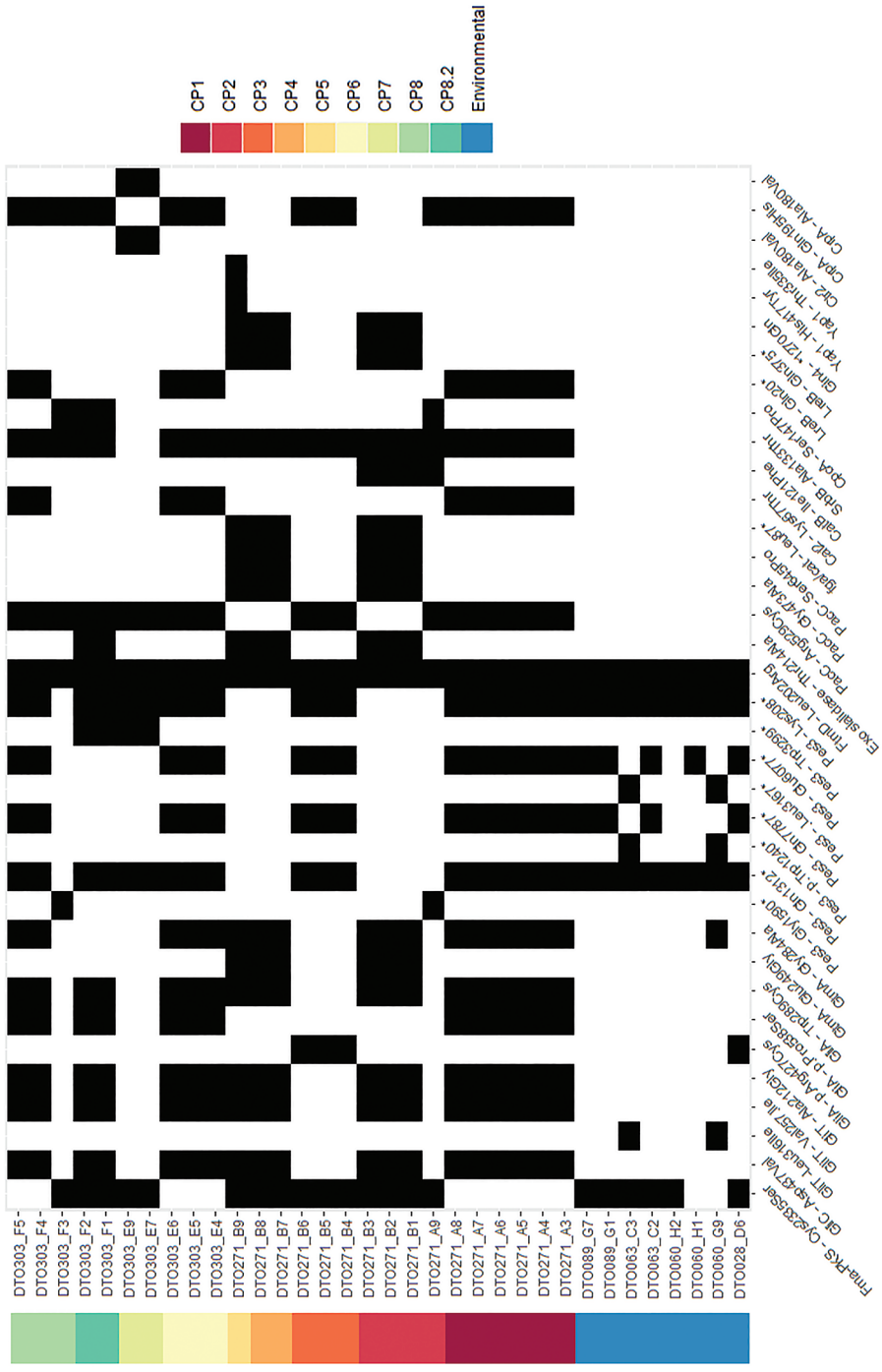

Figure 4. View of the SNPs in Table 4 per strain. SNPs present in an isolate are indicated by black shading of the cell. The origin of the isolates are depicted as a coloured bar to the left.

Phenotyping

Growth of the dog isolates was assessed during oxidative (hydrogen peroxide), pH, and copper stress.

Hydrogen peroxide sensitivity of fungal isolates

Dog isolates were on average more sensitive to H_2O_2 than the reference strain Af293 (data not shown). Moreover, differences in H_2O_2 sensitivity were observed between isolates from the same fungal plaque. Six isolates (DTO 271-A6 /E5/B4/E7/B7/B9; clades A, B1, C, D2) derived from canine patients (CP1, CP3-CP7, respectively) were very sensitive to H_2O_2 (inhibition zones > 35 mm) as compared to Af293 (~27mm) and the other dog isolates (inhibition zones > 27 mm and < 35 mm) derived from different canine patients (CP1, CP3-CP7, respectively) (Figure 5). SNPs could not be related to H_2O_2 sensitivity. For example, the SNP conferring a stop codon (Leu87*) in the catalase-peroxidase fgaCat (Table 5, Supplementary material S2) was present in isolates DTO 271-B1/B2/B3/B7/B8/B9 (clade D) but only two of them were considered as highly sensitive (inhibition halo > 35 mm) (Figure 5). A similar situation was observed for the SNP in the conidial catalase Cat2 (Afu8g01670) (Table 5).

Fungal growth at different pH values

High variation in growth was observed between dog isolates when cultured at pH 5.0, 6.5, 7.2 or 8.0. Notably, 21 and 3 dog isolates showed >20% more growth at pH 8 and pH 5, respectively Especially, growth at pH 5 for 24 h was very poor for the vast majority of fungal strains. However, after 48 h of growth the difference in colony diameters was considerably reduced when compared to the other pH values (Figure 5). The Arg529Cys SNP found in PacC could also not explain the different growth behavior of isolates. For instance, this SNP was found in several isolates but only DTO 271-B9 did not grow after 24 hours at pH=8.0 (Figure 5).

Copper stress

Isolates from the environment did not grow at all on high copper medium during a 4 day period (data not shown). In contrast, growth at high copper concentration was observed after 4 days in 15 out of 26 dog isolates. These colonies were generally small except for DTO 303-E9 (CP7) that was similar to Af293 (Figure 5). Apparently, these two latter strains are the most resistant but 14 other isolates also showed resistance to high copper. SNPs causing amino acid substitutions in the copper transporters Ctr2 (Afu3g08180) and CrpA (Afu3g12740) are present in isolates DTO 303-E7/E9 (Table 5, Figure 3) that show high copper resistance after 24 and 240 h of growth, respectively. This suggests relationship between copper resistance and the presence of the SNPs.

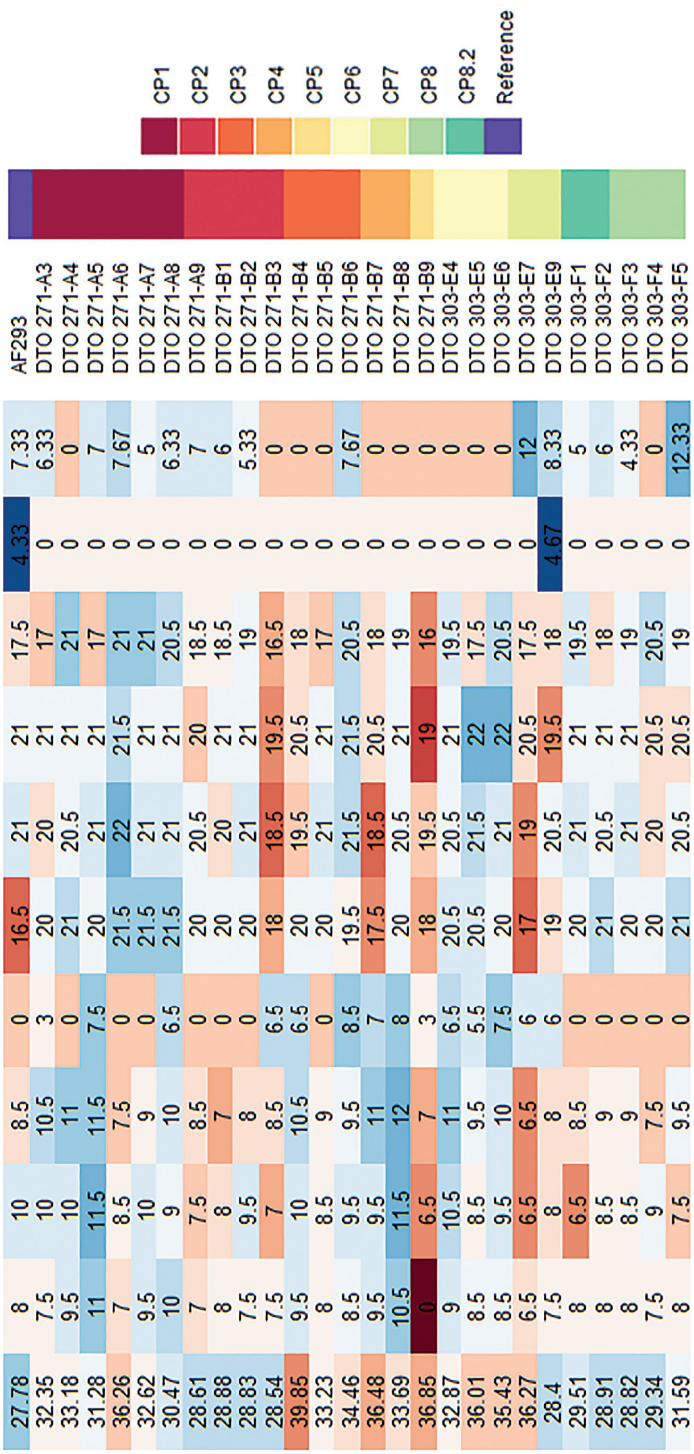

Figure 5. Overview of phenotyping. the color of the cells corresponds to a z-score of the test, (red = poor growth, blue = good growth, white = average growth). Note that in the case of H_2O_2 the colour relates to the size of the inhibition halo with a red color indicating large inhibition. Average measurements per strain are depicted in each cell. The colored bar to the right indicates the origin of the isolate.

DISCUSSION

We here report the analysis of SNPs in isolates of *A. fumigatus* from the environment and from dogs suffering from SNA to address whether mutations contribute to in host adaptation in dogs. A total of 28 dog and environmental isolates contained on average around 45.000 non-synonymous (ns)SNPs. Notably, 6 isolates from a total of 3 dogs contained approximately 2.5-fold more mutations. Remarkably, phylogenetic analyses indicated that the high SNP variants also group together suggesting that they share a relatively larger set of SNPs. It should be noted that we also found 2 high SNP isolates in 63 published *A. fumigatus* genomes . (Data not shown). As such, high SNP variants are not limited to isolates to dogs with SNA. Limited increase of mutations can be advantageous during infection by allowing the selection of variants that are better adapted to grow and survive for instance in the sino-nasal environment. A similar mechanism has been described for *Pseudomonas aeruginosa.* Isolates of this bacterium from patients with cystic fibrosis (CF) showed the presence of hypermutable strains in 37% to 55% of the patients during the course of infections [54, 55]. Mutations in the DNA repair genes *mutS* and *mutY* were shown to cause the hypermutable (mutator) phenotype [56], which was proposed to be benificial to increase fitness under the stress that occurs in CF lung tissue [55].

The presence of high SNP isolates in SNA patients suggest that the dog sinus represents a stressful environment that allows the generation of 'hypermutable strains'. This may explain why 157 nsSNPs were found in 29 genes involved in DNA repair (Supplementary material S7). Yet, none of the nsSNPs had a disruptive high impact on the amino acid sequence level (for example stop codons), but 65 out of the 157 missense SNPs were present exclusively in the 6 high SNP isolates. These high SNP isolates accumulated on average 87 nsSNP in the DNA repair genes while the total average for the other isolates was 40 (Supplementary material S7). Whether the missense mutations in the various DNA repair genes indeed affect and reduce the activity of the DNA repair systems needs to be addressed. However, it is tempting to speculate that the combination of a stressful oxidative environment in the sino-nasal area in combination with a reduced DNA repair activity resulted in the generation of high SNP isolates in dogs. Notably, mutations in the *A. fumigatus* DNA repair kinases Atma (Afu5g12660) and AtrA (Afu4g04760) were sensitive to UV light but more resistant to ROS stress and voriconazole [57]. Thus, a defective DNA repair system can have a trade-off by generating variants with a lower fitness. This may explain why the dog isolates were more sensitive to peroxide.

Fungi use diverse products for defence and nutrient acquisition [58, 59]. Amongst others, secondary metabolites (SM) like toxins, pigments and antibiotics are produced by modular protein complexes encoded by SM clusters. Genome-wide analysis of *A. fumigtatus* indicated strain variation of the structure of SM clusters and 5 different types of polymorphisms were distinguished that can affect the function of these clusters [28]. Remarkably, dog isolates present a high enrichment of variants of high impact in such clusters, while environmental isolates did not. For

example, stop codons in *pes3* were found as well as changes in amino acids in enzymes involved in the production of fumagillin, fumitremorgin and gliotoxin. Some of these metabolites have been shown to have a role in infection and loss of function thus is expected to affect virulence. Yet, it should be realized that loss of function occurs in a fungal plaque with related strains that can complement a phenotype. It may thus be that the 'beneficiary' is sparing energy, while being surrounded by other isolates publicly providing this good. Such a dependent relationship has been proposed as the Black Queen Hypothesis (BQH) [60]. BQH initially tried to explain the absence of oxidative stress genes such as catalases in *Prochlorococcus sp*. These cyanobacteria depended on heterotrophic helpers for survival. The latter produced 'leaky' catalases that not only detoxified the cytoplasm of the producing strain but also lowered the hydrogen peroxide concentration in the extracellular environment [60]. BQH may also occur in fungal plaques as in six out of nine fungal plaques a highly sensitive hydrogen peroxide isolate was found. BQH may also explain the presence of SNPs in genes related with detoxication (GliT,GliA) and regulation of gliotoxin synthesis (GtmA).

Binding proteins like lactoferrin and calprotectin limit availability of ions like manganese, zinc, iron and copper for pathogens in te host [61]. On the other hand, by increasing the local concentration of metals like copper the immune system creates a toxic environment for the pathogen [62]. SNPs were found that can potentially affect copper adaptation; a subset of isolates was less sensitive to high copper possibly caused by changes in export via CrpA or import via Ctr2. The observed increase in copper resistance might also affect resistance to transition-metal mediated hydroxyl radical production [63]. Copper accumulates in macrophages to kill phagocytosed micro-organisms [62]. Yet, hyphae in the biofilms will not be effectively taken up. Possibly, these hyphae are exposed by high copper concentration due to lysis of immune cells. Transition metal ions have a toxic effect by their role in the conversion of peroxide into hydroxy radicals. It is known that this conversion is more effective at low pH because of increased solubility of copper and iron [64, 65]. Thus, increased pH, such as found in the fungal plaques in dogs (i.e. pH 8), will reduce the rate of production of such radicals. Growth at pH 8 of the dog isolates is not compromised and in general even better as compared to Af293 wich was isolated from a human patient suffering from invasive aspergilliosis . This, together with the SNP in PacC in a large set of isolates from dogs suggests adaptation to pH at alkaline growth conditions in sino-nasal fungal plaques [19].

Phylogenetic analysis showed that most of the genotypes identified by STR*Af* overlap with the SNP data set. Two isolates (DTO 271_B9 and DTO 303_F3) did not match the micro-satellite analysis and the SNP data revealed that these isolates were more variable than anticipated. This suggests that variation in microsatellites is not predictive for total genome variation.

Together, we here report the existence of genetic and phenotypic variability of isolates from the same SNA patient. Results indicate that fungal variants appear during growth at the sino-nasal mucosal surface due to selection. We propose that

a fungal biofilm at the sino-nasal mucosal surface is composed of a mixture variants of *A. fumigatus* as a result of in-host adaptation. Future experiments targeting the impact of the detected SNP should focus on the fitness and stability of fungal isolates to address DNA repair defects, production of SM compounds and functionality of the affected genes and corresponding products.

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SUPPLEMENTARY MATERIAL.

Supplementary material S1. Media used for growth tests.

Supplementary material S2. Sequencing coverage and variant content per strain.

Supplementary material S3. Distribution of different types of SNPs in dog and environmental isolates. Green and blue bar represent the number of SNP/INDELS that are different (alternative alleles) and equal (reference alleles) to the reference. Red bar = number of sites that were not detected in the variant calling software

Supplementary Material S4. SNP density pattern observed between high SNP strains and the rest of the strains. two high SNP strains are showed(DTO 271-B2/B9) and DTO 303-F3 which showed the lowest amount of biallelic SNP

Supplementary Material S5. Full association data, between white/green phenotypes, origin of the isolates (Dog environmental) and SNP data.

Supplementary Material S6. nsSNP Occurring in genes related with secondary metabolism, stress response, and transcription factors involved in pathogenesis**.**

Supplementary Material S7. nsSNP occurring in genes related with DNA repair

CHAPTER 6

The sino-nasal war: general discussion

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Non-invasive aspergillosis in the sino-nasal area of dogs (SNA) resembles human chronic non-invasive rhinosinusitis that can be caused by several fungal species [1]. Knowledge of SNA is limited and most studies have focused on diagnostics and treatment of dogs [2, 3] and on the local immune response [4-8]. In this thesis I analyzed the genomes, expression patterns and phenotypes of *A. fumigatus* isolates from individual SNA patients. **Chapter 2** describes a detailed characterization of these fungal isolates. It is shown that each canine patient is infected by a single genotype of *A. fumigatus*. This contrasts human patients where multiple genotypes are recovered from the infected area. The fact that humans and animals continuously inhale conidia of *A. fumigatus* makes the finding of several genotypes in human patients more easy to explain when compared to the situation in the dog. Interestingly, the fungal isolates from the SNA patients do show a large variation in phenotypes. For instance, they demonstrated reduced sporulation and/or pigmentation as well as changes in conidial structure and pigment formation. The observation that isolates with a single genotype shows a variety of phenotypes can be explained by fungal in-host adaptation.

Chapter 3 describes fungal gene expression in the SNA biofilms from canine patients infected by *A. fumigatus*. This study represents the first transcriptome analysis using RNA sequencing of a natural *A.fumigatus* fungal infection. It is shown that 6 % (514) of the *A. fumigatus* genes are expressed in a highly variable manner. This could be due to differences in environmental conditions in the host as well as by epigenetic changes and evolving mutations. Genes encoding secondary metabolites and genes involved in stress response and interaction with the host were part of the genes with highly variable expression. Together, these results suggest that *A. fumigatus* adapts to the stress imposed by the host.

The expression analysis described in **Chapter 4** agrees with previous observations [4-6] showing that both the innate immunity and the Th1 response are involved in the mucosal immune response. Most remarkable is the absence of Th17 response in SNA as evidenced by the absence of production of the pro-inflammatory cytokines IL-17, IL-21 and IL-22 that play important roles in controlling infections [9]. By binding to their cognate receptors these cytokines induce expression of other pro-inflammatory cytokines, attract neutrophils to the site of infection and induce the production of antimicrobial peptides [10]. IL-17 is the landmark cytokine of the Th17 response that is required to protect the mucosa from infection by a variety of microbial species [11] including *Candida* [12], *Cryptococcus neoformans* [13] and *Pneumocystis jiroveci* [14]. Together, the transcriptome analysis suggests that a dysregulation of the T-helper cell response contributes to the chronic state of SNA.

Chapter 5 provides evidence for extensive genetic and phenotypic variability between fungal strains from the same genotype isolated from dogs. Mutations were found in genes related to copper transport, adaptation to pH and oxidative stress. Furthermore, a group of isolates presented a remarkable high number of SNPs probably caused by mutations in genes related to DNA repair. SNP analysis furthermore indicated that isolates from different patients shared high impact SNP

that were not detected in environmental isolates. I propose that there is a positive selection for these SNPs during early stages of infection in the sinus. High impact SNPs were found in gene clusters involved in secondary metabolism and in a variety of stress-related genes including catalases and genes involved in hypoxia and pH response. Stress assays indicated a large variability of fungal growth, even between isolates of the same fungal biofilm carrying shared SNPs in components of the response to the stress conditions within the dog. Future research should address whether these differences in growth also occur when mixed with the other isolates of the same biofilm. This should reveal whether these strains complement each other.

Fungal in-host adaptation

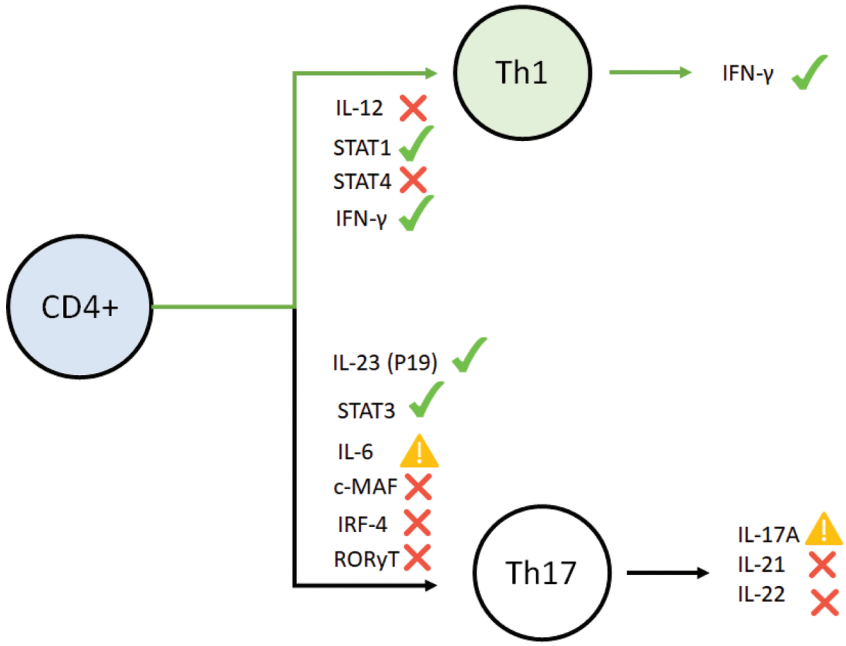
The results described in this Thesis show that the sino-nasal mucosal surface is a war zone between *A. fumigatus* and the host tissue. Immuno-chemistry showed a high infiltration of macrophages and neutrophils at the site of infection during SNA [4]. This was also found in this study (**Chapter 4**). The infiltration of the lamina propria can be explained by the high expression of IL-8 and alarmins (**Chapter 4**). These cells combat the fungal biofilm using nutritional immunity, and inflammatory and Th1 responses. On the other hand, adaptive responses of *A. fumigatus* enable growth and contribute to the suppression of the host immune response. This includes generation of immune-modulatory metabolites, adaptation to nutritional stress, and phenotypic and genetic plasticity.

The existence of genetic variants within a fungal biofilm is expected to be the result of selection in the stressful environment of the dog. Different factors can shape the genetic variations. Stress conditions may depend on the location within the biofilm. As a result, a mixture of variants can evolve that are spatially distributed. These variants may complement each other when also traits are acquired that make them more sensitive to the host environment. Thus, a symbiotic relationship may evolve between the isolates inside the fungal plaque. A trade-off between loss of function and survival in symbiotic systems is described as the black queen hypothesis (BQH). Generation of individuals with an increased frequency of mutation can be advan-tageous for the pathogen in a stressful environment [37]. As such, the situation in SNA would be similar to the one encountered in chronic infections like cystic fibrosis [38]. This phenomenon has previously been described for *Pseudomonas aeruginosa* [38-40], *Candida albicans* [40] and *Cryptococcus neoformans* [41, 42] and this is the first report for *A. fumigatus*. The increased mutation rate in our study may be due to a defective DNA repair system as indicated by the finding that the genes involved in this system show increased incidence of biallelic non-synonymous SNPs. In addition, formation of heterokaryons in the fungal plaque may increase the presence of alternative alleles [43, 44] because the total number of SNPs is the sum of the SNPs in each type of nucleus in the mycelium. Similarly, increased copy number of genes may cause increased SNP rates as was previously shown in *A. fumigatus* [45].

Code red: a continuous state of war

Expression of genes involved in the immune response of the dog to SNA have been studied using qPCR [5, 6], micro-arrays [7] and RNAseq (**Chapter 4**). The former studies suggested an over-reactive pro-inflammatory Th1 response during SNA. This adaptive immune response is characterized by the production of Interferon-gamma (IFN-γ). In addition, the interleukin IL-6 was upregulated in dogs with SNA [5]. IL-6 is related to the Th17 response that promotes antifungal properties of neutrophils [15] and is required for optimal fungal clearance at the mucosal level [12, 16]. Microarray analysis showed no differential expression of genes involved in the Th17 response [7] (Figure 1). This may be explained by the fact that these studies used different dog breeds. The fact that these studies did not analyze individual patients but made use of pools of RNA of different patients makes it difficult to find the cause of the difference in the qPCR and micro-array studies. In contrast, I did analyze individual patients (**Chapter 4**) enabling an analysis of individual and breed-specific immune responses. In agreement with the qPCR studies [5, 6] we found up-regulation of factors involved in the Th17 response (i.e STAT3 and the Il-23 subunit P19). Yet, the expression of IL-17 was detected in only one Saint Bernard patient (CP7). Thus, the anti-fungal Th17 response seems to be affected in SNA. Since patient-specific immune response were observed, future research should focus on differential gene expression studies of naturally infected dogs with SNA using non-infected dogs of the same breed as a control, or the same patient after recovery.

There might be several reasons why the Th17 response is absent. Activation of this response requires activation of dendritic cells (DC) and antigen presentation. We cannot exclude that DC do not detect fungal antigens. However, we expect that fungal hyphae that might start invasive processes are killed via neutrophils in the lamina propria releasing antigens. A defective Th17 response can result in a reduced recruitment of neutrophils to the site of infection. However, strong or prolonged activation of the Th17 response can also cause the opposite effect resulting in excessive inflammation and immune pathology [12, 17]. Kynurenines may be involved in the dysregulation of the Th17 response at the mucosal surface and in the establishment of the chronic status of SNA in dogs (**Chapter 5**). These molecules result from the degradation of tryptophan via indoleamine 2,3-dioxygenase (IDO1) and play a role in the control and establishment of fungal infections [18]. Both host and *A. fumigatus* have IDO1 genes and can thus produce kynurenine. It has been reported that kynurenines produced by *A. fumigatus* can inhibit IL-17 production in human monocyte-derived macrophages [19]. Furthermore, the formation of host derived kynurenine on Paracoccidioidomycosis was shown to modulate the immune response such that a state of disease tolerance was created, thereby preserving host fitness without pathogen clearance [18, 20]. Together, I propose that in the case of SNA, host and pathogen kynurenine modulates the immune response which results in the absence of the Th17 response creating a state of disease tolerance explaining the chronic nature of the infection.



Gene	Peeters 2006/2007 qPCR	Vanherbergen 2012 array	This study RNAseq (mean TPM)
IRF-4	NA	ND	ND
STAT1	NA	ND	14.69
STAT3	NA	NDE	312.68
STAT4	NA	ND	ND
RORγT	NA	ND	ND
c-MAF	NA	ND	ND
IL-6	UP	ND	ND
IL-12	NA	NDE	ND
IL-23			
P19	up	ND	95.4
IL-12 P40	up	ND	ND
IL-21	NA	ND	ND
IL-17 (A/F)	NA	ND	ND*(IL-17A expression in only one dog sample)
IL-22	NA	ND	ND
IFN-γ	NA	ND	19.97

Figure 1. Expression of genes involved in the Th1/Th17 response in SNA. Green marks and red crosses indicate expression and absence of expression, respectively, in our RNAseq study, while yellow warning signs indicate discordance between reported studies. ND, NDE, and NA mean that expression was not detected, that differential expression was not detected, and that expression was not analyzed, respectively.

The continuous pro-inflammatory reaction in the dog epithelium can be stimulated by the release of S100 proteins. These proteins have pleiotropic effects on nutritional immunity and induction of pro-inflammatory responses [21]. Moreover, expression of pattern recognition receptors (PRR) involved in the innate immune response to *A. fumigatus* (**Chapter 4**) like TLR (toll-like receptor) 2 and 4 [22], Mincle (CLEC4E) [23], and Dectin-1 (CLEC7A) [24] accompanied by high expression of IL-8, which can be stimulated by IL-1α, hyphal antigens [25, 26], and Dectin-1 [26] contributes to the continuous activation of a proinflammatory reaction and a Th1 response. The fact that IL-10 was shown to be expressed in SNA may result in a dampened Th1 response that would prevent excessive tissue damage. Moreover, it can contribute to the chronic state of the infection as suggested previously [6]. At this point, we do not know how changes in the microbiota in the sino-nasal area affect or contribute to SNA. Changes in the bacterial population in the canine nasal area were studied previously and dogs with nasal diseases, excluding dogs with SNA, did show for example reduction in *Moraxella* spp and *Pasteurellaceae* were significantly increase [27].

Four dog breeds were previously reported to be more prone to SNA suggesting that a breed specific defect in immunity exist. The gene pool of dog breeds is closed since breeding does not occur with other dog varieties. This reduces the degree of heterozygosity. Indeed, individual within breeds share high homology in their genomes [28]. A recent genomic analysis of 1346 dogs representing 161 breeds showed that 91% of breeds (146/161) formed single, breed-specific nodes and 29 multi-breed clades. A relation between pedigree and susceptibility to musculoskeletal, gastrointestinal, urogenital, nervous and respiratory defects were previously reported [29] as well as various types of cancer. For example, greyhounds are susceptible to develop osteosarcoma during which overexpression of IL-8 and constitutive expression of STAT3 is found [30]. Possibly, breeds differ in their ability to create a disease tolerogenic state. Breeds that reach such a state more easily might have a higher chance to develop a chronic sino-nasal infection. More research is required to further pinpoint breed-specific immunological defects related to SNA.

The absolute unit: *A. fumigatus* responses during chronic infection.

A. fumigatus is able to grow in many different environments and can cause infections in many different organisms demonstrating its ability to adapt easily. Using whole-genome sequencing (WGS) (**Chapter 5**), stress bio-assays (**Chapter 5**), and RNA-seq (**Chapter 3**) I identified high variability in genetic and phenotypic traits within fungal isolates from the same canine patient. This variability appears to be related to in-host adaptations. Transcriptome data from the host showed expression of genes related to nutritional immunity (**Chapter 4**), suggesting that in SNA the host-pathogen interface is depleted of metals like iron and zinc. In particular, ns-SNPs were found in genes related to detoxification of reactive oxygen species (ROS) produced by the host, and in genes involved in the pH and stress response. These host environmental factors can play a role in the selection of variants during in-host

adaptation. One particular example is copper transport. NsSNPs were found in the copper exporter CrpA and the copper importer Ctr2 in some strains that were able to grow on high concentrations of copper (**Chapter 5**). Additionally, expression data from the fungal plaques (**Chapter 3**) showed that CrpA had a highly variable expression between all four fungal plaques which also carried isolates with the nsSNP in the mentioned gene. This suggests that high copper concentrations at the mucosal surface impose a selection of copper-resistant variants. It is not clear how high copper concentration may develop but the metal may be released from macrophages that are known to accumulate copper. Copper is a transition metal that is used by the macrophages to produce hydroxyl radicals in a Fenton reaction to kill intracellular pathogens [31, 32]. Cytotoxic activity of secondary metabolites like gliotoxin might be responsible for the release of the copper into the environment thus increasing copper concentration at the mucosal surface.

SNA is typified by high expression of secondary metabolites of *A. fumigatus*. For instance, the full expression of the gliotoxin, neosartoricin and hexadehydroastechrome clusters was found (**Chapter3**). Gliotoxin has several immune-suppressive activities by activation of the NF-κβ pathway, killing of immune cells and inhibition of H_2O_2 production in macrophages [33]. Gliotoxin can thus be responsible for killing mucosal epithelial cells and the immune cells that are recruited at the site of infection. Furthermore, gliotoxin together with neosartoricin can suppress the local adaptive immune response via inhibition of T-cell proliferation [34]. This may be strengthened by expression of IDO1 in *A. fumigatus* that results in the production of the immune-suppressive kynurenine (see above). In addition, I showed expression of the genes involved in the production of fungal oxylipins/prostaglandins (PPOA/B/C). Prostaglandin PGE2 was previously shown to inhibit the differentiation of Th17 cells [40]. Altogether, multiple fungal mechanisms appear to be present to suppress the mucosal immune response.

The alkaline pH of the fungal plaque can be explained by the production of ammonia by the fungus due to catabolism of amino acids [35] and / or to the action of the enzyme agmatinase (**Chapter 3**). The change of local pH was previously described as a general strategy of human and plant pathogens to promote invasion and virulence [35]. Alkaline pH lowers the solubility of the transition metals copper, iron and zinc thus reducing the production of hydroxyl radicals and reduction of oxidative stress at the mucosal surface [37].

Looking from a safe distance: Model of SNA.

Based on the results described in this Thesis, a model of the interaction between host and pathogen during SNA is proposed (Figure 2). The initial infection with conidia in the sino-nasal area results in outgrowth of the fungus into a mature fungal biofilm. The interaction between this developing biofilm and the mucosal immune system ends up in a continues war in which the immune system tries to eliminate the infection with a controlled inflammatory reaction. However, fungal production of immune-modulating compounds results in dampening of innate and Th1 responses and in the absence of the Th17 response. This results in a state of disease tolerance resulting in chronic infection. The fungus in the biofilm is exposed to different types of stress such as nutritional and oxidative stress. This is proposed to invoke selection of strains resulting in in-host adaptation yielding genetic and phenotypic variants. Increase of mutational rates due to changes in DNA repair machinery might be a mechanism to increase the adaptation. Increase in copper resistance and raising the environmental pH by the fungus seems a strategy to escape the copper and oxidative stress at the mucosal surface. Production of SM helps in immune suppression and increases cytotoxicity resulting in necrosis at the mucosal surface. Prolonged growth results ultimately in large amounts of epithelial tissue and bone damage allowing the fungus to reach the brain which is lethal.

Debriefing: conclusion and future perspectives.

The results presented in this Thesis contribute to the knowledge of non-invasive infections caused by *A. fumigatus*. The data obtained indicate that this fungus presents a remarkable degree of genotypic and phenotypic variation within the dog. This allows this fungus to adapt and survive in the hostile environment of the nose epithelium of dogs with SNA. Several fungal mechanisms have been identified that can contribute to immune-modulation, while secondary metabolites also seem to play a crucial role during infection.

Sequencing genomes via single molecule real-time (SMRT) sequencing, *de novo* genome assembly, and bisulfite sequencing is required to obtain more information about structural changes in the genome, either at level genome organization as well as genome modifications (epigenetic changes). Furthermore, with improved genome data reanalysis of the RNA-seq data from the fungal plaques is possible which allows identifying expression of genes in a fungal isolate specific way. Moreover, co-cultivation of fungal isolates from the same biofilm in stress assays might confirm that these strains complement each other, thus compensating for acquired mutations that actually reduce the fitness of an individual strain. Analysis of differential gene expression in dogs with SNA and non-infected controls of the same breed will allow deeper analysis of breed-specific gene expression and identification of breed-specific genetic defects which results in increased sensitivity to SNA.

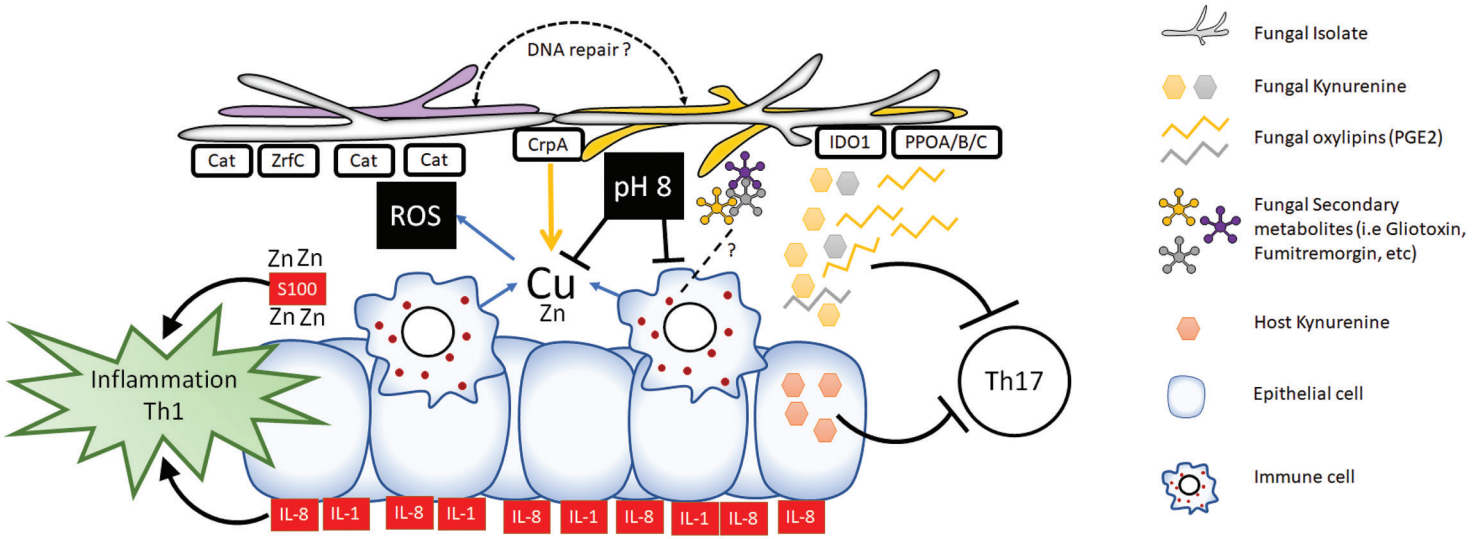


Figure 2. Sino-nasal aspergillosis (SNA) model. Molecules (Icons), genes (white and red boxes representing fungal and host genes, respectively) and processes (black boxes) implicated in the infection process are depicted. Different genotypes/phenotypes of *A. fumigatus* are indicated as mycelial structures with different colors. Links described in this thesis are depicted with dashed arrows. Activation/production/export are depicted with full arrows (black = process, blue=host, yellow=fungi). Inhibition of processes are depicted as black blunted lines.

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Appendix

Nederlandse samenvatting

Niet-invasieve aspergillose in het sino-nasale gebied (gebied van de neus en de neusbijholtes) van honden (SNA) lijkt op menselijke neusbijholteontsteking die veroorzaakt kan worden door verschillende soorten schimmels. De kennis over SNA is beperkt en het meeste onderzoek richt zich op de diagnostiek, de behandeling en de lokale afweerreactie. In dit proefschrift analyseerde ik de genomen, genexpressie en fenotypes van *A. fumigatus* isolaten die afkomstig zijn van honden met SNA. In **Hoofdstuk 2** wordt beschreven dat elke hond met SNA geïnfecteerd was met een enkel *A. fumigatus* genotype terwijl in menselijke patiënten meerdere genotypen in de longen werden gevonden. Omdat mensen en dieren continu conidia van *A. fumigatus* inademen zou men bij zowel bij de hond als bij de mens verschillende genotypes in patiënten verwachten. Echter, in het geval van de hond domineert schijnbaar een genotype. De isolaten uit dezelfde hond bleken wel een grote variatie in fenotypes te vertonen zoals verminderde sporulatie en / of pigmentatie van de sporen. Dit fenomeen verklaar ik door aanpassingen van de schimmel tijdens de groei in de gastheer.

Hoofdstuk 3 beschrijft genexpressie van *A. fumigatus* in de biofilms van honden met SNA. Dit is de eerste genexpressieanalyse met behulp van RNA-sequencing van een natuurlijke *A. fumigatus*-schimmelinfectie. De analyses laten zien dat 6% (514) van de *A. fumigatus* genen op een zeer variabele manier tot expressie worden gebracht. Deze variatie kan het gevolg zijn van verschillen in omgevingsfactoren in de gastheer, maar ook door epigenetische veranderingen en genomische mutaties. Genen die coderen voor secundaire metabolieten, stressresponse en *host-pathogen* interacties bleken zeer variabele genexpressie te hebben. De resultaten geven aan dat *A. fumigatus* zich aanpast aan de stressfactoren in de gastheer.

In **Hoofdstuk 4** wordt de genexpressie beschreven van het weefsel van de hond dat in contact staat met de schimmel in het SNA gebied. De analyse bevestigt eerdere waarnemingen dat zowel de aangeboren immuniteit als de Th1-respons betrokken zijn bij de mucosale afweer. Het meest opmerkelijk is de afwezigheid van een Th17-response wat bleek uit de afwezigheid van de cytokines IL-17, IL-21 en IL-22. Door binding aan receptoren induceren deze cytokines expressie van andere pro-inflammatoire cytokines, trekken neutrofielen aan naar de plaats van infectie en induceren de productie van antimicrobiële peptiden. De resultaten beschreven in dit hoofdstuk wijzen op een ontregeling van de T-celrespons waardoor een chronische schimmelinfectie kan ontstaan.

Hoofdstuk 5 beschrijft de grote genetische en fenotypische variabiliteit tussen schimmelisolaten afkomstig uit dezelfde honden. Er werden onder andere mutaties gevonden in genen die verband houden met kopertransport en met aanpassing aan pH en oxidatieve stress. Een deel van de isolaten uit honden hadden een opmerkelijk hoog aantal *single nucleotide polymorphisms* (SNPs), die waarschijnlijk veroorzaakt worden door mutaties in genen die coderen voor het DNA herstelsysteem. Het hoge aantal SNPs werd niet waargenomen in isolaten die hun oorsprong

vinden in het binnenmilieu van huizen. Dit wijst op een positieve selectie voor deze SNPs, waarschijnlijk tijdens de vroege stadia van infectie in de hond. Mutaties met hoge impact werden vooral gevonden in genclusters die coderen voor secundaire metabolieten en in genen die betrokken zijn bij de oxidatieve stress, hypoxie en pH-response. Stress-assays duidden ook op een grote variabiliteit van schimmelgroei, zelfs tussen isolaten afkomstig van dezelfde biofilm. Meer onderzoek is nodig om de relaties tussen SNPs en groei te analyseren.

Schimmel aanpassing in de gastheer

De resultaten beschreven in dit proefschrift laten zien dat het sino-nasale oppervlak een oorlogsgebied is tussen *A. fumigatus* en de gastheer. Door limitatie van nutriënten, het aangeboren immuunsysteem en inductie van een Th1-respons wordt de schimmelgroei bestreden. Zo is er een hoge infiltratie van macrofagen en neutrofielen in het bindweefsel onder het epitheel dat kan worden verklaard door de hoge expressie van IL-8 en alarmins (**Hoofdstuk 4**). *A. fumigatus* past zich hierop aan waardoor groei mogelijk is en de immuunreactie van de gastheer worden onderdrukt. De schimmel doet dit door de productie van immuun modulerende metabolieten, via aanpassing aan voedingsstress en fenotypische en genetische plasticiteit. Het bestaan van genetische varianten binnen dezelfde biofilm is waarschijnlijk het resultaat van selectie in de stressvolle omgeving van de hond. Verschillende factoren kunnen de genetische variatie beïnvloeden, zoals de lokale omstandigheden in verschillende delen van de biofilm. Er ontstaat hierdoor een mengsel van varianten die ruimtelijk zijn verdeeld. Deze varianten kunnen elkaar aanvullen wanneer ook eigenschappen worden verkregen die ze juist gevoeliger maken voor factoren uit de gastheeromgeving. Zo kan een symbiotische relatie ontstaan tussen varianten in de biofilm. Een verhoogde mutatiefrequenties ten gevolge van mutaties in DNA herstelmechanismen zijn eerder beschreven in andere microorganismen maar dit is de eerste keer dat ze zijn beschreven voor *A. fumigatus* wordt gezien. Naast schade aan DNA herstelmechanismen kan de vorming van heterokaryons in de schimmelbiofilm de aanwezigheid van alternatieve allelen verhogen omdat het totale aantal SNPs de som is van de SNPs in elk type kern in het mycelium. Daarnaast kan een verhoogd aantal genkopieën een oorzaak zijn.

Code rood: een voortdurende staat van oorlog

Expressie van genen die betrokken zijn bij de afweerreactie van honden met SNA zijn eerder bestudeerd met behulp van qPCR, microarrays en nu met RNAseq (**Hoofdstuk 4**). De eerdere studies lieten vooral een Th1-reactie zien, die gekenmerkt wordt door de productie van interferon-gamma (IFN-γ). Ook werd verhoogde expressie van IL-6 bij honden met SNA waargenomen die nodig is voor de Th17-respons en die neutrofielen stimuleert. Deze reactie is belangrijk om de schimmelinfectie op het mucosale niveau te bestrijden. Microarray-analyse toonde eerder geen veranderingen aan in

de expressie van genen die betrokken zijn bij de Th17 response. Dit is mogelijk te verklaren doordat RNA werd bestudeerd dat een mengsel was van patiënten van verschillende hondenrassen. Mogelijk reageren hondenrassen namelijk verschillend op een SNA infectie. In **Hoofdstuk 4** heb ik individuele patiënten geanalyseerd waardoor analyse van individuele maar ook rasspecifieke afweerreactie mogelijk was. In overeenstemming met eerder onderzoek werd expressie van slechts enkele factoren die betrokken zijn bij de Th17-respons (d.w.z. STAT3 en de Il-23-subeenheid P19) aangetoond. Expressie van IL-17 werd slechts gedetecteerd in één hond, een Sint Bernard. Omdat mogelijk een ras-specifieke afweer werd waargenomen zou toekomstig onderzoek zich moeten richten op verschillen in genexpressies binnen rassen tussen honden met en zonder SNA. Het belang hiervan wordt geïllustreerd door het feit dat men gevonden heeft dat vier hondenrassen meer vatbaar zijn voor SNA, wat suggereert dat er een ras-specifiek defect in immuniteit bestaat. Dergelijke rasspecifieke verschillen kunnen ontstaan door de gebruikte fokmethodes waardoor dieren minder heterozygoot zijn.

Er kunnen verschillende redenen zijn waarom de Th17 response afwezig is. Activering van deze response vereist activering van dendritische cellen (DC) en presentatie van antigeen en dit proces zou mogelijk ook minder goed kunnen verlopen. Echter, schimmelhyfen kunnen invasief zijn maar dan direct gedood worden via neutrofielen in het bindweefsel onder het epitheel waardoor schimmelonderdelen vrijkomen die door DCs kunnen worden gezien. Een defecte Th17-response zal resulteren in vermindering van neutrofielen op de infectieplaats. Een sterke of langdurige activering van deze reactie kan echter ook het tegenovergestelde effect veroorzaken, wat resulteert in een overmatige ontsteking en bijbehorende weefselschade. Kynurenines kunnen betrokken zijn bij de ontregeling van de Th17-respons aan het slijmvliesoppervlak en daarbij een chronische infectie laten ontstaan (**Hoofdstuk 5**). Kynurenines ontstaan door afbraak van tryptofaan via indoleamine 2,3-dioxygenase (IDO1). Zowel gastheer als *A. fumigatus* hebben IDO1 genen en kunnen dus kynurenine produceren. Er is eerder aangetoond dat kynurenines die door *A. fumigatus* zijn geproduceerd de IL-17-productie in menselijke monocyt-afgeleide macrofagen kunnen remmen. Tegelijkertijd, gastheer-afgeleide kynurenines ten gevolge van een paracoccidioidomycosis bleken de afweerreactie zodanig te moduleren dat een toestand van ziektetolerantie werd gecreëerd, waardoor de fitheid van de gastheer behouden bleef zonder het pathogeen op te ruimen. Ik stel dan ook voor dat in het geval van SNA, gastheer en pathogeen kynurenines de afweereactie zodanig moduleren dat dit resulteert in de afwezigheid van de Th17-respons waardoor een toestand van ziektetolerantie wordt gecreëerd die de chronische aard van de schimmelinfectie kan verklaren.

Reacties van *A. fumigatus* tijdens chronische infectie

A. fumigatus kan in veel verschillende omgevingen groeien en infecties veroorzaken bij verschillende organismen omdat het zich gemakkelijk kan aanpassen. Via het sequencen van het genoom, SNP analyse en stress-bio-assays (**Hoofdstuk 5**) en RNA-seq (**Hoofdstuk 3**) identificeerde ik zoals eerder genoemd een hoge variabiliteit in genetische en fenotypische kenmerken binnen schimmelisolaten van dezelfde hond. Deze variabiliteit lijkt verband te houden met aanpassingen. Analyse van genexpressie (**Hoofdstuk 4**) gaf aan dat in SNA op het gastheer-pathogeen grensvlak metalen zoals ijzer en zink zijn uitgeput. Tegelijkertijd kunnen bepaalde metalen ook een hogere concentratie hebben zoals het geval lijkt te zijn voor koper. SNPs werden gevonden in het koper exporteiwit CrpA en het koper importeiwit Ctr2 van *A. fumigatus.* Diverse isolaten konden dan ook groeien bij hoge concentraties koper (**Hoofdstuk 5**). Genexpressiegegevens van de schimmelbiofilms (**Hoofdstuk 3**) lieten tevens zien dat CrpA een zeer variabele expressie had. Dit suggereert dat hoge koperconcentraties aan het mucosale oppervlak een selectie van koperresistente varianten levert. Het is niet duidelijk waarom er hoge koperconcentratie op dit oppervlak aanwezig zijn. Mogelijk komt het metaal vrij uit macrofagen waarvan bekend is dat ze koper accumuleren. Koper is een overgangsmetaal dat door deze cellen wordt gebruikt om intracellulaire pathogenen te doden door het produceren van hydroxylradicalen via de Fenton-reactie. Cytotoxische activiteit van secundaire metabolieten zoals gliotoxine kan daarnaast verantwoordelijk zijn voor het vrijkomen van het koper in de omgeving.

SNA wordt gekenmerkt door hoge expressie van genen die coderen voor secundaire metabolieten van *A. fumigatus*. Zo werd bijvoorbeeld expressie van de gliotoxine-, neosartoricine- en hexadehydroastechroomclusters gevonden, de laatst component is een derivaat van tryptofaan met een rol in het ijzer metabolisme van de schimmel (**Hoofdstuk 3**). Gliotoxine heeft verschillende immuunonderdrukkende activiteiten door activering van de NF-κβ-route, het doden van afweercellen en de remming van H_2O_2-productie in macrofagen. Gliotoxine kan dus verantwoordelijk zijn voor het doden van mucosale epitheelcellen alsmede immuuncellen die worden gerekruteerd op de plaats van infectie. Bovendien kan gliotoxine samen met neosartoricine de lokale adaptieve afweereactie onderdrukken door remming van T-celproliferatie. Dit kan worden versterkt door expressie van IDO1 in *A. fumigatus* en productie van het immuunonderdrukkende kyneurine (zie hierboven). Bovendien toonde ik expressie van de genen aan die betrokken zijn bij de productie van schimmeloxylipinen/prostaglandines (PpoA/B/C). Eerder werd aangetoond dat prostaglandine PGE2 differentiatie van Th17-cellen remt. Alles bij elkaar lijken dus meerdere schimmelmechanismen actief te zijn om de mucosale immuunrespons te onderdrukken. Dit wordt nog versterkt door de alkalische pH van de schimmelplak. Deze hoge pH kan worden verklaard door de productie van ammoniak door de schimmel als gevolg van katabolisme van aminozuren en/of de werking van het enzym agmatinase (Hoofdstuk 3). De verandering van de lokale pH werd eerder bes-

chreven als een algemene strategie van menselijke en plantaardige pathogenen om invasie en virulentie te bevorderen. Alkalische pH verlaagt de oplosbaarheid van de transitiemetalen koper, ijzer en zink, waardoor de productie van hydroxylradicalen en dus vermindering van oxidatieve stress op het mucosale oppervlak wordt bereikt.

Vanaf een veilige afstand: een model van SNA

Op basis van de resultaten die in dit proefschrift worden beschreven, wordt een model van de interactie tussen gastheer en pathogeen tijdens SNA voorgesteld. De initiële infectie met sporen in het sino-nasale gebied resulteert in uitgroei van een spore (sporen) van een bepaald genotype. De interacties tussen deze ontwikkelende biofilm en het mucosale afweersysteem leidt tot een voortdurende oorlog waarin het immuunsysteem de infectie probeert te elimineren met een gecontroleerde ontstekingsreactie. De schimmel produceert echter immuunmodulerende verbindingen wat resulteert in het dempen van de aangeboren afweer en de Th1-immuunrespons en de afwezigheid van de Th17-respons. Dit resulteert in een toestand van ziektetolerantie die resulteert in een chronische infectie. De schimmel in de biofilm wordt blootgesteld aan verschillende soorten stress, zoals voedings- en oxidatieve stress. Dit leidt tot selectie van genetische en fenotypische varianten die beter zijn aangepast aan de gastheer. Verhoging van de mutatiefrequentie ten gevolge van veranderingen in het DNA-herstelsysteem kan een mechanisme zijn om snellere aanpassing te krijgen. Verhoging van de koperresistentie en verhoging van de lokale pH lijken dergelijke aanpassingen te zijn. Daarnaast worden SMs gevormd die de afweer onderdrukken en cellen doden, resulterend in necrose op het mucosale oppervlak. Langdurige groei resulteert uiteindelijk in grote hoeveelheden epitheelweefsel en botschade waardoor de schimmel de hersenen kan bereiken die dodelijk is.

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Curriculum vitae

Ivan Daniel Valdes Barrera was born on 25th October ,1987 in Bogota, Colombia. He followed his primary and secondary education at Colegio Mayor de Nuestra Señora del Rosario in Bogota Colombia and graduated in 2004. In January 2005 he began his bachelor's in Biology at the Military University Nueva Granda in Bogota, Colombia obtaining His diploma in 2010. In 2011 he was awarded with a young researcher fellowship from the Colombian administrative department of science technology and innovation (COLCIENCIAS) to continue research for two years at the same university (UMNG) where he focused on two main research lines, : fungal plant pathogens and applied phytochemistry. Later in 2012 he was awarded a graduate assistant scholarship Los Andes University which allowed him to complete his Master's in Biological sciences. During this period he did an internship at the mycology and phytopathology laboratory (LAMFU) under the supervision of PhD Silvia Restrepo. His Master's project was focused in the metabolic reconstruction of the Oomycete *Phytophtora infestans*. Ivan Obtained his MSc diploma in 2014. In September 2014 he moved to Utrecht, The Netherlands and started his PhD within the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of Prof. Dr Han Wosten and Dr. Hans de Cock. During his PhD he studied the phenotypic , genomic and transcriptomic aspects of *Aspergillus fumigatus* isolates coming from fungal plaques isolated from dogs suffering from sino-nasal aspergilliosis (SNA).The results of his research are described in this thesis

List of Publications

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