Characterisation of allergic reactivity to fungi in a Zimbabwean population

A thesis submitted for the degree of *Doctor of Philosophy*

Lorraine Tsitsi Pfavayi

Kellogg College, University of Oxford

Michaelmas 2022

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Abstract

While fungal diseases are a growing global problem, there remains a paucity of epidemiological data in most developing countries. Hence, this thesis aimed to investigate the epidemiology, immunology, and aetiology of fungal allergic diseases in Zimbabwe.

This study demonstrated that the burden of fungal diseases in Zimbabwe is high (14%), with tinea capitis being the most prevalent condition. The country's background sensitivity to fungal allergens and the effect of host-related factors was unknown. Therefore, this thesis further investigated the prevalence of fungal sensitisation and reactivity among Zimbabwean children and how the gut mycobiome composition affected these, whilst considering the effects of host age, gender, and *Schistosoma haematobium* infection status. The prevalence of fungal sensitisation to ≥1 fungal species was 96%, and the metagenomic analysis of the gut mycobiome showed that the mycobiome comprised <1% of the sequenced gut microbiome. There was no association between the mycobiome and fungal reactivity, or the host factors studied. Interestingly, an increase in *Aspergillus, Tricholoma,* and *Periglandula* abundance was associated with schistosome infection.

Due to the high prevalence of fungal sensitisation, this thesis further identified and characterised fungal proteins that were immunoreactive against serum samples from fungal-sensitised children. Furthermore, the utility of a specific *Aspergillus fumigatus* allergen (Asp f 2) peptide in the differential diagnosis of fungal allergy was evaluated. As a result, novel immunogens from fungi were discovered, potentially increasing the number of known fungal allergens. The Asp f 2 peptide was demonstrated to be an inadequate indicator for diagnosing fungal allergy in the population.

Taken together, the findings of this thesis add to the ongoing global discussions about the burden of fungal diseases. Furthermore, it provides novel data on several important aspects of fungal allergy that must be evaluated and validated since they may have implications for the care of allergic individuals.

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Associated Publications

Chapter 1: The Pathogenesis of Fungal-Related Diseases and Allergies in the African Population: The State of the Evidence and Knowledge Gaps
Status: Published in International Archives of Allergy Immunology (February 2020)
Authors: Lorraine T Pfavayi (LTP), Elopy N Sibanda (ENS), Francisca Mutapi (FM)
Authors' contributions: LTP did the literature search and wrote the first draft of the review. LTP, FM, and ENS revised successive drafts of the manuscript and approved the final version

Chapter 3: Determining the burden of fungal infections in Zimbabwe
Status: Published in Scientific Reports (June 2021)
Authors: Lorraine T Pfavayi (LTP), David W Denning (DWD), Stephen Baker (SB), Elopy N Sibanda (ENS), Francisca Mutapi (FM)
Authors' contributions: FM and DWD conceptualized and designed the study. LTP did the literature search, selected the studies and extracted relevant information. LTP analysed the data with input from DWD and FM. LTP wrote the first draft of the paper. LTP, FM, DWD, SB, and ENS revised successive drafts of the manuscript and approved the final version.

Chapter 4: Fungal allergic sensitisation in young rural Zimbabwean children: Gut mycobiome and seroreactivity characteristics
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Authors: Lorraine T Pfavayi (LTP), Elopy N Sibanda (ENS), Stephen Baker (SB), Mark Woolhouse (MW), Takafira Mduluza (TM), Francisca Mutapi (FM)
Authors' contributions: FM, TM and EN conceived the study. LTP, FM, TM, and EN conducted the fieldwork. LTP conducted the laboratory work and analysed the data with input from FM. LTP wrote the first draft of the paper. LTP, FM, ENS, SB, TM and MW revised successive drafts of the manuscript and approved the final version.

Chapter 5: The Identification and Characterization of Immunoreactive Fungal Proteins Recognized by Sera from Zimbabweans Sensitized to Fungi
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Authors' contributions: FM, TM and EN conceived the study. LTP, FM, TM, and EN conducted the fieldwork. LTP curated the field data. LTP and RB conducted the laboratory work. LTP analysed the data with input from RB and FM. LTP wrote the first draft of the paper. LTP, FM, ENS, SB, TM and MW revised successive drafts of the manuscript and approved the final version. LTP, RB, FM, ENS, SB, TM and MW revised successive drafts of the manuscript and approved the final version.

Contents

List of figures

List of tables

Abbreviations

ABPA	Allergic broncho-pulmonary Aspergillosis
AIDS	Acquired immune deficiency syndrome
AIT	Allergen Immunotherapy
ANCOM	Analysis of composition of microbiomes
ANOVA	Analysis of variance
APC	Antigen-presenting cells
ART	Antiretroviral therapy
BCA	Bicinchoninic acid
BGI	Beijing Genomics Institute
bp	Base pair
BSA	Bovine serum albumin
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CPA	Chronic pulmonary aspergillosis
CrAg	Cryptococcal antigen
CRD	Component resolved diagnosis
DTT	Dithiothreitol
ECL	enhanced chemiluminescent
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionization
ESI-MS	Electrospray ionization-mass spectrometry
FDR	False discovery rate
Fmoc	9-fluorenylmethoxycarbonyl
GAFFI	Global Action Fund for Fungal Infections
GIT	gastrointestinal tract
GLM	General linear model
GRADE	Grading of Recommendations, Assessment, Development and Evaluations
HAZ	Height–for–age Z-score
HIV	Human immunodeficiency virus
HMM	Hidden Markov Model
HMP	Human Microbiome Project
HRP	Horseradish peroxidase
HPLC	High-performance liquid chromatography
IA	Invasive Aspergillosis
ICU	Intensive Care unit
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC2	Type 2 Innate lymphoid cells
IQR	Interquartile range
IRIS	Immune Reconstitution inflammatory syndrome
ISAC	Immuno-Solid phase Allergy Chip
KMA	K-mer alignment

LC	Liquid chromatogram
MALDI	Matrix-assisted laser desorption ionization
MDA	Mass drug administration
MHC	Major Histocompatibility Complex
MS	Mass spectrometry
MSn	Tandem mass spectrometry
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multidimensional scaling
NGS	Next generation sequencing
ns	Not significant
NTD	Neglected tropical disease
OI	Opportunistic Infection
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PDB	Protein data bank
PERMANOVA	Permutational multivariate analysis of variance
PSAC	Preschool-aged children
RAST	Radioallergen absorbent test
SAFS	Severe Asthma with fungal sensitisation
SD	Standard deviation
SDG	Sustainable Development Goals
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
spp.	Species
SPT	Skin prick test
STH	Soil-transmitted helminths
SSA	Sub-Saharan Africa
TB	Tuberculosis
TBS	Tris buffered saline
TGF-β	Transforming growth factor beta
TBST	TBS buffer containing Tween 20
TFA	Trifluoroacetic acid
Th2	CD4+ helper T cell type 2
Th1	CD4+ helper T cell type 1
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	Regulatory T-cell
TSLP	Thymic stromal lymphopoietin
UNAIDS	Joint United Nations Programme on HIV/AIDS
UV	Ultraviolet
WASH	Water, sanitation and hygiene
WAZ	Weight–for–age Z-score
WHO	World Health Organisation

Chapter 1 Introduction

This chapter has been published as a review article in the International Archives of Allergy and Immunology journal [1]. A copy of the publication is included in **Appendix D.**

1.1 Background

Fungi are ubiquitous in nature; hence exposure to airborne spores is virtually constant throughout the year [2-4]. They are important disease-causing agents which can directly cause diseases such as cryptococcal meningitis [5], *pneumocystis* pneumonia [6], pulmonary aspergillosis [7-9], or indirectly act as allergens which can induce or exacerbate respiratory diseases such as asthma [10]. These diseases pose a significant but often neglected burden on public health [9, 11].

In recent years, the frequency and severity of fungal infections have increased substantially [12, 13] due to the global AIDS epidemic and growing numbers of susceptible individuals, such as those undergoing organ transplants, immunosuppressive therapies and surgery [9]. These infections often occur in resource-limited countries in Africa, South America, and Southeast Asia [14].

The rise in the prevalence of fungal diseases is partially attributed to climate change, with global warming thought to favour the propagation of fungal spores [12, 15]. Although fungi are a common and integral part of the environment, the impact of fungal diseases on the entire ecosystem can be devastating [9, 16-18]. Thus, fungi are regarded as a current and future public health problem which should not be underestimated [19].

This chapter summarises the current epidemiology of fungal diseases, including fungal allergies in Africa. The chapter also discusses allergy risk factors, diagnostics as well as management of allergies. The chapter then concludes with the rationale and specific aims

of the thesis focusing on some of the uncertainties identified in the following review of the literature regarding the epidemiology of fungal diseases, including allergic disorders, risk factors and prospects for improved diagnosis and management of allergic patients.

1.2 Public health burden of fungal-related diseases

The annual global mortality due to fungal infections is estimated to be over 1.6 million [20, 21]. Despite this, the association between fungal pathogenesis and the adverse health sequelae remains poorly characterised, especially in Africa [1]. This is partly because disease frequently develops in patients with multiple morbidities, including immunodeficiencies [22, 23].

Globally, mucosal infections affect more than 100 million people [20], whilst over a billion suffer from fungal skin infections [9, 24] with an age-standardised disability-adjusted life year (DALY) rate of 54.86 per 100 000 [25]. To date, more than 10 million people have succumbed to severe fungal allergies and one million die per year due to fungal infections [8]. As of 2021, the global mortality owing to fungal infections was greater than that of malaria [26] and was equivalent to that of tuberculosis (TB) [20, 27]. Nonetheless, the public health impact of this relatively silent cause of morbidity and mortality has not been adequately addressed.

In Africa, the precise prevalence of fungal diseases is unknown due to limited access to healthcare infrastructure and fungal disease-specific health expertise [28]. However, the high prevalence of HIV [11] and pulmonary TB [27] cases in most African countries has led to a large number of cases of opportunistic fungal infections [29]. These fungal infections have been observed in most African countries in studies carried out by the Global Action Fund for Fungal Infections (GAFFI) [29, 30]. However, the epidemiology of allergic diseases due to fungal exposure has not been fully elucidated [31].

1.3 Medical disorders associated with fungi

As previously highlighted, fungi cause several diseases, which have been subdivided into six groups: acute invasive and life-threatening infections; chronic fungal infections; mucosal infections; superficial infections; allergic diseases and mycotoxicosis (see **Figure 1.1**). These diseases range from superficial infections to fatal systemic mycoses, in addition to producing potent toxins [32, 33]. As the populations at risk continue to expand, so does the spectrum of opportunistic fungal pathogens infecting these patients.

Acute invasive and life-threatening infections

Invasive fungal infections are generally uncommon, except in certain groups of patients with immune deficits, and they are scarce in those with apparently normal immune systems. Clinician experience, combined with the availability of precise, rapid diagnostic testing, is the most important factor determining survival from invasive fungal infection.

Chronic fungal infections

Some fungi are persistent and defy curative treatment leading to long-term infections. In most cases, local trauma or damage is a key risk factor, such as skin inoculation of a particular fungus, such as those causing mycetoma.

Mucosal infections

Mucosal infection commonly referred to as thrush is caused by *Candida* species and occurs in the mouth or vagina. Thrush results in a marked inflammatory response leading to reddened (erythematous) mucosa and considerable irritation and discomfort. Thrush is often recurrent because *Candida albicans* is a commensal organism and only causes thrush when the microbiome balance is disrupted [34].

Superficial infections

Superficial fungal infections are benign affecting skin, scalp and nails and are caused by dermatophytes. These fungi can degrade keratin and have been established to belong to *Trichophyton, Epidermophyton* and *Microsporum* species. Dermatophyte infections are common in both sexes at all ages and have a worldwide distribution. Examples of these infections include tinea capitis and tinea pedis [35].

Allergic diseases

Fungal spores are not only associated with IgE-mediated type I allergies but also with a broad panel of other diseases [3, 36]. The primary diseases that affect individuals are allergic rhinitis [37], allergic conjunctivitis, allergic fungal sinusitis [38], atopic dermatitis [39] and asthma [40]. Other less common immune-mediated diseases are allergic bronchopulmonary mycoses (ABPM) [41] and hypersensitivity pneumonitis [42]. The broad panel of diseases results from the inhalation and ingestion of fungal spores and vegetative cells (hyphae) or contact with fungal cells.

Mycotoxicosis

The mid and hot tropical climates [43] in Africa provide favourable growth conditions for fungi species, and as such, it is possibly the most exposed of all continents [44]. In addition to the climatic conditions, factors such as poverty make it highly plausible for African people to consume mycotoxin-contaminated food. These mycotoxins are produced by some fungal species as secondary metabolites [45] and may occur in spores, mycelia, and the matrix in which fungi grow. The toxic effects of mycotoxins are termed “mycotoxicosis, which is characterised by damage to the cells of most major organs, endocrine and immune systems. Exposure to these mycotoxins results in neurotoxic, mutagenic, carcinogenic and teratogenic effects [46-48]. The severity of toxic effects depends on the type of mycotoxin, the duration and dose of exposure and the age, health and nutritional status of the individual affected [49].

Most contaminated food crops [50] are sometimes part of the main ingredients in weaning porridge [51]. Because of this, it has been suggested that exposure to mycotoxins may be a causative factor for stunting and malnutrition [52, 53] observed in some African children.

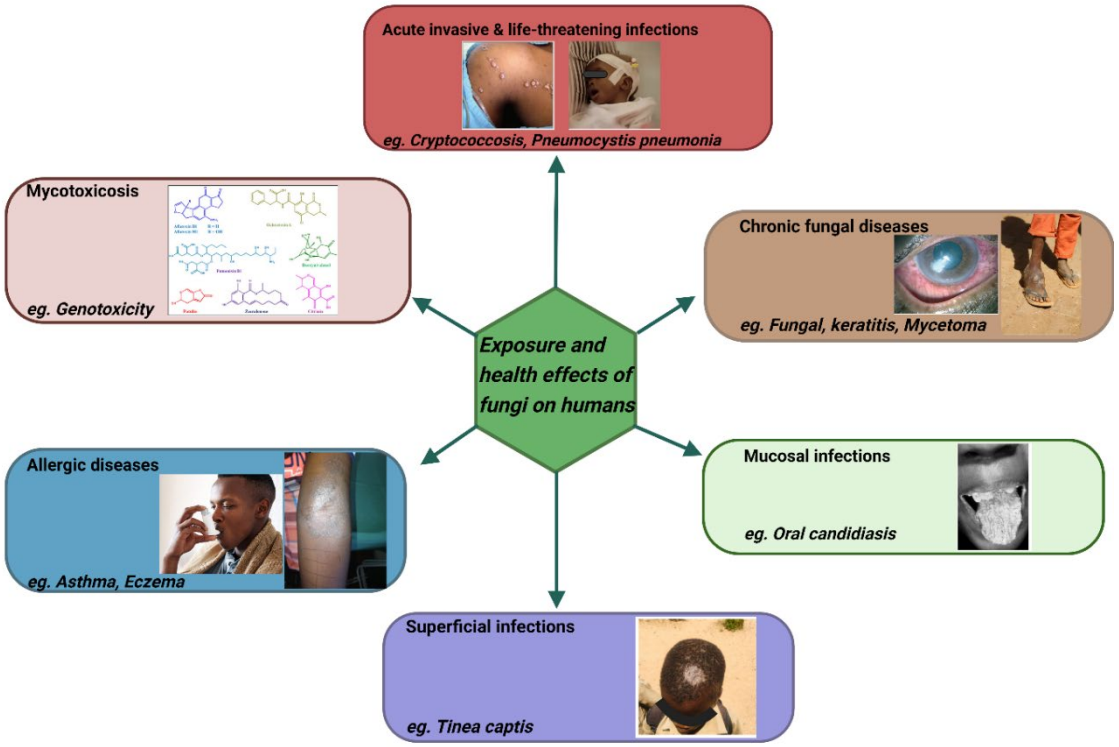

Figure 1.1: Groupings of fungal diseases

1.4 Allergy

The term “allergy” was first introduced into the medical literature by Clemens von Pirquet in 1906. This term was used to refer to an altered immune system reactivity to an antigen which may lead to protective immunity or hypersensitivity irrespective of whether it resulted in immunity or harmful effect [54]. Nowadays, it is used to describe the hypersensitive response of the immune system to foreign proteins commonly referred to

as allergens [55]. Environmental exposure to allergens is an important step in the aetiology and exacerbation of allergic conditions. However, even after extensive environmental exposure to specific aeroallergen sources, it has been shown that not all individuals develop an allergic disease, which is strong evidence of genetic predisposition [56-59] as well as other factors such as lifestyle [60, 61].

1.4.1 Classification of allergy

Gell and Coombs classified all hypersensitivity reactions into four basic types based on the effector cells and molecules produced during an allergic reaction [62], as illustrated in **Table 1.1** below. This was later reviewed by Rajan [63], who focused on the 'evolutionary drive' as a perspective on the nature of immunity in response to host invasion suggesting a possible fifth strategy (Stimulatory Hypersensitivity Type V). According to Rajan [63], the Type V hypersensitivity reactions are the T-helper (Th)1 and Th2 type reactions which cause the formation of granulomas, an example being Graves diseases [64]. However, some researchers classify this reaction as Type II rather than Type V [65].

In contrast to the antibody-mediated Types I – III and V, Type IV hypersensitivity reactions are characterised by the direct participation of cytotoxic and helper T cells. This classification considers events occurring at the molecular level (i.e., the primary manifestations of allergy), which may aggregate secondary effects that may be similar or overlapping in the case of any of the allergy types [66]. Hence, it is not always possible to distinctly identify the allergy type in a clinical scenario.

Table 1.1: Gell and Coomb's classification of types of allergic reactions

	Type I	Type II	Type III	Type IV
Immune reactant	IgE	IgG or IgM	IgG and IgM	T cells
Antigen form	Soluble antigen	Cell-bound antigen	Soluble antigen	Soluble or cell-bound antigen
Mechanism of action	Specific allergen induces cross-linking of IgE bound to mast cells and basophils with release of vasoactive mediators	IgG or IgM binds to cellular antigen leading to complement activation and cell lysis. IgG can also mediate ADCC with cytotoxic T cells, natural killer cells and neutrophils	Antigen-antibody complexes are deposited in various tissues inducing complement activation and resulting in an inflammatory response mediated by massive infiltration of neutrophils. Enzymes released from neutrophils damage tissues	Sensitised Th1 cells secrete cytokines, which activate macrophages or cytotoxic T-cells, which mediate direct cellular damage.
Examples of hypersensitivity reactions	Seasonal hay fever, Local and systemic anaphylaxis, asthma	Erythroblastosis fetalis, Blood transfusion reaction, ABPA	Systemic lupus erythematosus, Vasculitis glomerulonephritis	Contact dermatitis, Graft rejections

ADCC – antigen-dependent cellular cytotoxicity, ABPA – allergic bronchopulmonary aspergillosis

1.4.2 Epidemiology of all allergies

Globally, the prevalence of allergic diseases has increased over the past three decades. This increased prevalence of all allergic diseases suggests that the prevalence of atopy, defined as the genetic tendency to develop allergic diseases [67], has increased. This trend has been observed in epidemiological studies from Switzerland and Japan, showing an increased prevalence of atopy in children. In both these studies, the increased prevalence of atopy was due to increased sensitisation to a variety of allergens and not to one particular allergen [68]. The prevalence of sensitisation to allergens varies across regions worldwide due to either genetics or lifestyle habits [69]. However, due to a lack of diagnostic facilities and awareness in most African countries, there is a paucity of allergen prevalence data for this continent [70].

Risk factors for allergy

As reviewed by Gilles *et al*., individual genetic background influences the susceptibility of humans to allergic diseases [71]. This genetic background comprises several genes, each

with two specific allelic variants. These allelic variants are susceptible to modification by beneficial and harmful environmental factors and lifestyle factors. Furthermore, chronic exposure to pollutants, an unhealthy diet, or psychosocial stress have been suggested to induce epigenetic modifications in a subset of the allergen susceptibility genes. The resultant altered expression pattern is then passed on to the subsequent generation (see **Figure 1.2**), causing an increased prevalence of atopy [71].

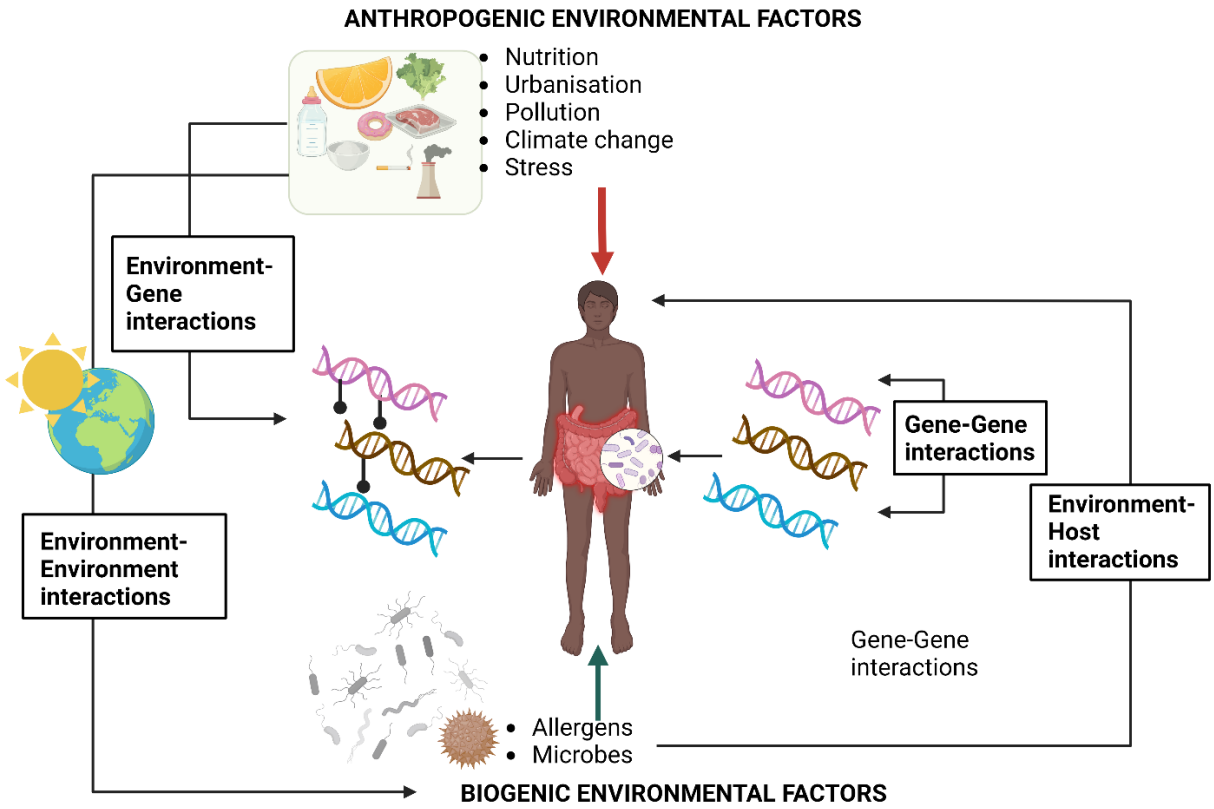

Figure 1.2: Factors influencing allergy development.
Allergic diseases are influenced by genetic, environmental and lifestyle factors. Several genes contribute to the disease phenotype (gene-gene interactions). Environmental and lifestyle factors modify the expression of genes via epigenetic mechanisms (environment-gene interactions). Priming of the innate immune system occurs by shaping the body's composition ′s microbiota. This, in turn, affects immune homoeostasis and predisposition to hypersensitivity in the adult (environment-host interaction). Finally, anthropogenic pollutants and climate change-related factors negatively affect human health either directly, by enhancing tissue inflammation and increasing comorbidities (environment-host interactions) or indirectly by modifying allergen carriers, as shown for pollen-producing plants (environment-environment interactions). Adapted from Gilles *et al.*, [71]. Created with BioRender.com

1.4.2.1 Scientific Hypotheses to explain rising global allergy incidence and prevalence

As previously stated, the prevalence of allergic disease is increasing worldwide, particularly among children [72]. Several population studies in sub-Saharan Africa [73, 74] have found that urban people have a higher prevalence of allergic diseases than rural people [74-79]. It is thought that improved hygiene, reduced pathogen exposure during childhood, and the adoption of a so-called "westernised" lifestyle are responsible for this rise in this region [80], as proposed by the hygiene hypothesis [81, 82].

The Hygiene hypothesis

In 1989, Strachan proposed this hypothesis after observing that the rate of hay fever was consistently negatively associated with family size and household birth position [83]. Thus this hypothesis implies that the concept of allergies is not only genetic but is also environmental [71]. For example, a history of measles infection was shown to be associated with a reduced risk of skin prick test positivity in Children of Guinea-Bissau [84]. However, this hypothesis has been challenged by studies showing that early mycobacterial infections or Bacillus Calmette Guerin (BCG) vaccination does not affect subsequent development of atopy [85, 86]. Therefore, as alternatives to the hygiene hypothesis, Rook proposed the 'old friends' hypothesis [87], Noverr and Huffnagle the ‘microflora hypothesis’ [88] and Haahtela the 'biodiversity hypothesis' [89].

Old friend’s hypothesis

Rook and colleagues proposed the old friend's hypothesis, which describes the co-evolution of microbes and macro-organisms, with the evolution of the human immune system [87]. Similar to the hygiene concept, it suggests that these organisms are necessary for healthy immunological development [87, 90]. For instance, research in Gabon reported that schoolchildren diagnosed with schistosomiasis, a parasitic infection

caused by helminth parasites of the genus Schistosoma, had lower levels of allergen reactivity than their uninfected peers [91].

Microflora hypothesis

The microflora hypothesis suggests that early life disturbances due to factors such as antibiotic use, infection, or diet disrupt the normal microbiota-mediated mechanisms of the gut microbiota. Consequently, the microbiota can promote immunological tolerance and bias the immune system toward a state that promotes hypersensitivity disorders [88].

Biodiversity hypothesis

The biodiversity hypothesis, proposed by Tari Haahtela, suggests that an enriched human microbiome promotes immune balance and protects from allergy and other inflammatory disorders [89].

Epithelial barrier hypothesis

Recently, the 'epithelial barrier hypothesis' was proposed by Cezmi Akdis [92]. This hypothesis posits that disturbance of the epithelial barriers by damaging agents linked to industrialisation and urbanisation cause tissue inflammation and microbial dysbiosis. Therefore, play a role in many chronic non-communicable diseases such as allergies [92].

The three hypotheses (hygiene, microflora and biodiversity) focus on the role of the microbiota in conditions involving inappropriate immune responses and postulate that changes in the composition of the microbes we encounter are the main reason for disease development and pathology [92]. Thus, all these hypotheses suggest that the microbiota have a role in allergy development and will be further investigated in this thesis.

1.4.2.2 Helminths and allergic diseases

Helminth infections and allergic disorders are both public health burdens, with the former being highly prevalent in developing tropical countries while the latter has been suggested to be more prevalent in developed countries and urban areas of developing countries [93, 94]. Paradoxically, while efforts and resources are put together to control and ultimately eliminate helminth infections in developing countries [95], it is hypothesised that the decline in childhood infections, including helminth infections, in developed countries is responsible for, or at least associated with, the rise in allergy and other immune disorders [96, 97].

To date, helminth parasites including soil-transmitted helminths as well as both *Schistosoma haematobium* and *S. mansoni* are thought to induce regulatory immune responses that may dampen the clinical manifestations of atopy [91, 98-100]. However, this 'protective' effect of helminth infection on atopy remains controversial as some epidemiological field studies report mixed results on the effects of helminth infections on allergen-specific IgE in endemic areas [101-103]. These conflicting results could possibly be explained by a number of factors such as host genetics, different helminth species and intensity, time and duration of infection [104, 105]. However, there is paucity of studies investigating these mechanisms in human populations despite results from animal studies [106]. Thus to contribute to this knowledge gap, the effect of *S.haematobium* infection in fungal sensitisation was investigated in **Chapter 4** of this thesis.

1.5 Proposed explanations for the link between the microbiome and disease

The human body harbours diverse species of bacteria, fungi, protists, archaea and viruses that contribute to the host biology known as the microbiota [107, 108]. The microbiome comprises the genomes of the gut, skin and other mucosal environment

microbes, their products, and the surrounding environment [109]. Although all body sites are colonised , the highest microbial numbers are found in the gut [110] which is the focus of this thesis. Recent microbiome research has shown that the gut microbiome plays a vital role in maintaining barrier integrity and influences various host functions, including nutritional responses, metabolism and host immune system [111-113]. As a result, it has been proposed that the gut microbiome plays a role in health and disease.

1.5.1 The gut microbiota: diversity and disease risks

The gut microbiome composition has been suggested to be relatively stable in healthy individuals [114]. However, it can be altered by several factors like antibiotic exposure, diet, disease and birth mode, resulting in susceptibility to non-infectious diseases such as obesity [115], asthma and allergies [116-118] and type 1 diabetes [119]. Therefore, understanding gut microbiota development in early life could provide insight into how its progression impacts immune development and lead to microbial-based therapeutics that target disease prevention at an early age. The several factors influencing early life microbiota and the role of microbiota alterations in disease risks are summarised in **Figure 1.3** below.

The importance of gut microbiota for mammalian immune development has been illustrated in germ-free (GF) mice studies. In these studies, it was observed that the lack of a microbiota resulted in reduced Peyer's patches, fewer plasma cells, and increased susceptibility to pathogen invasion compared to conventionally raised mice [120, 121]. Although GF murine models are valuable in mechanistic studies, these studies do not accurately represent human environmental exposure as they are performed in controlled environments [122].

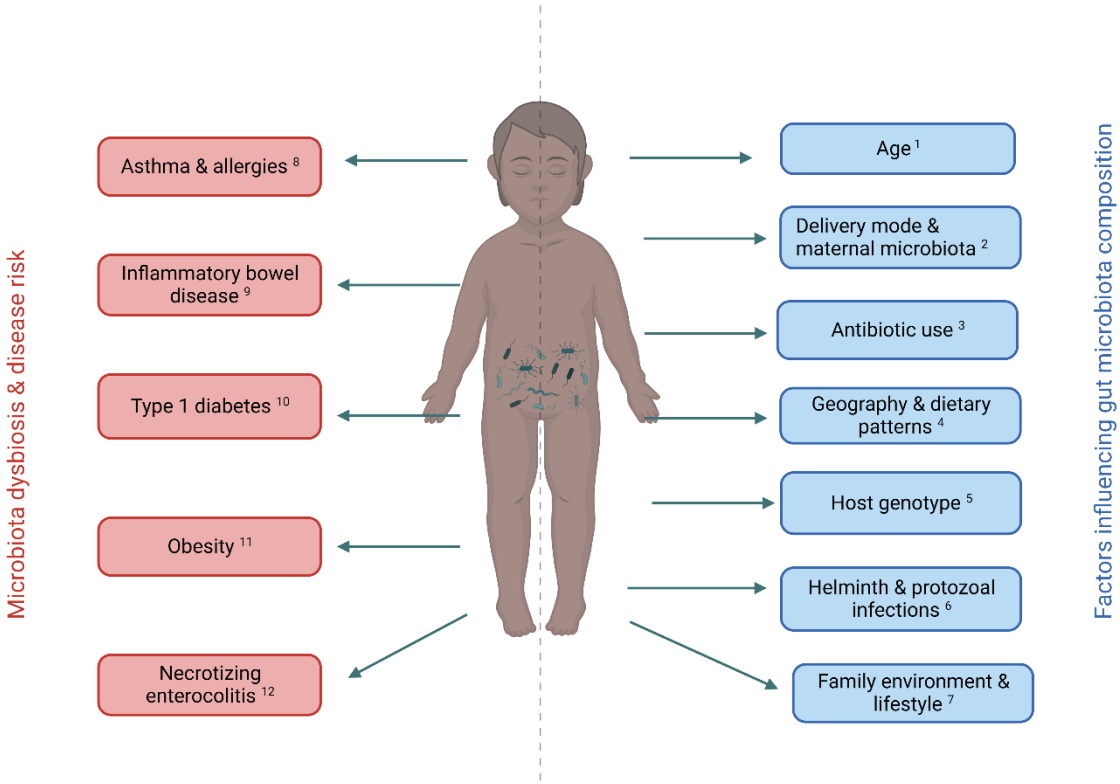

Figure 1.3: Factors influencing the child microbiota diversity, and risk of disease from microbiota alterations
Sources from 1 [123, 124]; 2 [125]; 3 [126, 127]; 4 [128-130]; 5 [131]; 6 [132]; 7 [133]; 8 [134, 135]; 9 [136, 137]; 10 [119]; 11 [138, 139]; 12 [140].

1.5.2 Gut mycobiome and disease

Prior research on the role of the microbiome has centred mostly on the bacterial microbiota. However, the characteristics of other important microbiome components, such as fungi, remain undefined in disease. Fungi residing in the gastrointestinal tract (GIT), collectively termed the mycobiome, account for approximately 0.1% of all gut microbiota [141]. Empirical studies have suggested that the gut mycobiome is crucial to maintaining gut homeostasis and systemic immunity [142]. This has been observed in a study by Jiang *et al.*, [143] where commensal fungi, *Candida albicans* or *Saccharomyces cerevisiae*, could alleviate gut mucosal injuries from dextran sodium sulphate (DSS)-induced colitis in mice and exhibited immune modulatory properties [143].

Similarly to the gut bacteria, the gut mycobiome structure varies depending on age, gender, diet, disease status and antibiotic use [144]. Dysbiosis of the gut mycobiome has been implicated in various diseases such as Inflammatory bowel disease [145-148], Crohn's disease [149], Autism [150], as well as Rett syndrome [151]. Benito-Leon *et al*., [152] hypothesised that the gut mycobiome plays a role in multiple sclerosis, which was also observed in a case-control study [153]. However, further studies are necessary to comprehensively understand the role of the mycobiome in the pathophysiology of these diseases.

1.5.3 Gut mycobiome and allergy

There is mounting evidence that the mycobiome may have a role in the development of allergic reactions. For example, Wheeler and colleagues showed that prolonged oral treatment of mice with anti-fungal drugs increased the abundance of *Aspergillus*, *Epicoccum* and *Wallemia spp* in the gut and exacerbated the development of allergic airway diseases [154]. In the same study, the authors also reported that inducing alterations in the existing mycobiome could change the course of house dust mite (HDM)-induced allergic diseases. This suggests a functional role of the mycobiota in modulating immune function and the development of inflammatory disease [155]. Noverr *et al*., also demonstrated that mice develop allergic airway responses if their endogenous microbiota, including the mycobiome, is altered compared to those with normal microbiota [156, 157].

Furthermore, it has been observed in both human and experimental models that the prevalence of allergic diseases correlates with the widespread use of antibiotics [156-160]. This results in an alteration of the faecal microbiome, which leads to the overgrowth of yeast such as *Candida albicans* and secretes the potent prostaglandin-like immune response modulators involved in inflammation. Given the widespread use of antibiotics in African countries [161, 162] and the increasing prevalence of allergic diseases in this continent, there is a likelihood that gut mycobiome is involved in allergic diseases.

Most of these previous studies suggest a connection between the gut mycobiome and allergy in animal models. However, there is still a lack of studies in relevant model systems for human fungal disease; hence, the mechanism of pathogenesis remains unclear despite all the research progress made in experimental models. This is primarily due to the difficulties in transposing mice immunological responses into valuable human data [163].

1.6 Fungal allergy

Fungal allergies encompass a spectrum of conditions characterised by abnormal immunological responses to fungi. The fungal allergens can elicit hypersensitivity reactions including, Type I, Type III and Type IV and these may act together to mediate the pathogenesis of different allergic diseases. A schematic illustration of these reactions is shown in **Figure 1.4**, but the specific allergens responsible for symptoms remain poorly characterised [164-166]. This is further complicated by the capability of fungi to colonise and germinate in the respiratory mucosa. Thus having a more significant impact on the patients' immune system than other respiratory allergen sources [167], predisposing a susceptible patient to severe allergic disease [168].

1.6.1 Allergy mechanism

Allergic reactions usually exacerbate IgE production specific to a given allergen but may also be mediated by IgG. There are three steps to the allergic response: the sensitisation phase, the effector phase, and the late phase response [169].

Sensitisation phase

An antigen-presenting cell (APC), such as dendritic cells (DCs), presents the allergen to a naive CD4+ T helper lymphocyte. These cells ingest and digest the antigen [170] to

produce ‘epitopes’, defined as specific antigenic peptides/regions within the intact antigenic protein to which immune cells bind to evoke an immune response. The processed epitopes are then transported to the APC surface, where they are conjugated with MHC type II molecules (MHC-II) to form an MHC-II-epitope complex. This complex is then presented to Th cells that bind to the complex via the T cell receptor (TCR), leading to T cell activation [171], Th cell division and production of Th2 cells. These effector Th2 cells generate the cytokines IL-3, IL-4, and IL-13 which stimulate B cells to switch immunoglobulin heavy chain class to the ε type, thus stimulating the production of allergen-specific IgE antibodies [172]. These antibodies bind to high-affinity IgE receptors (FcεRI) on the surface of mast cells and basophils.

Effector phase

A new encounter with the allergen results in the cross-linking of the IgE bound to the FcεRI surface receptor. Th2 cells secrete IL-13 and express CD40 ligand, which, together with IL-4, promote IgE class switching and stimulate the expression of CD23 and the release of soluble CD23 (sCD23). The sCD23 is known to up-regulate IgE synthesis and secretion, thus exacerbating the IgE response [173]. The cross-linking results in the secretion of mediators such as histamine, proteases, and heparin which act on different cell types to cause rapid inflammatory responses such as vasodilatation, increased vascular permeability and smooth muscle contraction, resulting in allergic symptoms. These symptoms include urticaria, angioedema, bronchospasm, vomiting, diarrhoea, and hypotension [174].

Late phase

The late-phase reaction is caused by the activated mast cell's induced synthesis and release of mediators such as leukotrienes, chemokines, and cytokines. These mediators

promote neutrophil, eosinophil, and Th2 cell influx [175]. Eosinophils play a crucial function in the late phase of this immune response by releasing inflammatory mediators such as leukotrienes, prostaglandins, eosinophilic cationic and major basic proteins. These contribute to the widespread tissue damage, which appears clinically as persistent mucus secretion, oedema development, and bronchial hyper-reactivity.

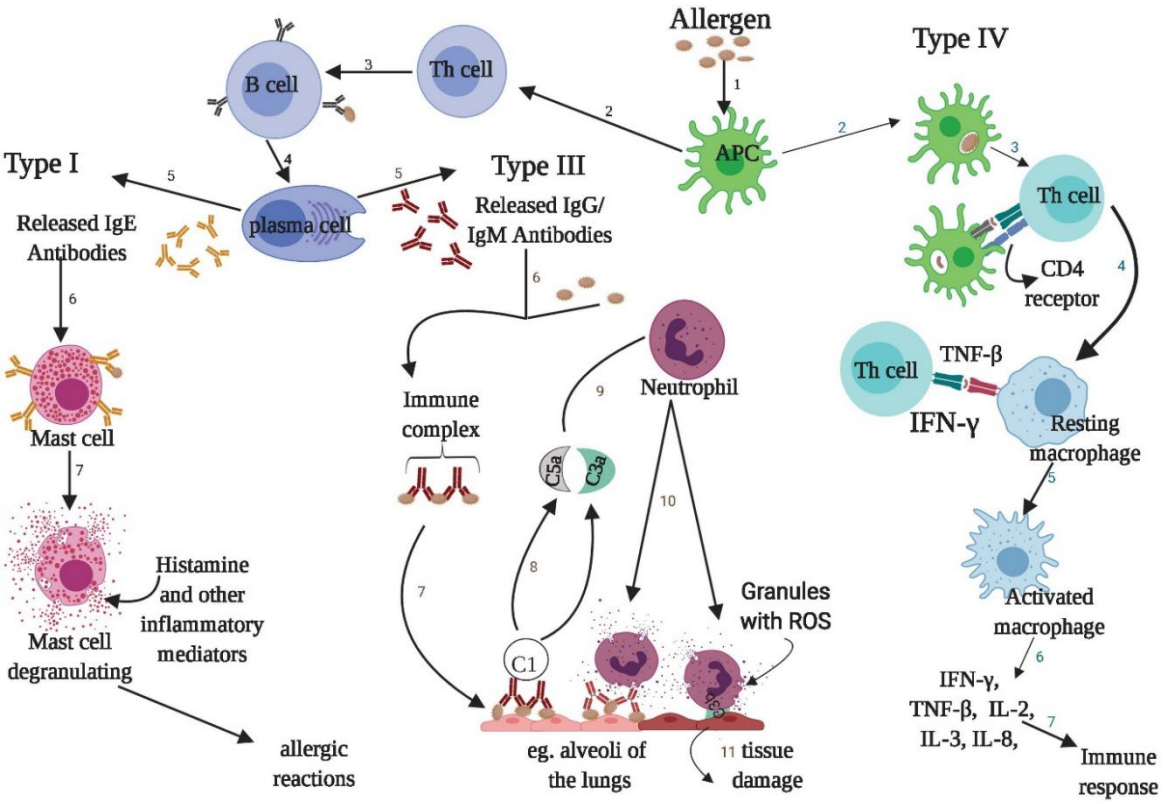

Figure 1.4: Mechanisms of hypersensitivity reactions involved in fungal allergy
In the Type I hypersensitivity reaction, the mechanism of action involves preferential production of IgE (5), in response to allergens and the primary cellular component in this hypersensitivity is the mast cell (6). In Type III hypersensitivity reactions primary components are soluble immune complexes and complement (C3a and 5a) and the neutrophils cause the injury. In Type IV hypersensitivity reactions, the damage is caused by activated macrophages. Diagram adapted from Rajan [63].

Cells and cell-mediators involved in fungal allergic inflammation

Significant progress is currently being made in understanding the mechanistic pathways by which fungi cause or exacerbate allergic diseases such as asthma. Fungal cell wall

components such as β-glucans, chitin and proteases are the main source of pathogen-associated molecular patterns (PAMP) and are recognised by both pattern recognition receptors (PRRs) as well as protease-activated receptors (PARs) on the host cells [176]. These cell wall components have been suggested to be widely conserved across the fungal kingdom, and as they are absent in humans, they are the ideal targets for immune recognition [177]. When exposed to the cell wall components, the epithelial cells mount an immune response against these by releasing chemokines, cytokines and antimicrobial peptides [178].

Fungal proteases induce inflammatory responses by compromising mucociliary clearance, altering the permeability of the epithelial barrier and activating innate immune responses leading to asthma development [179, 180]. The β-glucans induce IL-6, IL-8, and CCL-20 from airway epithelial cells through Dectin-1 receptor [181, 182]. While chitin induces inflammatory responses characterised by IL-17, IL-23 and TNFα [183] as well as induces the expression of IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). This activates the innate lymphoid cells (ILC2s) [184] to express IL-5 and IL-13, which leads to eosinophilia [185] and the accumulation of alternatively activated macrophages.

ILC2s have been shown to contribute to the initiation and persistence of fungus-mediated allergic immune responses in mice [186, 187], suggesting that they have a role to play in fungal allergy. It has been suggested that repeated exposures to fungi allergens lead to the induction of Th1, Th2 and Th17 reactions as well as chronic airway inflammation [188-190] (see **Figure 1.5)**. However, the mechanism that explains how airway exposure to fungal allergens results in increased production and secretion of pro-type 2 cytokines, such as IL-33, leads to activation of ILC2s and other inflammatory cells in airway mucosa is partially understood [186]. Therefore, further studies are required to better understand the mechanistic pathways involved in the pathogenesis of fungal allergy.

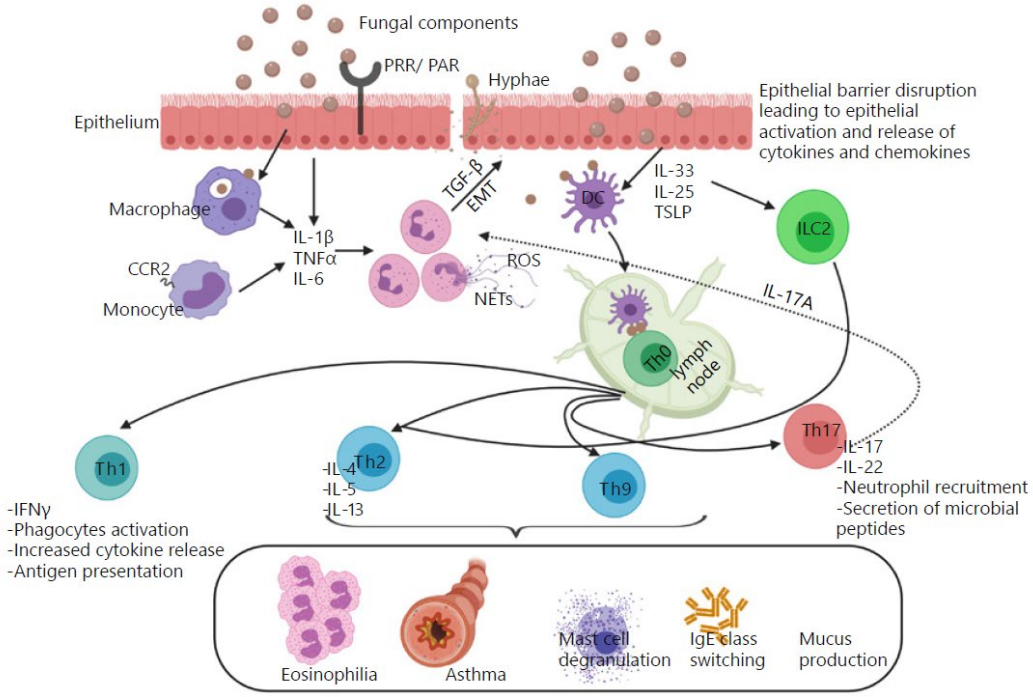

Figure 1.5: Cells and cell-mediators involved in allergic fungal inflammation
The epithelium is exposed to proteolytic enzymes from fungi, which digest proteins of the epithelial layer, making it more porous. Exposure to fungal components induces the selective release and production of IL-33, IL-25 and TSLP by the airway epithelial cells. TSLP, IL-33 and IL-25 activate ILC2s to produce type 2 cytokines such as IL-5 and IL-13, initiating allergic inflammation. TGF-β: transforming growth factor beta; EMT: epithelial-mesenchymal transition; IFN-γ: Interferon-gamma. Adapted from references [185, 187, 191-197].

1.7 Allergens from fungi and sensitisation

Many allergens can be defined as small soluble proteins or glycoprotein molecules of molecular weight 5-100kDa that induce allergic disease via several immunological pathways. The proteins that make up allergens are heterogeneous and have many biochemical functions that may include proteolytic enzymes and transport molecules, yet a large proportion of proteins have still not been functionally characterised. While the nature of an allergen is generally defined in terms of the induction of an IgE response, the molecules stimulate a much broader immune response, including via receptors on T-cells, other antibody isotypes (IgG, IgA) and through innate mechanisms [198]

Allergy may be induced through numerous exposure routes, including inhalation, ingestion, injection, dermal or indirectly via the placenta or breast milk. The common allergens in a population are determined by the molecules that are common in the environment; thus, factors including geography, season, occupation, lifestyle and social settings are important determinants.

Sensitisation to allergens from various environmental sources is the general precursor to developing a respiratory disease, including allergy and asthma in genetically predisposed individuals. Fungal allergens are seasonal, though they can be found throughout the year. Fungi, along with the house-dust mite [199, 200], animal dander [201, 202], cockroach [203] and pollen [204, 205] allergens, are the most widely recognised sensitising sources that have been implicated to have a causal role in the development of sensitisation. However, due to the heterogeneity between fungal species and the variations between allergen extracts as well as the methods of analysis; associations between fungal sensitisation and the severity and incidence of respiratory disease have often been difficult to interpret and remain poorly understood [206, 207].

The most commonly studied and characterised allergens belong to the Ascomycota phylum and originate from *Aspergillus fumigatus, Alternaria alternata,* and *Cladosporium herbarum.* The allergenic proteins of these fungi [208] can induce sensitisation and result in immune-mediated diseases such as asthma [209, 210], allergic bronchopulmonary mycoses [211-213] and/or hypersensitivity pneumonitis [214, 215]. Other fungal species such as *Penicillium chrysogenum* and *Epiccocum nigrum* (*purpurascens)* have also been implicated in fungal sensitisation.

Currently, the fungal allergen extracts used for skin prick test (SPT) and provocation tests are typically crude, un-purified, and highly variable, as a result of fungal growth being dependent on specific temperature, nutrients, moisture and light parameters.

Consequently, when these conditions vary slightly or are limited, the expression of many proteins is altered. To adapt to these modified conditions, the fungus responds by synthesising a different assortment of proteins, resulting in variable fungal allergen extracts [216].

Prevalence of skin prick reactivity to fungi has been shown to range from 3% to 58% [217-222]. These results highlight the differences between distinct populations and thus emphasize the need for studies to characterise sensitisation patterns in different regions of the world [223, 224]. The heterogeneity in the prevalence of reactivity can be accounted for by variations in airborne fungal concentrations in different sampling environments, the selection criteria for study participants, the source, and the batch of commercial fungal extracts.

Although progress has been made in characterising and identifying clinically relevant allergens, the fungal allergen repertoire has not yet been characterised in an African population. However, to improve diagnosis [225] and the management of fungal patients globally, fungal allergens in different geographic locations need to be identified. **Tables 1.2** and **1.3** below represent the fungal allergens identified and officially named according to the WHO/IUIS nomenclature (http://www.allergen.org), as well as those found on the Allergome database [226] from *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum, Penicillium chrysogenum* and *Epicoccum nigrum* (*purpurascens).*

Table 1.2:Species-specific Allergens

Allergen source (Species)	Allergen	Molecular weight range(kDa)	Protein family	References
Alternaria alternata	Alt a 15*	50-58	Serine proteases	[227]
	Alt a 10*; Alt a 8*	28-53	Dehydrogenases	[228, 229]
	Alt a 4*	57	Disulfide isomerases	[228, 230]
	Alt a 7*	22	Flavodoxins	[228, 230]
	Alt a1*	11-45	Unknown	[228, 231]
Aspergillus fumigatus	Asp f 23*	44	Ribosomal proteins	[232]
	Asp f 17*	19.42	Galactomannan proteins	[232]
	Asp f 34*	19-20	Cellwall proteins	[233]
	Asp f 10*	34-35	Aspartic proteases	[232, 234]
	Asp f 15*	15-16	Cerato platanins	[232]
	Asp f 9*	33.7	Glycosyl hydrolases	[232, 235]
	Asp f 5*	42-43	Metallo proteases	[232]
	Asp f 2*	34-37	Fibrinogen binding proteins	[232]
	Asp f 1*	16-18	Ribonucleases	[230]
	Asp f 4*; Asp f 7*	11-45	Unknown	[232]
Cladosporium herbarum	Cla h 9*	50-58	Serine proteases	[227]
	Cla h 8*; Cla h 10*	28-53	Dehydrogenases	[228, 229, 236]
	Cla h 7*	22	Flavodoxins	[228]
	Cla h HCh1	10.5	Hydrophobins	[237]
	Cla h2*	11-45	Unknown	[231]
Penicillium chrysogenum	Pen ch 20	68	N-acetyl-glucosaminidase	[238]
	Pen ch 31		Calreticulin	[239]
	Pen ch 33	16	Unknown	[239]
	Pen ch 35	36.5	Transaldolase	[3]

**These allergens have been approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Committee [240]. All the other allergens can also be found in the Allergome database [226].*

Cross-reactivity and autoreactivity

Fungi polysensitisation (sensitisation to multiple fungi) or cross-reactivity is frequently observed in clinical cases. Cross-reactivity can be seen when IgE antibodies initially directed against a given allergen also bind to a structurally related allergen from another allergenic source [241]; thus, it results from shared B-cell epitopes among homologous proteins. This makes the precise identification of a given fungal allergen challenging. This is further complicated by the fact that fungi share several potentially allergenic epitopes, making an accurate diagnosis of a specific fungal allergy difficult [242].

Cross-reactivity may be analysed by various techniques, such as immunoblots, Enzyme-linked immunosorbent assay (ELISA) and radioallergosorbent test (RAST) inhibition. For example, Tee *et al*., [243] identified considerable cross-reactivity between *Alternaria* and *Cladosporium* by studying cross-reactivity between different fungal genera using RAST-inhibition. Component-resolved diagnostic (CRD) techniques [244] have been utilised to map a patient's allergen sensitisation at the molecular level using pure natural or recombinant allergenic molecules in place of allergenic extracts [245], hence avoiding the cross-reactivity problems.

Due to the similar epitopes between fungal and human proteins [61], such as manganese superoxide dismutase [100], thioredoxin, cyclophilins, and acid ribosomal proteins, fungi have been shown to contribute to auto-reactivity against self-antigens. The underlying process is thought to be molecular mimicry [61, 101], which is responsible for the persistence of severe chronic allergic disorders such as atopic dermatitis [102]. Currently, the evidence to link fungal exposure to the induction of autoimmune diseases is debatable. Studies by Miyoshi *et al*., and Myllykangas-Luosujarvi *et al*., [246, 247] suggest that fungal proteins have a role to play in autoimmune diseases. However, further studies are needed to establish fungi's role in autoimmune diseases' immunopathology.

Table 1.3: Cross-reactive Allergens

Allergen source (Species)	Allergen	Molecular weight range(kDa)	Protein family	References
Alternaria alternata	Alt a 6*	45-48	Enolases	[228, 230]
	Alt a 12*;Alt a 5*	11-12	Ribosomal proteins	[228, 230]
	Alt a 3*	65-90	Heat shock proteins	[228, 231]
	Alt a TCTP	18-22	Translationally Controlled Tumour proteins	[248]
	Alt a NTF2	13-14	Nuclear transport factors	[249]
Aspergillus fumigatus	Asp f 22*	45-48	Enolases	[250]
	Asp f 11*; Asp f 27*	16-20	Cyclophins	[251, 252]
	Asp f 6*	22-25	Manganese superoxide dismutases	[232, 253, 254]
	Asp f 8*	11-12	Ribosomal proteins	[255]
	Asp f 12*	65-90	Heat shock proteins	[228]
	Asp f 3*	17-19	Peroxisomal proteins	[256]
	Asp f 13*;Asp f 18*	32-34	Serine proteases	[257, 258]
	Asp f 28*;Asp f 29*	10-12	Thioredoxins	[242, 259]
	Asp f GST	26	Glutathione-S-transferases	[260]
Cladosporium herbarum	Cla h 6*	45-48	Enolases	[250, 261]
	Cla h 12*;Cla h 5*	11-12	Ribosomal proteins	[242, 255]
	Cla h TCTP	18-22	Translationally Controlled Tumour Proteins	[262]
	Cla h NTF2	13-14	Nuclear transport factors	[249]
Penicillium chrysogenum	Pen ch13	34	Alkaline serine protease	[3]
	Pen ch18	32	Vacuolar serine protease	
***Epicoccum nigrum* (*purpurascens*).**	Epi p1	30	Serine proteases	[3]

**These allergens have been approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Committee[240]. All the other allergens can also be found in the Allergome database [226].*

1.8 Diagnosis of fungal allergic disease

The clinical tests available for diagnosing an allergy to a specific allergen can be divided into those conducted *in vivo* or *in vitro*. *In vivo* methods include SPT, intradermal tests and inhalation challenges. *In vitro* methods are further divided into allergen specific-IgE assays or basophil degranulation assays. RAST which is the original allergen specific-IgE assay has since been replaced by the trademarked ImmunoCAP assay [263]. More recently, a multiplex chip technology has been developed to offer a more comprehensive allergy diagnosis; however, it is not widely used [264]. Basophil degranulation assays which are less commonly used, include the basophil activation test and histamine release assay [265-268]. These clinical tests are described below.

1.8.1 In vivo methods for allergy diagnosis

SPT and intradermal testing

SPT is commonly used to demonstrate an immediate IgE-mediated allergic reaction [269]. It is a valuable diagnostic tool as it is robust, easy to administer and an efficient means of screening a patient for allergy to the tested allergens [270-272]. A European SPT panel for specific inhalant allergens is commonly used and includes birch, grass mix, *Alternaria*, cat, dog and house dust mite [273]. Although the results from an SPT should be used in conjunction with a clinical history and/or serum-based immunoassays, its ease of use means it remains a cornerstone in allergy diagnosis.

Inhalation challenge

Although used worldwide, this technique is only conducted in a limited number of centres with specialist equipment and adequately trained healthcare professionals. During the inhalation challenge, the patient is exposed to the suspected allergen under controlled conditions and with close observation [274]. Their lung function is measured during and following the exposure and compared to their response to a control substance, usually methacholine. This technique is useful for occupational asthma diagnosis as the suspected causative allergen may not be available as an SPT reagent [274, 275].

1.8.2 In vitro methods for allergy diagnosis

Allergen specific-IgE assays

Methods for determining the presence of allergen specific-IgE from patients' serum rely on allergosorbent solid-phase technology [172]. Compared with *in vivo* testing, these methods are more standardised and automated. However, the results from serum-based assays are not immediate, less translatable to the patient (SPTs provide visual evidence), more expensive, and require blood sample collection [276].

The radioallergosorbent test (RAST)

RAST uses a radioactive isotope, Iodine$_{125}$, as a detection substrate for determining the quantity of allergen specific-IgE in patient serum [277]. A gamma scintillation counter gives a percentage of the total counts of radioactivity observed. Because of the recognised hazard of using radioactive isotopes, this detection method has largely been replaced with fluorophore substrates; however, the basic principles of allergen and specific-IgE binding remain the same.

Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay, commonly known as the ELISA, is a well-established technique used to quantify specific proteins, such as antigens or antibodies. Similar to RAST, the principle of an ELISA is an antigen-antibody interaction where the specific antibodies associate or bind to their target antigen. Only when the interaction occurs can the substrate bind to the enzyme; thereby, substrate conversion can be observed, and a positive result can be obtained. This technique is usually used in research settings. A major drawback of this assay in measuring specific IgE is that all specific antibodies, irrespective of their isotype, can become bound to the plate. For example, specific-IgG antibodies compete with specific-IgE for binding to the allergen [278].

Singleplex ImmunoCAP assay

The ImmunoCAP system, developed by Thermo Fisher Scientific in Uppsala, is now the most used system to quantify allergen specific-IgE. It is a fully automated fluorescent enzyme immunoassay (FEIA) that tests for specific-IgE to over 650 allergens. In the assay, allergens are covalently bound to a cellulose solid phase and react with allergen specific-IgE in patient serum. An enzyme-labelled anti-IgE antibody is used to bind to specific-IgE, and a developing agent is added, producing fluorescence when bound to the

enzyme. The measure of fluorescence represents the quantity of allergen specific-IgE present in the serum.

The ImmunoCAP assay has several advantages. As the allergen is fixed in excess to the cellulase solid phase, it allows for the complete binding of specific-IgE antibodies, resulting in high sensitivity. It has good specificity as there is no interference from competitive inhibition by allergen-specific IgG antibodies. However, the ImmunoCAP assay does not distinguish between binding to separate allergen components which may have greater or lesser clinical relevance [279]. Due to this, irrelevant positive results may be obtained if allergen specific-IgE binds to proteins in the allergen extract that have no clinical relevance. This is the case for glycoproteins in some allergens with an α-1,3-fucose epitope structure, also known as 'cross-reactive carbohydrate determinants'. These are present in pollens and venoms and are a well-defined cause of false-positive results in allergy diagnosis [280].

Multiplex ImmunoCAP chip technology

Recently, a multiplex ImmunoCAP specific-IgE assay became available. This microarray chip technology allows for the simultaneous measurement of specific IgE from one serum sample to a broad spectrum of individual allergen components from different sources. Trademarked as an ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC), the device contains allergen components immobilised in a polymer-coated microarray [264]. It uses the same FEIA technique to measure specific IgE as the ImmunoCAP assay described above. The use of ISAC in clinical diagnosis provides a detailed patient specific-IgE antibody profile to numerous allergen components, using only a minute amount of serum (40 µL). Although the chip could be used as a screening tool to conduct several measurements simultaneously, it is costly, thus limiting its potential use [279]. Determining a patient's specific-IgE profile offers the opportunity for more personalised treatment and immunotherapy against specific allergen components.

Basophil activation test

The basophil activation test is used occasionally in clinical diagnosis of allergy but is more commonly used in the allergy and immunology research sector [281]. The test is also used to monitor the progress and success of allergen immunotherapies [282]. The test relies on specific-IgE bound to the surface of basophils causing granules to be released in the presence of the suspected allergen.

Histamine release assay

The histamine release assay similarly relies on the use of whole blood and detecting the release of mediators from activated basophils in the presence of the suspected allergen. It can be conducted using a glass-fibre fluorometric method, where released histamine binds to the solid phase. All other blood constituents are washed away, and the trapped histamine is eluted by increasing the pH. A complex is then formed with o-phthaldialdehyde, which can be quantified fluorometrically [267, 283].

1.9 Management of allergic diseases

Accurate diagnosis of the allergens causing allergy disorders provides therapeutic potential for allergen-specific therapies, such as allergen avoidance and immunotherapy. The first consideration in dealing with allergic patients is avoidance. However, allergen avoidance is particularly contentious when it comes to aeroallergens and respiratory allergic disorders [284].

Currently, the management of allergies in acute cases includes antihistamines, non-steroidal anti-inflammatories and corticosteroids to suppress eosinophilic airway inflammation. These are uniformly applied to all cases, irrespective of the underlying cellular and cytokine profile, resulting in poor disease management. Besides being just palliative, they may further complicate the disease outcome.

To date, the only disease-modifying approach is allergen-specific immunotherapy (AIT), in which allergy-inducing molecules are used for de-sensitisation [285]. This approach involves administering increasing doses of an allergen to a patient to achieve clinical and immunological tolerance over time. Allergen injection immunotherapy induces T cell tolerance by several methods, including decreased allergen-induced proliferation, alteration of secreted cytokines, stimulation of apoptosis, and the production of regulatory T cells (Tregs). This results in reduced inflammatory cells and mediators in the affected tissues, the production of blocking antibodies, and the suppression of IgE [286]. AIT is widely used for bee and wasp, house dust mites, pollen and pet allergies [287]. To date, there is limited evidence supporting the use of AIT in managing fungi-induced asthma and allergic rhinitis. An exception is *Alternaria alternata,* as this is the only standardised allergen extract available [288].

1.10 Rationale of the research described in this thesis

Presently, there is a dearth of data on serious fungal infections in sub-Saharan Africa (SSA), including Zimbabwe. The existing literature reveals several case reports of fungal infections, most of which are outdated (reported 20 years ago) [289, 290]. These fungal diseases cause increased morbidity, mortality and economic costs, particularly in the setting of HIV/AIDS, TB, and poverty prevalent in SSA. Furthermore, existing data supports that there is an increasing incidence of allergies. These allergies are far more prevalent than TB or HIV, affecting 25 to 30% of the SSA population, resulting in significant morbidity, employment absenteeism, loss of quality of life, and sometimes, fatal outcomes [291].

The relevance of the hygiene hypothesis in Africa requires contextual investigation. There has been a tendency to assume that ‘absence of evidence means evidence of absence’

when it comes to immune dysfunction diseases such as allergies and autoimmunity in Africa. The lack of data on these conditions in Africa [222] is partly due to poor, or lack of appropriate diagnostics as well as limited awareness [28]. Recent reports from different African countries indicate that allergic conditions are common; however, their frequency and associated triggers are rarely documented.

Allergies due to fungal allergens are also the least studied. Previous studies in some parts of SSA determined the prevalence of fungal sensitisation amongst secondary referral patients [292, 293]. Thus, only those with severe symptoms that warranted specialist consultation and had the financial capacity to afford specialist care were included. Consequently, the cost barriers meant only a small proportion of affected individuals were captured in the studies [1]. Therefore, studies representative of the population are needed to investigate the prevalence of these diseases in the African population.

Generating empirically based knowledge on the above will provide evidence to inform health workers of the scale of fungal diseases, including allergies in an African setting, as well as inform the diagnosis and management of fungal allergic disorders. This is of increasing importance, particularly for patients who may present with co-morbidities.

Allergic sensitisation and inflammation studies of human populations and experimental studies in animal models point to interactions between the external environment, the microbiome, and immune function in early life as causing an underlying predisposition to allergic sensitisation [294]. The studies report that an alteration in the mycobiome [295] is associated with the development or exacerbation of allergic conditions such as asthma [154, 156, 157, 296]. However, only a limited number of studies have been carried out in human populations, highlighting the need to extend further the present knowledge regarding the relationship between the human mycobiome and fungal allergy, which would give insight on the pathogenesis of fungal-induced allergies.

To date, a comprehensive characterisation of gut mycobiome is lacking in the African population. Investigating the gut mycobiome in fungal sensitisation and/ or allergy could reveal associations between gut mycobiome structure and sensitivity and/ or tolerance to fungal allergens, as well as the number of unculturable fungal species whose influence on fungal sensitised and/ or allergic people is yet unknown. These findings could then inform the development of appropriate diagnostics and interventions for fungal allergic diseases, particularly those occurring as co- or multi-morbidities. This is critical for African health systems where the growing burden of non-infectious diseases must be managed on a background of endemic and epidemic infectious diseases.

1.11 Thesis aims

The overall aim of this study is to characterise the epidemiology, immunology, and aetiology of fungal allergic diseases in Zimbabwe.

The specific objectives of this thesis were as follows:

i. To determine the burden of fungal diseases in Zimbabwe
ii. To characterise the structure (abundance and diversity) of the gut mycobiome and to determine the relationship between fungal sensitisation/ seroreactivity and gut mycobiome. The effects of host age, sex and *S. haematobium* infection were also determined.
iii. To identify and characterise the fungal proteins recognised by serum samples from fungal-sensitised Zimbabwean patients
iv. To investigate the diagnostic relevance of an fibrinogen binding protein (Asp f 2) peptide in fungal allergy

1.12 Thesis outline

This chapter describes the current understanding of the epidemiology of fungal diseases including fungal allergies in Africa, the knowledge gaps and how the work presented in this thesis contributes to this knowledge base.

Chapter 2 outlines the study design related to the specific aspects of this study, as well as methodological considerations related to fieldwork and laboratory analysis. The main statistical methods used to analyse the data described in this thesis are also outlined.

In contributing to data on the global burden of fungal diseases, **Chapter 3** determines the prevalence and incidence of fungal infections in Zimbabwe using local and international registries as well as epidemiological data from other countries outside of Africa.

Chapter 4 characterises the structure of the gut mycobiome of Preschool-aged children (PSAC) and describes the relationship between the gut mycobiome and fungal sensitisation/seroreactivity. It further describes the effect of host age, sex and *S. haematobium* infection on fungal sensitisation/seroreactivity.

Chapter 5 identifies and characterises the fungal proteins recognised by serum samples from fungal-sensitised individuals.

Chapter 6 provides an investigation of the utility of *A. fumigatus* (Asp f 2) peptide in the differential diagnosis of fungal allergic diseases.

Chapter 7 summarises and discusses the major findings presented in this thesis in broader terms. It further provides suggestions for further work.

Chapter 2 Methods

2.1 Introduction

The study designs and methods detailed here, describe all the procedures used in this PhD project for investigating the epidemiology, immunology and aetiology of fungal sensitisation in a Zimbabwean population.

2.2 Literature review

To determine the burden of fungal diseases in Zimbabwe (**Chapter 3**), an exhaustive search of epidemiological publications using PubMed, Web of Science, EMBASE and Google Scholar was carried out. The relevant publications were retrieved and the quality of the papers was assessed via an adapted Grading of Recommendations, Assessment, Development and Evaluations (GRADE) score [297] approach. The detailed method is in **Chapter 3.**

2.3 Fieldwork studies

2.3.1 Ethical approval

Ethical and Institutional approval were received from the Medical Research Council of Zimbabwe (MRCZ/A/1964) and the University of Edinburgh (fmutapi-0002), respectively. Permission to conduct the study in the region was obtained from the Provincial Medical Director. At the beginning of the study, all participants and parents/guardians of participating children had the aims and procedures of the project explained fully in the local language, Shona. Written consents were obtained from participants and parents/guardians of participating children before enrolment into the study. Only compliant participants were included in the study, and all recruited participants were free to withdraw at any time. Each participant was allocated a unique case number, and all data collected from an individual was identified by case number.

2.3.2 Study designs

This was a descriptive cross-sectional study based on data obtained from two studies;

a. **Paediatric schistosomiasis study:** this was a field study where the overall health impact of paediatric schistosomiasis in children aged five years and below was investigated. Within this broader framework, the structure of the gut mycobiome was characterised and related to fungal sensitisation and seroreactivity (**Chapters 4-5**).

This study was carried out Shamva District, Northeast Zimbabwe [298] . This region is *S.haematobium*-endemic with >50% endemicity, while the prevalence of *S. mansoni* and soil-transmitted helminths (STH) is low (<15%) [299]. It is one of seven districts in the Mashonaland Central province of Zimbabwe, with a population of about 165, 641; according to the 2022 national census [300]. Shamva has a humid subtropical, dry winter climate and the main activity in the area is subsistence farming. The inhabitants from the study area are of similar ethnicity (Shona) and socioeconomic background.

b. **Allergy, asthma study:** was conducted at Asthma, Allergy and Immune Dysfunction Clinic in Harare. Within this study, a peptide from *A. fumigatus* Asp f 2 was used to assess its utility in the differential diagnosis of fungal allergy (**Chapter 6**).

These two studies are summarised in **Figure 2.1** below.

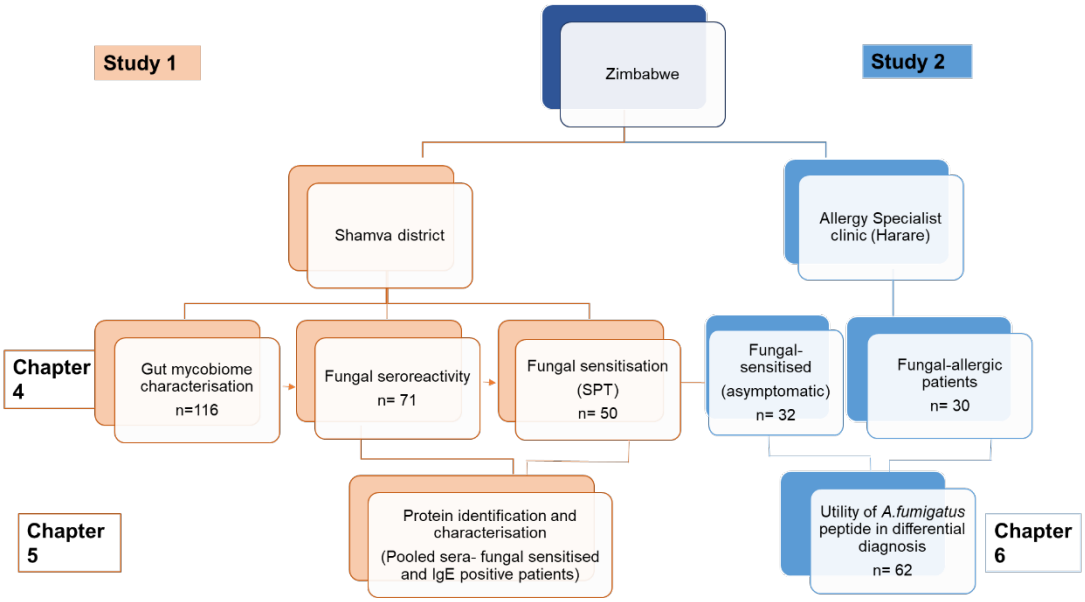

Figure 2.1: Summary of the two cross-sectional studies carried out in this thesis

2.3.3 Sample collection, processing and storage

In this thesis, blood, urine and stool samples were collected in the field. The collected blood samples were processed for serum for serological / immunoassays in **Chapters 4-6**. The urine and stools samples were collected as part of the Paediatric schistosomiasis study to screen for *S. haematobium*, *S. mansoni* and soil-transmitted helminths (STH). Additionally, stool samples were also collected and DNA extracted for the characterisation of the gut mycobiome in **Chapter 4**. The sera and the extracted stool DNA were stored at -20 °C in the field and transferred to a -80 °C freezer in the laboratory at the University of Zimbabwe. The samples were then shipped on ice to the University of Edinburgh and stored at -80 °C until use. Specific details of sample collection, processing and storage are in **Chapter 4**.

2.3.4 Parasitology

Following the standard urine filtration method [301], urine samples were examined microscopically for *S. haematobium* infection. To screen for *S.mansoni* and STH stool

samples collected were processed using the Kato–Katz thick smear method [302]. These two methods are detailed in **Chapter 4**.

2.3.5 Field data handling and cleaning

In terms of fieldwork, I was involved in the research methods and procedures described to obtain the data required for this study. Prior to any statistical analyses, data was cleaned and verified to correct any inconsistencies.

2.4 Clinical studies

2.4.1 Skin Prick Tests (SPT)

SPTs / scratch tests are widely used in clinical diagnostics and research [303-305]. The test is conducted according to international standards for testing [269, 273], where a small amount of possible allergens are applied to a patient's forearm. The patient's skin is then scratched so that the allergens can react with the skin, and if they are allergic to the substance, a reddish, elevated bump with a red ring around it will appear, which can be measured. This bump may also be severely itchy [306]. SPTs are relatively inexpensive and easy to perform. Furthermore, they yield rapid and visible results, which are informative to the patient. However, interpretation is difficult in patients with eczema or dermatographism, and the use of antihistamines may suppress the wheal and flare reaction [307]. In **Chapter 4**, SPTs were performed to determine the prevalence of fungal sensitisation in the study population.

2.5 Laboratory studies

2.5.1 Protein quantification

2.5.1.1 Determination of protein concentration

Protein concentration determination is essential and frequently used in protein biology, molecular biology, and other scientific fields before proceeding with protein isolation,

purification, and analysis. To date, several protein estimation methods have been developed, including the Biuret, ultraviolet (UV) absorption, Lowry, bicinchoninic acid (BCA), and Bradford assays [308]. In comparison to others, each approach has advantages and limitations.

In Chapter 4, protein content in all the fungal extracts used was determined using the Bradford assay [309], which encompasses various preparations of the dye Coomassie Brilliant Blue G-250 used for protein quantitation purposes. This assay is incompatible with most detergents typically employed to solubilize membrane proteins. However, it was chosen because of its high sensitivity, ease of use and low cost of the reagents [310].

2.5.1.2 Enzyme-linked Immunosorbent assay (ELISA)

ELISAs are a standard and robust method for detecting the presence and concentration of antibodies or antigens [311, 312]. The technique is based on a specific antigen-antibody reaction [313]and to date there are four types of ELISAs; direct, indirect, sandwich and competitive and these are summarised in **Figure 2.2**.

In all ELISAs, the most important step is immobilisation of the target antigen, which is achieved through either direct adsorption to the test plate or indirect attachment of a capture antibody to the plate. The antigen is then either directly (conjugated primary antibody) or indirectly (such as conjugated secondary antibody) detected [314]. The assay permits for the sensitive and selective quantification/qualification of antigens such as proteins, nucleic acids, and peptides [315]. Additionally, it is sensitive due to enzyme amplification strategies [316]. However, it is a time-consuming method, and insufficient blocking of the surface of the antigen-immobilised microtiter plate increases the likelihood of false-positive or false-negative results [313]. In this thesis, Indirect and Sandwich ELISA were used in **Chapters 4** and **6** respectively.

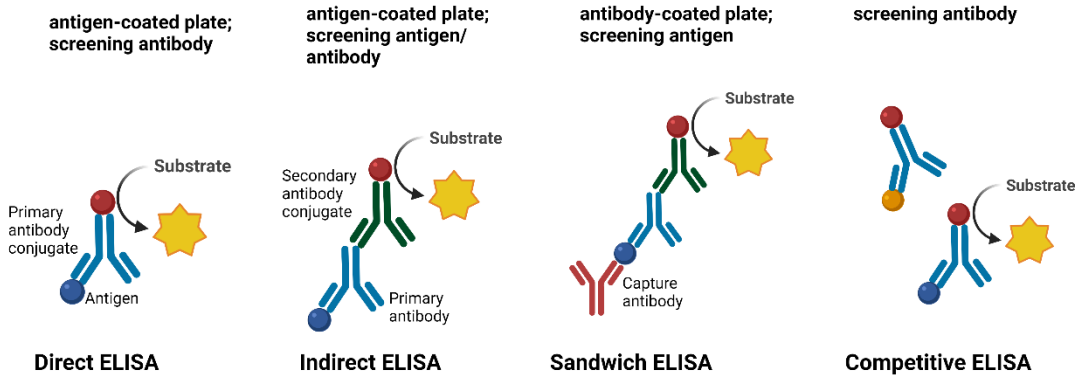

Figure 2.2: The different types of ELISA
Direct, indirect, sandwich, and competitive. Created using BioRender.com

2.5.2 Protein identification

2.5.2.1 Protein separation/ sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE method [317] is the most widely used electrophoresis technique. In this method, the protein mixture is denatured by heating at 95°C in excess SDS which is an ionic detergent that denatures and binds to proteins to make them uniformly negatively charged, and a reducing reagent (β-mercaptoethanol) is employed to break disulphide bonds. The use of the SDS results in the separation of proteins by molecular weight (MW) only [318]. Consequently, when a current is supplied, all SDS-bound proteins will migrate through the gel toward the positively charged electrode. Due to the sieving effect of the gel matrix, proteins with lower MW pass through the gel matrix more rapidly than those with higher MW [319]. Once separated by electrophoresis, proteins can be detected in a gel with various stains, transferred to a membrane for Western blotting detection, and excised for mass spectrometric analysis. In **Chapter 5** crude fungal extracts were separated using this method.

Protein staining

After electrophoresis, proteins can be visualised using various in-gel detection methods such as Coomassie blue, silver nitrate, Sypro ruby, Deep Purple, and DIGE. Coomassie blue is traditionally the most widely used dye [320]. However, according to Neuhoff *et al*.,

[321], it has low sensitivity in protein detection. In contrast, silver nitrate has higher sensitivity but could interfere with protein analysis by mass spectrometry (MS) [322].

Principally, protein staining has four steps: water wash to remove electrophoresis buffers from the gel matrix, fixing of the gel with acid/ alcohol to limit diffusion of protein bands from the matrix, staining of the gel and de-staining to remove excess dye from the gel matrix background. However, depending on the particular staining method, two or more of these functions can be accomplished with one step. For example, Coomassie blue formulated in an acidic buffer can effectively fix and stain in one step. This dye was used in **Chapter 5** enabling the use of the stained proteins in downstream analysis.

2.5.2.2 Western blotting

Western blotting is a fundamental technique for detecting and characterising proteins [323-325]. The method allows one to identify particular proteins by utilising the specificity of antibody-antigen interaction. This technique is powerful since it combines electrophoretic separation of proteins with immunological identification.

Principally, western blotting has three main elements: separation of proteins, transfer to a solid support and visualisation of the target protein using primary and secondary antibodies [326]. Western blots are sensitive and specific, capable of detecting picogram concentrations of protein in a sample [327]. However, this technique is only possible if a primary antibody against the target protein is present. To detect post-translational alterations such as phosphorylation of target proteins, phosphorylated residue-specific antibodies are required. In addition, many antibodies may have off-target effects by interacting with other proteins [328]. **Figure 2.3** summarises the western blot process.

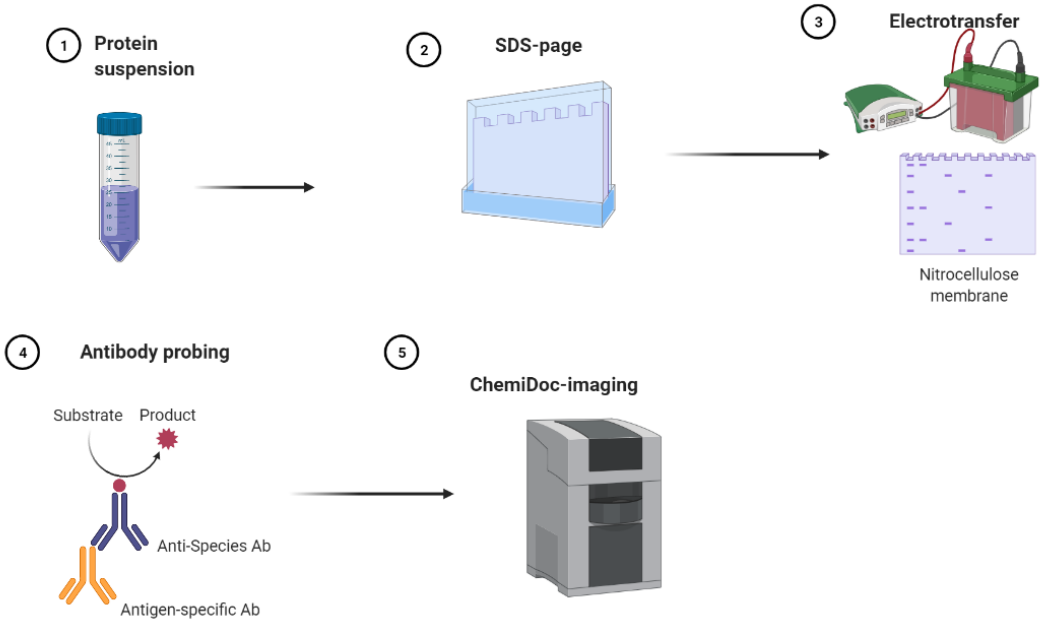

Figure 2.3: Overview of a western blot protocol
Protein suspension to be separated (1); proteins are separated by molecular weight using SDS-PAGE (2) and electrotransfer of proteins to a membrane (3). The membrane is incubated with an antigen-specific primary antibody (Antigen-specific Ab), followed by a conjugated secondary antibody (Anti-species Ab) (4). The luminescence emitted from the substrate binding to the conjugate antibody is captured (5). Created using BioRender.com

2.5.2.3 Mass spectrometry (MS)

Mass spectrometry (MS) is an invaluable technique used to detect, identify and quantitate molecules based on their mass-to-charge (m/z) ratio [329, 330]. It is versatile as it can search for the most abundant proteins in a sample or be targeted to identify specific species of interest. The great advantages of mass spectrometric sequencing include the high sensitivity, the rapid speed of the analysis, the large amount of information generated and the ability to detect post-translational modifications [331-333].

Protein identification via MS is usually carried out in the form of whole-protein analysis ("top-down" proteomics) or analysis of enzymatically or chemically produced peptides ("bottom-up" proteomics) [334]. In this thesis, the "bottom-up" approach was taken whereby the proteins in the fungal extract were separated prior to enzymatic digestion (**Chapter 5**), followed by further peptide separation online coupled to tandem mass spectrometry (**Figure 2.4**) as illustrated in the flowchart below (**Figure 2.5**).

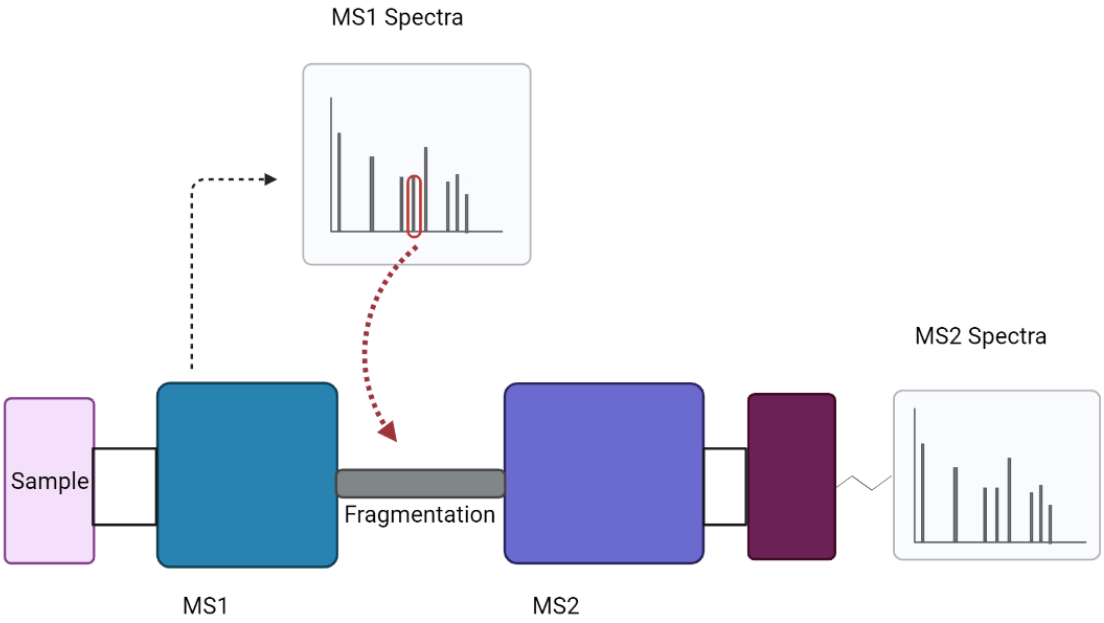

Figure 2.4: Diagram of tandem mass spectrometry (MS/MS).
A sample is injected into the mass spectrometer, ionized, accelerated and analysed by mass spectrometry (MS1). Ions from the MS1 spectra are then selectively fragmented and analysed by the second stage of mass spectrometry (MS2) to generate the spectra for the ion fragments. While the diagram indicates separate mass analysers (MS1 and MS2), some instruments utilise a single mass analyser for both rounds of MS. Adapted from ThermoFisher Scientific [335]. Created using BioRender.com

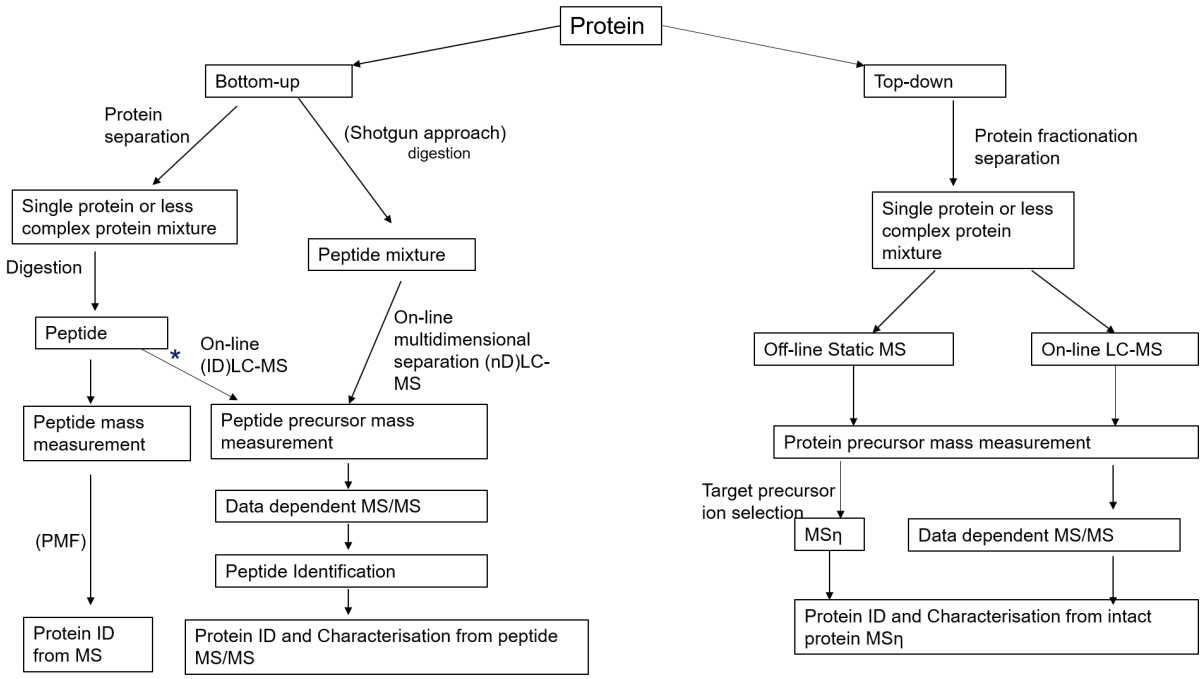

Figure 2.5: Strategies for MS-based protein identification and characterisation.
Proteins extracted from the fungal samples were analysed by the bottom-up method *. Adapted from Konstantinell [336]

2.5.2.4 Peptide synthesis

Peptide synthesis methods are conveniently divided into solution and solid-phase peptide synthesis (SPPS) [337]. SPSS was used in this thesis (**Chapter 6**). This method involves attaching the peptide chain to a stable solid phase. During synthesis, which includes de-protection, activation, and coupling steps, the peptide sequence remains bound to this resin while other by-products are removed by filtration and washing. After the peptide has been synthesised, it is separated from the solid phase using a cleavage procedure and is purified and characterised in the free solution [338].

2.5.3 Next generation sequencing (NGS)

NGS, also referred to as massively parallel, high throughput (HTS) or deep sequencing, has vastly increased the throughput of genetic sequencing compared with previous methods. Multiple platforms are available, utilising different methods [339-341]. Principally, NGS has four main process elements these are: sample pre-processing (DNA extraction and purification), library preparation, sequencing and data analysis as shown in **Figure 2.6** [342].

NGS is capable of producing sequencing reads in a timely, sensitive, and cost-effective manner. However, it requires large amounts of data storage, sophisticated bioinformatics systems, and fast data processing infrastructures, all of which can be expensive [343]. In **Chapter 4**, this method was used to characterise the gut mycobiome.

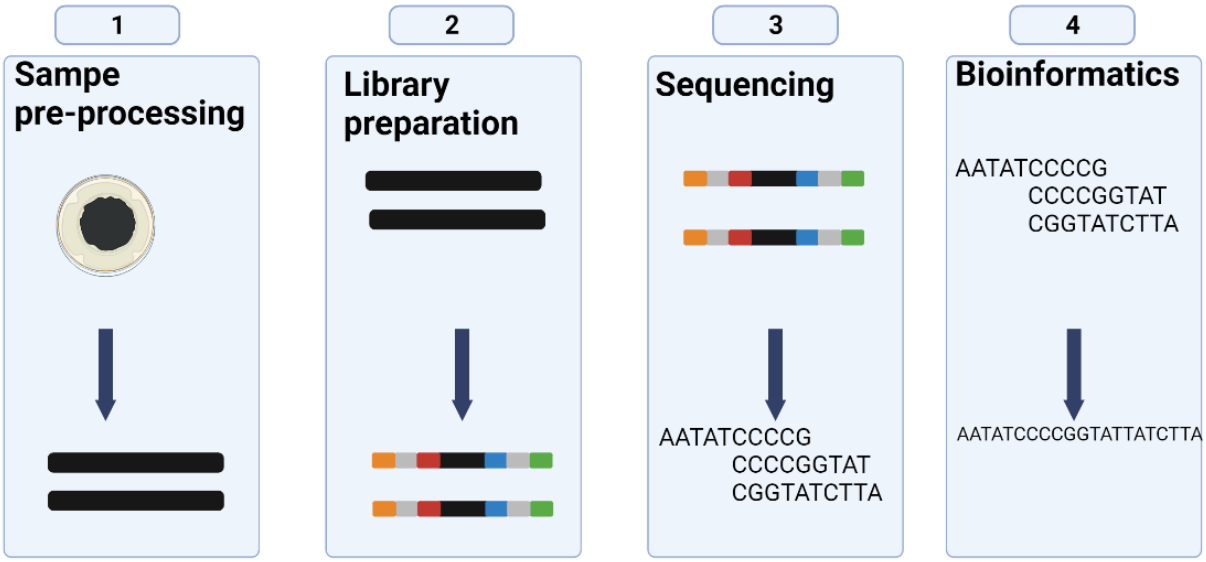

Figure 2.6: Next Generation Sequencing generic workflow.
First, the nucleic acid sample is extracted from the sample (e.g. stool, etc.). Library preparation yields a population of DNA fragments of defined lengths with defined oligomer sequences at both ends to be compatible with the applied sequencing technique. Afterwards, the actual sequencing run on the respective system takes place. A bioinformatics analysis pipeline processes the resulting sequence data to obtain the relevant information. Adapted from [342]. Created using BioRender.com

2.6 Statistical approaches

Data were analysed using the statistical package for social sciences (SPSS) version 22 (IBM Corp.) and with the R software (www.bioconductor.org; www.r-project.org) (R Development Core Team 2011) unless otherwise stated. Data visualisation was performed with Rstudio v3.6.1 and GraphPad Prism versions 7.02 and 8.2.0 (GraphPad Software, Inc.). Whilst the specific statistical methods used to answer each research question are detailed in each chapter, an overview of the statistical approach used is discussed below.

2.6.1 Parametric tests

In parametric tests, an assumption is made that the data has a normal distribution. Where data were normally distributed, parametric statistics were used. In cases where data were aggregated or skewed, the data was transformed to meet normality assumptions in order

to conduct parametric tests. Where transformed data did not meet the assumptions of parametric tests, appropriate non-parametric alternatives were used.

Parametric techniques used for data analyses include the following:

(i) Descriptive statistics were used for exploratory data analyses and to provide summaries on measures of central tendencies (i.e. mean), along with measures of dispersion, including the range, standard deviation (SD), and standard errors (SE).

(ii) The student's t-test was used to compare the means between two independent groups.

(iii) A general linear model (GLM) was used to investigate the relationship between variables of interest to predict an outcome and, where appropriate, account for potential confounders. The method is flexible in that it allows the use of predictor (explanatory) variables measured on different scales (continuous and categorical), as was the case with the work presented in this thesis. This included the analysis of variance (ANOVA) and linear regression models, consisting of categorical and continuous variables.

2.6.2 Non-parametric tests

Where normality assumptions were not met, or in cases where discrete or categorical data were involved, non-parametric statistics were used.

(i) Descriptive statistics were used for exploratory data analyses and to provide summaries on measures of central tendencies (i.e. median), along with measures of dispersion, including the range and interquartile ranges (IQR),

e.g. the median age of the study population. Categorical data were summarised as absolute numbers and percentages.

(ii) The generalised linear model was used to model categorical responses or dependent variables against independent variables. This is an extension of the GLM, which takes into account instances where response variable distributions did not meet normality assumptions. This included the binary logistic regression.

2.6.3 Multivariate analyses

Where analysis of data involved more than one dependent or response variables which were related or correlated with each other (e.g. in this thesis, antibody profiles), multivariate analyses were used. Below, the multivariate analysis methods used in this thesis are outlined.

2.6.3.1 Non-Metric Multidimensional scaling (NMDS)

To facilitate combined analysis of multiple antibody responses in the context of their inter-dependency, aggregated secretion patterns within populations, and the quantitative differences between types, data reduction and ordination method (Non-Metric Multidimensional scaling (NMDS)) adapted for use in heterogeneous data sets with variables measured in a range of different scales was used [344]. This method provides an ideal means of identifying patterns of antibody responses ('antibody profiles') because they group antibody variables according to their co-variance with each other and relative dissimilarities between participants [344] rather than by their absolute quantities. The added advantage of NMDS is the reduced number of comparisons made and thus reduces the risk of false positive results (type 1 error) [345].

2.6.3.2 Permutational multivariate analysis of variance (PERMANOVA)

To determine if there was any variation across a space of a multivariate dissimilarity measure in response to one or more independent variables while making no distributional assumptions [346], PERMANOVA was used.

2.6.4 General considerations

To control for multiple significance testing and reduce the likelihood of type 1 error, the false discovery rate (FDR) approach was used [347] unless otherwise stated, 95% confidence intervals (95% CIs) were calculated using exact binomial tests for all frequencies. Standard error was calculated to accompany every result, summarised using the geometric mean value.

Chapter 3 Burden and management of fungal diseases

Part of this work has been published in the Scientific Reports journal [348] and a copy of the publication is included in **Appendix D.**

3.1 Introduction

In recent decades, there has been a significant increase in fungal diseases worldwide [349, 350]. Over one billion people suffer from fungal diseases, accounting for over 1.6 million deaths and contributing to many diseases' poor and fatal outcomes [19]. Evidence shows that fungal diseases are largely responsible for a silent epidemic as they are frequently hidden diseases that cause substantial morbidity and mortality [9] with more people dying from fungal diseases than either malaria or TB annually [20, 26, 351]. Nonetheless, their impact is not widely appreciated compared to other diseases, and research on fungal diseases is neglected.

In resource-constrained countries, the absence of diagnostic tools and insufficient training of healthcare professionals (as discussed in **Chapter 1**) hinder the proper diagnosis and management of fungal diseases. This often results in the misdiagnoses of fungal diseases which mimic and co-exist with conditions such as HIV, TB and bacterial infections. The lack of proper diagnosis and management of fungal diseases has led to poor epidemiological data. Concerted efforts are beginning to address this, for instance, Global Action Fund for Fungal Infections (GAFFI) has been raising awareness of, and collecting worldwide fungal disease data [350]. To date, they have assisted in the estimation of the burden of fungal diseases in more than 80 countries, covering over 5 billion people globally (**Figure 3.1**) [350, 352]. Furthermore, the World Health Organisation (WHO) expanded the list of neglected tropical diseases (NTD) to include Mycetoma,

chromoblastomycosis and other deep mycoses [29, 353] and issued new guidelines for prevention and management of cryptococcal meningitis [354].

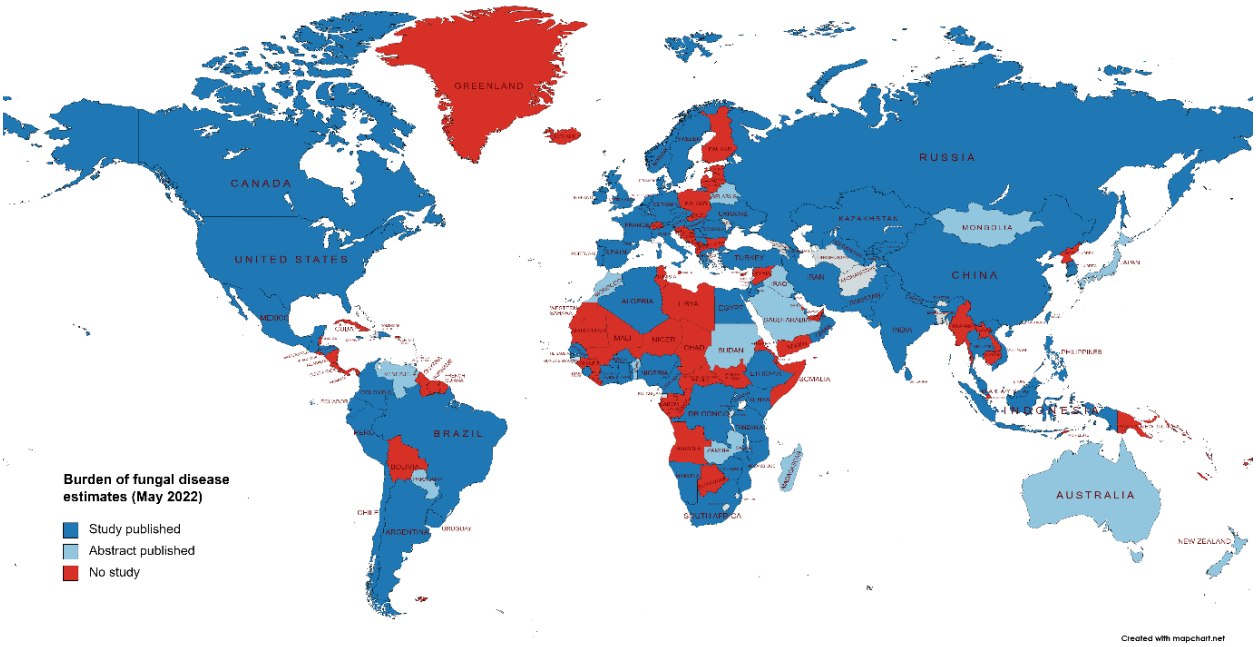

Figure 3.1: Map showing countries with estimates of fungal diseases burden by May 2022.
Adopted from GAFFI [350]. Created with mapchart.net

The prevalence of fungal infections varies by geographic region, socioeconomic status, and the proportion of individuals with underlying conditions. Zimbabwe is a low income country with a population of approximately 15 million [355], and currently has no officially reported data on fungal infections. This situation impedes the collection of epidemiological data and conceals the true incidence and prevalence of fungal diseases in the country.

Moreover, Zimbabwe's health system has faced significant challenges [356], such as the demand for healthcare services for the control of HIV and TB as well as other endemic infections such as malaria and schistosomiasis. Recent outbreaks of COVID-19, typhoid fever and, cholera [357-359], have further strained Zimbabwe's health system. This has impacted Zimbabwe's progress towards Universal Health Coverage, a goal Zimbabwe

has been aiming for over a decade [360], thus also affecting Sustainable Development Goal (SDG) 3 of ending poverty and reducing inequalities [361].

Fungal infections including histoplasmosis, mycetoma, chromoblastomycosis, sporotrichosis, cryptococcal meningitis and tinea capitis [362-366] have been reported in Zimbabwe but , the prevalence and/ or incidence of these and other fungal diseases in the country is unknown. Therefore, a number of questions remain unanswered and these include; are fungal diseases rare in Zimbabwe or is it just a case of lack of awareness resulting in low clinical index of suspicion? Which fungal diseases are common and which populations are affected? What are the health implications of these fungal infections in the population on current and future health?

3.2 Study aim

The aim of this chapter was to determine the burden of fungal infections in Zimbabwe using available epidemiological data and approaches from published work. This will indicate the current fungal disease status in the country to inform health policy and disease awareness. Furthermore, the findings from this study will inform research for improved diagnosis, management and treatment of patients with fungal diseases, while also contributing to the global fungal diseases estimation data.

3.3 Methods

3.3.1 Search strategy and selection criteria

As mentioned in **Chapter 2**, to determine the fungal burden, data was retrieved from published papers obtained via a review of literature published in the databases: PubMed, Web of Science, EMBASE and Google Scholar [348]. The search terms used are shown in **Table 3.1** below.

Table 3.1 : Search terms used to retrieve data publications

First search: fungal infection, fungal burden, fungal epidemiology, Zimbabwe, Southern Africa, and Africa
Second search: This included the same search as above as well as the following diseases: *Cryptococcus*/cryptococcal, *Candida*/thrush, *Aspergillus*/aspergillosis, histoplasmosis, asthma, leukaemia, chronic obstructive pulmonary disease (COPD), *Pneumocystis* pneumonia (PCP)/*Pneumocystis jirovecii* pneumonia /*Pneumocystis carinii* pneumonia, chronic pulmonary aspergillosis (CPA), aspergilloma, allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitisation (SAFS), tinea capitis/ringworm

Figure 3.2 shows a flowchart of literature review process. The fungal diseases included in this study are summarised in **Table 3.2** and the quality of the papers retrieved was assessed via the adapted GRADE score [297] approach as detailed below (**Table 3.4**)

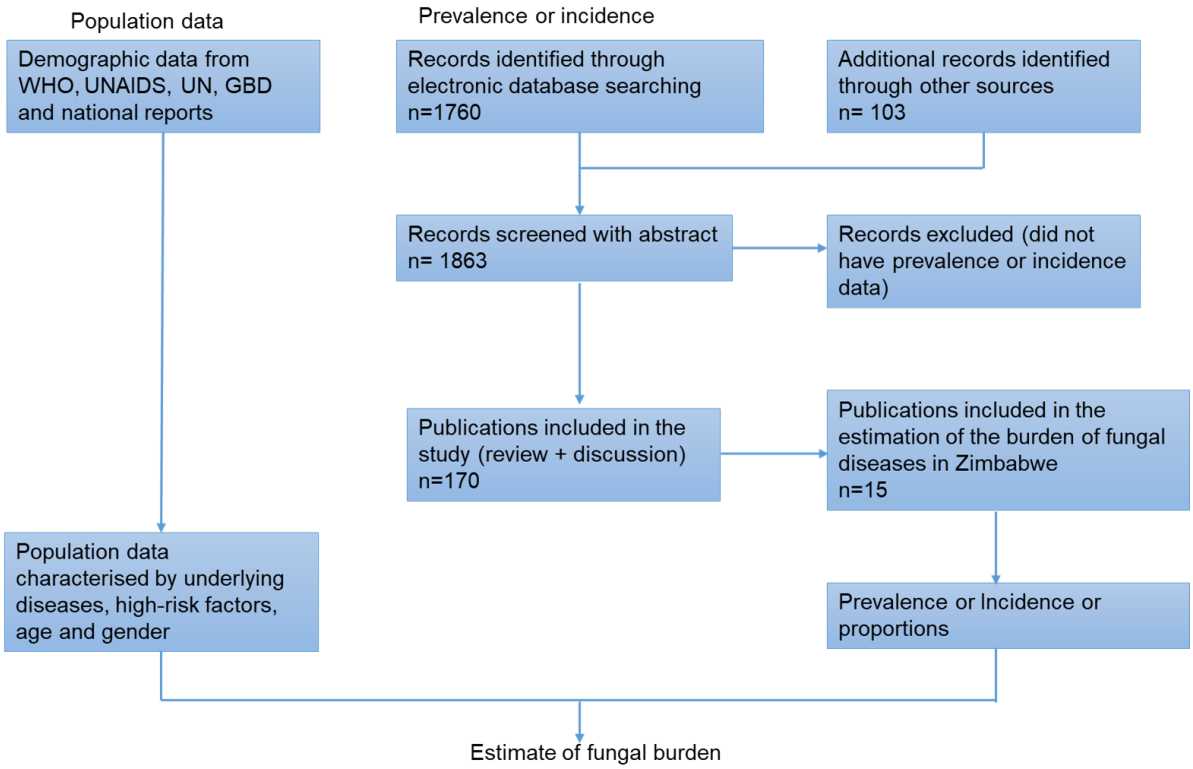

Figure 3.2: Flowchart of literature review for the burden of fungal diseases in Zimbabwe.
Reports published in English between January 1950 – January 2022 were searched. WHO, World health Organization, GBD, Global Burden of Disease, injuries and risk factor Study; UNAIDS, the Joint Nations Program on HIV/AIDS; UN, United Nations Population Division

Table 3.2: Summary of fungal diseases included in this study

Fungal infection	Fungal responsible	Diagnosis	Treatment	Reference
Candidaemia invasive candidiasis	*Candida spp.*	Blood culture PCR Tissue biopsy Candida antigen Antibody test	Caspofungin Micafungin Anidulafungin Amphotericin B Fluconazole Voriconazole	[367]
Intra-abdominal candidiasis (Candida peritonitis)	*Candida spp*., particularly *C. albicans*	Culture & Microscopy	Fluconazole Amphotericin B	[368]
Cryptococcal meningitis	*Cryptococcus neoformans*	Cryptococcal antigen testing (CrAg), Culture	Amphotericin Fluconazole Flucytosine	[354]
Pneumocystis pneumonia (PCP)	*Pneumocystis jirovecii* (formerly *carinii*)	PCR Beta1,3-D-glucan Histology	*First line therapy:* Cotrimoxazole with corticosteroids *Second line therapies*: Pentamidine Clindamycin & Primaquine	[369]
Invasive aspergillosis	*A. fumigatus* and other *Aspergillus spp.*	CT scans Aspergillus antigen PCR on blood Sputum Bronchoalveolar lavage or Histology (if possible)	Voriconazole (most effective) Amphotericin B Micafungin Caspofungin Posaconazole Itraconazole	[370]
Disseminated histoplasmosis	*Histoplasma capsulatum*	Histoplasma antigen test	Amphotericin B Itraconazole	[371]
Mucormycosis (Zygomycosis)	*Rhizopus spp.* *Mucor spp.*	Microscopy Histology	Amphotericin B Posaconazole	[372]
Allergic bronchopulmonary aspergillosis (ABPA)	*A. fumigatus* (>95%) and other *Aspergillus spp*.	Aspergillus IgG and IgE antibodies SPT Eosinophilia PCR Culture	Oral corticosteroids Inhaled steroids Oral itraconazole	[373-375]
Severe asthma with fungal sensitisation (SAFS)	*A. fumigatus* *P. chrysogenum* *C. herbarum* *A. alternata* *C. albicans* *Trichophyton spp.* and others.	SPT Specific IgE test for any fungus	Itraconazole Azoles (but not fluconazole)	[164, 373]

Oesophageal candidiasis	*C. albicans*	Endoscopy with microscopy Culture Biopsy Barium swallow	Fluconazole Amphotericin B Anidulafungin Caspofungin Micafungin Itraconazole Posaconazole	[376]
Oral candidiasis (oral thrush, oropharyngeal candidiasis (OPC))	*C. albicans* (>98%) Other *Candida spp*.	Culture & Microscopy Inspection of the mouth (showing white plaques or erythematous patches)	Nystatin Amphotericin B Clotrimazole Chlorhexidine Azoles	[376, 377]
Chronic pulmonary aspergillosis (CPA, aspergilloma)	*A. fumigatus*	*Aspergillus* IgG testing *Aspergillus* precipitins Radiology PCR	Azoles	[378]
Recurrent vulvovaginal candidiasis (RVVC) Vaginal thrush Candida vulvovaginitis	*C. albicans* *C. glabrata*	Microscopy & Culture	Clotrimazole Nystatin pessaries Fluconazole Itraconazole Flucytosine	[379, 380]
Tinea capitis (including kerion and favus)	*Microsporum spp.* *Trichophyton spp.*	Microscopy	Terbinafine Itraconazole Griseofulvin	[381, 382]
Sporotrichosis	*Sporothrix spp*.	Skin biopsy with both Histology & Culture	Potassium iodide & Itraconazole Terbinafine Amphotericin B Micafungin	[383]
Chromoblastomycosis (chromomycosis)	Melanised (black fungi) fungal spp. *Fonsecaea pedrosoi* *Cladophialophora carrionii*	Skin scrapings using 10% KOH & Parker ink or calcofluor white Biopsy tissue sections (stained with hematoxylin and eosin, PAS, and silver stains)	Cryosurgery Itraconazole Terbinafine Posaconazole	[384]
Blastomycosis	Blastomyces dermatitidis	Culture (sputum/ skin biopsy) Antigen test	Itraconazole Amphotericin B	[385]
Fungal keratitis	*A. flavus* *A. fumigatus* *Fusarium spp.* *C. albicans*	Microscopy & Culture PCR	Natamycin 5% Amphotericin B 0.15% Voriconazole 2-3% (50µg/ml) Azoles	[386]
Mycetoma (eumycetoma)	*Madurella spp.* *Scedosporium apiospermum*	Biopsy Culture & Microscopy	Itraconazole Voriconazole	[387]

3.3.2 Country profile

Zimbabwe is a landlocked country in Southern Africa, between the Zambezi and Limpopo Rivers, with Botswana, Mozambique, South Africa and Zambia as its neighbours. As of May 2022, the Zimbabwean population was estimated to be 15.99 million, with 40.9% adults [388] and 1.3 million people living with HIV/AIDS (PLWH) [389]. National TB data were obtained from the World Health Organization (WHO) [27]. National prevalence data for lung cancer, chronic obstructive pulmonary disease (COPD), diabetes and incidence data for acute myeloid leukaemia (AML) were obtained from the 2019 Global Burden of Disease study [390]. Population data required to calculate the burden of fungal diseases is shown in **Table 3.3**.

Table 3.3: Country's profile. Populations and rates required to calculate the burden of fungal-related diseases in Zimbabwe.

		Patient numbers and rates	Source
Demographics	Total population	15,994,000	[388]
	Children (< 15 years),	6,540,000	
	Total number of adults,	9,454,000	
	Adult women	4,747,727	
HIV/AIDS	Current total HIV/AIDS	1,300,000	[389]
	Children with HIV	72,000	
	Proportion of diagnosed cases on ARVs	91%	
	Number of diagnosed cases receiving ARVs	1,200,000	
	Proportion of those on ARVs who fail or have ARV resistance	11%	[391]
	Number of diagnosed cases not receiving ARVs	100,000	
	Annual new AIDS cases (at risk of OIs)	14,286	
	AIDS-related deaths	19,800	
Respiratory diseases	Pulmonary tuberculosis annual incidence (survivors)	10,620	[27] [392]
	Prevalence of asthma in adults	2.28%	[393]
	COPD prevalence (all GOLD stages)	7.8%	
	COPD hospital admissions	130,991	
Lung cancer		744	[394]
Diabetes		8.5%	[395]
Leukaemia	AML	230	[394]

COPD, chronic obstructive pulmonary disease; GOLD, Global initiative for Obstructive Lung Disease; ARV, antiretroviral; OI, opportunistic infection; AML, acute myeloid leukaemia.

The methodology used in this thesis was adapted from previously published approaches by Leading International Fungal Education (LIFE) (https://www.life-worldwide.org/) [350, 396] which have been employed in other countries. Thus, to calculate the burdens of cryptococcal meningitis (CM), candidiasis and *Pneumocystis* pneumonia (PCP), HIV data was used. Asthma data was used to calculate the burden of ABPA. While COPD and TB data were used to calculate the burden of CPA. Burdens of candidaemia and *Candida* peritonitis were derived from critical care and/ or cancer patients' data.

The burden was calculated for the general healthy population and the 'at risk' populations, including HIV/AIDS patients, survivors of pulmonary TB, cancer, COPD, asthma and intensive care patients. The applied formulae are detailed below. National or local data were preferred, but where unavailable, data were extrapolated from other sources

3.3.3 Data quality assessment

Papers presenting the incidence or prevalence of any fungal disease were evaluated using an adapted GRADE score [297] based on the following criteria:

i) diagnostic accuracy, ii) study size (using a cut-off of >10 cases), iii) year of study, with more recent studies scoring higher, iv) type of publication, with original research article scoring more, v) methodology and vi) country, with studies from Zimbabwe scoring higher (**Table 3.4**). Those with an adapted GRADE mean score of >2 were deemed acceptable and enabled a minimum determination of the country's burden of fungal diseases (**Table 3.5**). Papers with a mean score <2 were excluded in determining the country's burden of fungal infections but are discussed in this chapter.

Table 3.4: Scoring system for modified GRADE criteria

Diagnostic	**Score**
PCR + laboratory + clinical + imaging	2
Culture, smear, histology	1
Clinical suspicion only	0
Patient sample size	Score
≥10	1
<10	0
Year of study	Score
<5 years	2
5-10 years	1
>10 years	0
Country (data used)	Score
Zimbabwe	2
Any other African country	1
Rest of the world	0
Methodology (well designed)	Score
Yes	1
No	0
Type of publication	Score
Research paper	2
Case study/ short reports	1
Review papers	0
Possible total score	**10**

Table 3.5: Modified GRADE score for the papers used for estimating burden of fungal diseases in Zimbabwe

Disease	Diagnostic accuracy	Patient sample size n>10	Up to date	Type of publication	Methodology	Country	Overall score	Ref
PCP	2	1	1	2	1	1	**8**	[397]
Histoplasmosis	1	1	1	1	0	2	**6**	[363]
Invasive aspergillosis	2	1	1	2	1	0	**6**	[398]
	1	1	0	1	1	0	**4**	[399]
	1	1	1	1	1	0	**5**	[400]
	0	1	1	1	1	0	**4**	[401]
Candidaemia	-	-	1	0	1	-	**2**	[402]
***Candida* peritonitis**	1	1	1	1	1	0	**5**	[403]
ABPA	-	-	1	0	1	-	**2**	[7]
SAFS	-	-	1	0	1	-	**2**	[404]
CM	-	-	2	0	1	-	**3**	[5]
RVVC	-	-	2	0	1	0	**3**	[405]
Tinea capitis	1	1	0	2	1	2	**7**	[362]
Mucormycosis	0	1	1	1	1	0	**4**	[406]
Fungal keratitis	-	-	1	0	1	-	**2**	[407]

CM, Cryptococcal meningitis; PCP, Pneumocystis pneumonia; CPA, Chronic pulmonary aspergillosis; ABPA, Allergic bronchopulmonary aspergillosis; SAFS, Severe asthma with fungal sensitisation; IA, Invasive candidiasis; RVVC, Recurrent vulvovaginal candidiasis

3.3.4 Determining fungal disease burden

The annual burden was calculated for all fungal diseases included in the study and presented as: i) absolute number of cases per year in the country and ii) annual rates. Following the methodology in previous studies in other countries[408-410], the absolute cases were presented as either incidence or prevalence depending on the type of infection. Using the absolute annual number of cases as the numerator and the entire Zimbabwean population as the denominator, the annual rates (incidence or prevalence) were calculated. Data used to identify the most accurate denominators to use for determining the burden was derived from published reports and these are summarised in **Table 3.6**. The 2022 Zimbabwean population (n = 15,994,000 [388]) was used regardless of the year from which the numerator data originated. Population demographics were derived from the United Nations population estimates, HIV data from The Joint Nations Programme on HIV/AIDS (UNAIDS) and all the other data was derived from WHO reports.

Prevalence levels were calculated for ABPA, SAFS, CPA, rVCC, and tinea capitis while annual incidence estimates were determined for the remaining infections. Due to paucity of data, I was not able to calculate both prevalence and incidence for each disease. Previous studies have reported that the risk of acquiring any opportunistic fungal infection increases with declining CD4 cell counts, particularly when below a threshold of <200 × 10^6/l and this usually occurs within 6–10.5 years from seroconversion in patients not on antiretroviral therapy (ART) [411-415]. Therefore, to determine the burden for HIV-related fungal diseases, a 7-year linear decline in CD4 count to <200 × 10^6/l, of those not on ART was used, doubled to reflect those on ART who fail with ARV resistance or default (at risk of opportunistic infections).

Table 3.6: Data and calculations for the determination of fungal disease burden in Zimbabwe made

Fungal disease	Underlying condition	Derived data	Calculations	Refs
CM	HIV/AIDS	7.1% of AIDS patients	$(Annual\ new\ AIDS\ cases\ \times 0.071)\ \times 2$	[5]
PCP	HIV/AIDS	11% PCP as newly diagnosed HIV/AIDS adults over 2 years	$(Annual\ new\ AIDS\ cases\ \times 0.11)\ \times 2$	[397]
IA	HIV/AIDS; COPD; Leukaemia; lung cancer	1.10% of AML patients develop IA, 2. Rate in non-AML same as in AML, 3.1.3% of admitted COPD patients, 4.2.6% of lung cancer patients, 5.4% of HIV/AIDS deaths	$(AIDS-related\ deaths\ \times 0.04)+(lung\ cancer\ \times 0.026)$ $+(AML\ patients\ per\ year\ \times 0.1\ \times 2)$ $+(COPD\ admissions\ \times 0.013)$	[398-401]
CPA	Tuberculosis, COPD	1.22% of those with and 2% of those without cavities after TB develop CPA 2. 67% of underlying disease of CPA is pulmonary TB	$(Pulmonary\ tuberculosis\ annual\ incidence\ \times 0.22\ \times 0.22)$ $+(Pulmonary\ tuberculosis\ annual\ incidence$ $\times 0.78\ \times 0.02)\times 3.152\ \times 1.5$	[7]
ABPA	Asthma	2.5% of adult asthmatics have ABPA	$(Asthma\ numbers\ in\ adults\times 0.025)$ $+(Cystic\ fibrosis\ in\ adults\ \times 0.15)$	[7, 416, 417]
SAFS	Severe asthma	33% of worst 10% of adult asthmatics.	$(Asthma\ numbers\ in\ adults\times 0.1)\ \times 0.33$	[404]
Candidemia		1.5/100,000 (mean of 2-11/100,000) with 30% in ICU (critical care and post-surgical patients) 2. 70% in cancer and other immunocompromised patients	$\left(\frac{Total\ population}{100000}\times 3.5\right)+\left(\frac{Total\ population}{100000}\times 1.5\right)$	[402]
Candida peritonitis	Pancreatitis, major abdominal surgery	1.1 patient with hospital-acquired (almost all post-operative) Candida peritonitis for every 2 patients with candidaemia, in ICU	$\left(\frac{Total\ population}{100000}\times 1.5\right)\ \times 0.5$	[403]
Oral candidiasis	HIV/AIDS	Occurs in 90% of untreated HIV patients over 2 years	$(Annual\ new\ AIDS\ cases\ \times\ 0.9)\times 2$	[418]
Oesophageal candidiasis	HIV/AIDS	1. 20% of patients not on ARVs, 2. 0.5% of those on ARVs	$(((Number\ of\ diagnosed\ HIV\ cases\ not\ receiving\ ARVs\ \times 0.2)$ $+(Number\ of\ diagnosed\ HIV\ cases\ receiving\ ARVs\ \times 0.05)))/2$	[419, 420]
RVVC (≥4x/year)		6% of adult women	$(Number\ of\ women\ between\ 15-50)\times 0.06$	[405]
Mucormycosis		It affects 2 per million of the population based on data from Europe	$(Total\ population\ \times 0.2/100000$	[406]
Histoplasmosis	HIV/AIDS	Based on literature		[363]
Tinea capitis		29% of children	$Number\ of\ children\ \times 0.29$	[362]

CM, Cryptococcal meningitis; PCP, Pneumocystis pneumonia; CPA, Chronic pulmonary aspergillosis; ABPA, Allergic bronchopulmonary aspergillosis; SAFS, Severe asthma with fungal sensitisation; IA, Invasive candidiasis; RVVC, Recurrent vulvovaginal candidiasis

As shown in **Table 3.5**, most of the data used to determine the burden of fungal diseases in Zimbabwe came from other countries, which introduces some inaccuracies when calculating the burden due to socioeconomic and geographical differences [421]. Thus **Table 3.7** below, summarises the limitations of using this data.

Table 3.7: Limitations of the data used to determine the burden of fungal diseases in Zimbabwe

Fungal disease	Limitations
Cryptococcal meningitis; Pneumocystis pneumonia;	Incidence of CM and PCP was not determined in non-HIV people or children and hence could have resulted in underestimation.
Chronic pulmonary aspergillosis; Invasive aspergillosis	Based on estimates of estimates, and may not be very accurate due to missing country data
Allergic bronchopulmonary aspergillosis (ABPA); Severe asthma with fungal sensitisation (SAFS)	Absence of fungal sensitisation data in the country is a major limitation on the prevalence reported. Furthermore, they may be some duplication between ABPA and SAFS, because sensitisation to *Aspergillus* is universal in ABPA, and some of these patients have severe asthma.
Candidemia, *Candida* peritonitis; Fungal keratitis Mucormycosis	No data in Zimbabwe and hence based on estimates of estimates, thus introducing substantial inaccuracies
Oral and Oesophageal candidiasis	Incidence of candidiasis was not determined in otherwise healthy people such as those receiving antibiotic therapy, which promotes the overgrowth of *Candida* [422]. Hence the reported incidence could be an underestimation
Recurrent vulvovaginal candidiasis (≥4x/year)	The use of the 6% rate implies that RVVC affects women with equal frequency and this is highly unlikely. Furthermore, it does not include women on hormone replacement therapy.
Histoplasmosis	Based on dated Zimbabwean data[363] , thus, the figure presented in this study may not accurately represent the country's current situation, due to decline in HIV prevalence.
Tinea capitis	The data used was derived from a dated Zimbabwean study[362]. Thus, the figure presented in this study may not accurately represent the country's current situation, probably due to changes over time

3.4 Results

This study determined that 2,240,402 (14%) Zimbabweans suffer from fungal infections each year (**Table 3.8**). **Figure 3.3** also depicts the rate of each fungal disease per 100,000 of people in Zimbabwe. Tinea capitis and RVVC were the most frequent fungal diseases in the population with a burden of 11,858.2 and 2,788.17 per 100,000 respectively.

Table 3.8: Estimated burden of fungal diseases in Zimbabwe

	Number of Infections per underlying disorder per year							
Infection	**None**	**HIV/AIDS**	**Respiratory disease**	**Cancer**	**Critical care + surgery**	**Totals**	**Burden**	**Rate/100,000**
CM		2,029				2,029	I	12.68
PCP		3,143				3,143	I	19.65
IA		792	19	50	1,703	2,564	I	16.03
CPA			8,775			8,775	P	54.87
ABPA			5,389			5,389	P	33.69
SAFS			7,113			7,113	P	44.47
Candidaemia				560	240	800	I	5.0
***Candida* peritonitis**					120	120	I	0.75
Oral candidiasis		25,714				25,714	I	160.77
Oesophageal candidiasis		62,857				62,857	I	393.0
RVVC (≥4x/year)	222,970					222,970	P	2,788.17*
Mucormycosis				32		32	I	0.2
Histoplasmosis		57				57	I	0.36
Fungal keratitis	2239					2,239	I	14
Tinea capitis	1,896,600					1,896,600	P	11,858.2
Total burden estimated	**2,121,809**	**94,592**	**21,296**	**642**	**2,063**	**2,240,402**		**15401.84**

** rate among all females*
I, Incidence; P, Prevalence; CM, Cryptococcal meningitis; PCP, Pneumocystis pneumonia; ABPA, Allergic bronchopulmonary aspergillosis; SAFS, Severe asthma with fungal sensitisation; CPA, Chronic pulmonary aspergillosis; RVVC, Recurrent Vulvovaginal Candidiasis

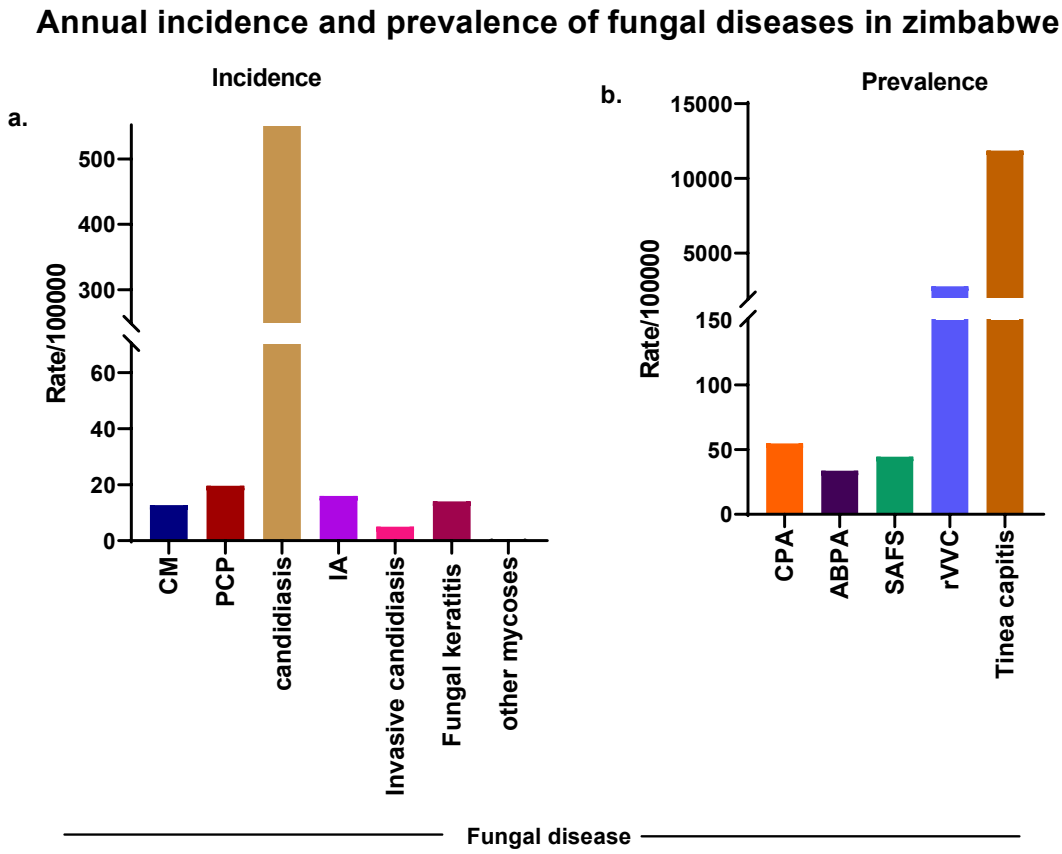

Figure 3.3: Annual incidence and prevalence of fungal infections in Zimbabwe. Bar charts representing the burden of fungal diseases per 100 000 people (a) incidence and (b) prevalence for each fungal disease with data available .CM, Cryptococcal meningitis; PCP, *Pneumocystis* pneumonia; candidiasis (oesophageal candidiasis); Invasive candidiasis (candidaemia and *Candida* peritonitis); IA, Invasive aspergillosis; other mycoses (histoplasmosis and mucormycosis); CPA, Chronic pulmonary aspergillosis; ABPA, Allergic bronchopulmonary aspergillosis; SAFS, Severe asthma with fungal sensitisation; rVVC, recurrent vulvovaginal candidiasis;

3.5 Discussion

There is a paucity of data on the burden of fungal diseases in Zimbabwe. This current study was conducted to determine the burden of specific fungal diseases in Zimbabwe. The calculated burden of 2,240,402 fungal disease cases was higher than most countries even in Africa, indicating significant morbidity and possibly mortality in Zimbabwe. Tinea capitis and RVVC were the most frequent fungal diseases in the population. While not life threatening, these two diseases can significantly decrease the quality of life for those affected and the social stigma can impact their work/ school performance. In order to improve population health and quality of life, health policy decisions must consider the enormous burden of these diseases [25].

Disease burden can be measured using the metrics of QALYs (Quality-Adjusted Life Year) and disability-adjusted life years (DALYs), providing a single measure of mortality and morbidity, used internationally for assessing health care interventions and treatments [423]. This is particularly important in public health, enabling policymakers to make informed decisions and the development of interventions to reduce global health disparities resulting from diseases. Yet, only DALYs of fungal skin diseases (54.86/100,000) has been calculated. Hence there is a need to calculate the metrics of all the other fungal infections to develop concerted and sustained global efforts toward reducing this burden [25].

Tinea capitis, which is a contagious dermatophyte infection, is common among children throughout Africa, particularly in areas with poor socioeconomic and sanitary conditions [424-427]. Africa's warm, humid climate enables the dermatophytes to thrive, resulting in high prevalence in these areas [24]. The main dermatophyte species endemic to Africa are *Trichophyton violaceum, Trichophyton soudanese* and *Microsporum audouinii* [428, 429]. This current study focused solely on tinea capitis due to its transmissibility, scarring

potential and occasional complications of kerion [430]. The data used was derived from a dated Zimbabwean study by Robertson and Wright [362]. Thus, the figure presented in this study may not accurately represent the country's current situation, probably due to changes over time. Nevertheless, it remains a common clinical problem.

Vulvovaginal candidiasis (VVC), commonly caused by *C. albicans* is a common gynaecological problem occurring among women globally [431, 432]. VVC prevalence rates range between 25%–40% [433, 434] in Zimbabwe, and here it was determined that 222,970 Zimbabwean women suffer from recurrent episodes (RVVC). This value may be conservative as data from hormone replacement therapy (HRT) which can precipitate RVVC [34], was omitted. As mentioned above, RVVC is not life threatening but has a negative impact on quality of life, resulting in anxiety, depression and a loss of productivity [435-437]. The burden of RVVC in Zimbabwe was the fifth highest among the Southern African Development Community (SADC) countries with available burdens [425, 438-440].

The prevalence of RVCC has been shown in a few studies to be higher in Africa than in other continents [437, 441, 442]. It has been hypothesised that genetic factors contribute to the susceptibility of Black/ African women to RVCC. Nonetheless, an exhaustive evaluation of the role of genetics in RVCC is still lacking. Similarly, other studies have suggested that host-related and behavioural factors could also have a role to play [443-445]. However, research is needed to elucidate the role of these factors in the development/ susceptibility of Black/ African women to RVCC.

Oral and oesophageal candidiasis are common in individuals with AIDS or other immunosuppressive conditions. Oral candidiasis is one of the most prevalent fungal opportunistic infections [446] in ART naïve immunosuppressed HIV patients [447, 448]

and was the most common opportunistic infection in Nigeria [449] and Uganda before the initiation of highly active antiretroviral therapy (HAART) [450]. Because of its bioavailability and efficacy compared to other antifungal drugs [451, 452], fluconazole is the drug of choice in treating oral candidiasis [447]. However, an increase in resistance of *Candida* species to fluconazole has been reported in some parts of Africa [453-456], which is significant because of the implications for morbidity and mortality rates [456, 457].

Cryptococcal meningitis remains a very common infection in patients with late-stage HIV infection, particularly in Africa and Southeast Asia. Despite the expansion of antiretroviral programmes, cases have not decreased in most SSA countries [438, 458]. Recent studies show that more cryptococcal patients are ART-experienced [459, 460]. In Uganda, 3% of ART-experienced patients with virological failure were cryptococcal antigen (CrAg) positive [461], with a background rate of 5-10% cryptococcal meningitis. Consequently, to determine the incidence of cryptococcal meningitis in Zimbabwe, in light of the fact that virological failure does not always translate to immunodeficiency, the at-risk population (ART naïve) was doubled, resulting in 2,029 cases of cryptococcal meningitis per year.

PCP is a life-threatening infection, mainly in HIV/AIDS patients, and unfortunately, most of these patients are undiagnosed, diagnosed late or misdiagnosed, particularly in resource-limited settings [290, 462]. However, when diagnosed early and successfully treated, survival rates are high. In western countries, the frequency of PCP has decreased compared to the start of the AIDS epidemic, but infection is inadequately documented in low-income countries due to the lack of diagnostic capability [369]. In Zimbabwe, the largest reported series cases of PCP among HIV-infected individuals with respiratory symptoms was 8 (22%) in 1989 [289]. Here it was determined that there are 3,143 cases (19.65/100,000) of PCP annually in Zimbabwe. Due to the lack of data, the incidence reported in this study was based on adults only, despite PCP being common in children

[463-465] and likely a significant contributor to the 3000 children who died of AIDS in 2019 [389].

CPA is a slowly destructive lung infection with marked systemic (fatigue, weight-loss) and pulmonary (breathlessness, productive cough, haemoptysis) features. It is almost indistinguishable from TB. In this study, the prevalence of CPA (54.87/100,000) was comparatively high in relation to other African countries [425, 438, 440] except South Africa, which had the highest prevalence at 176/100,000 [438]. The burden of CPA in Zimbabwe may reflect the country's high TB prevalence [466]; however, this must be confirmed by additional research studies. Currently, CPA is diagnosed using a combination of imaging and *Aspergillus* IgG antibody testing [467]. However, these are not routinely done in Zimbabwe, which might result in misdiagnosis, as observed in a previous study carried out in Nigeria where, 19% of CPA patients who were TB smear-negative and GeneXpert negative were incorrectly diagnosed as having pulmonary TB [468], resulting in inappropriate treatment.

In Zimbabwe, asthma is a significant public health issue that is frequently poorly managed [469]. Like most African countries, it appears to be more of a problem in urban areas than in rural areas [470]. However, this generalisation might be skewed as the rural population rarely goes to the hospital, and the urban areas have pollution issues which could trigger asthma symptoms [470]. There is uncertainty regarding the prevalence of asthma in Zimbabwe due to the different definitions of asthma, as illustrated by To *et al*., [393]. The authors reported that the prevalence of asthma in Zimbabwe is 2.28% in doctor-diagnosed asthma, 2.52% in clinical asthma and 5.48% in wheezing [393]. The current study calculated the prevalence using 2.28%, resulting in 215,551 cases.

Despite the absence of fungal sensitisation studies in Zimbabwe, the burden for ABPA and SAFS, collectively known as 'fungal asthma' and differs from allergic asthma, was

determined. In allergic asthma, bronchoconstriction can be alleviated using bronchodilators and inhaled glucocorticosteroids. In contrast, fungal asthma requires administering antifungal agents such as oral itraconazole [471-474] and voriconazole, which can only be prescribed following an accurate diagnosis. These antifungals decrease the fungal load, thereby thus reducing the stimulus for the ongoing inflammatory activity [475].

In 2020, Rapport *et al*., reviewed antifungals' role in managing patients with severe asthma [474], highlighting significant studies that confirm lower toxicity of treatment with azoles, particularly itraconazole for ABPA. The review further provided recommendations for using antifungal agents in patients with severe asthma, airway fungal infection and fungal colonisation [474]. This is important because inadequate management of fungal asthma can result in serious complications, including long-term steroid toxicity, bronchiectasis and CPA [404].

Disseminated histoplasmosis is a sub-acute infection usually diagnosed in HIV/AIDS patients and those undergoing solid-organ transplants [371]. In Africa, both *Histoplasmosis capsulatum var capsulatum* (HCC) and *Histoplasmosis capsulatum var duboisii* (HCD) co-exist, as is the case in Zimbabwe, where both cases have been reported [476]. HCC infection often mimics pulmonary TB and requires microscopy of bone marrow biopsy, antigen detection or PCR testing of blood/ long-term fungal culture. In contrast, *HCD* infection is easier to diagnose as cutaneous lesions are more frequent and easily accessible for diagnosis [477]. In total, 57 cases of disseminated histoplasmosis per year were reported.

Fungal keratitis, also known as mycotic keratitis or keratomycosis, is a severe, sight-threatening condition common in tropical and subtropical regions [407]. This condition tends to be poorly treated and has very high morbidity [478, 479]. According to recent

global data, the annual incidence of culture and microscopy-positive cases in Zimbabwe was 2,239. However, taking into consideration that culture and microscopy negative cases are usually cases of fungal keratitis in areas with high incidence [407], this number increases to approximately 3,153 cases.

Despite the high morbidity rate of mycetoma, [480] there are currently no control programmes in place, except for Sudan, where it is highly endemic [29, 353, 387]. Many African countries [481-487], including Zimbabwe [365], have reported cases of mycetoma. Similarly, chromoblastomycosis, sporotrichosis and blastomycosis have also been reported in Zimbabwe, albeit in dated reports. Since these dated reports were the only data available in the country, they were excluded from the current study because they could not be used to calculate a reliable, current burden.

To date, there have been no reports of disseminated *Emergomyces* infections in Zimbabwe despite reports from South Africa [488]. The lack of data could be due to a low index of clinical suspicion, limited diagnostic capacity and a deficiency of clinical and diagnostic expertise.

The majority of research on fungal infections in Zimbabwe was conducted more than 20 years ago [289, 362-364]. While the number of cases indicates susceptibility in the Zimbabwean population, they do not accurately reflect the current situation in the country, particularly in light of changes in the epidemiology of HIV, whose prevalence has gone down from more than 15% [356] to about 8.7%, with 85% of these individuals receiving ART [389]. However, the country's ongoing health and economic challenges in terms of public health priorities, clinical and laboratory expertise, and insufficient financial resources impede the early diagnosis and treatment of fungal infections. Contributing to high rates of morbidity and mortality [489].

Most serious fungal infections are opportunistic infections, so most of those affected are immunocompromised [9]. For instance, in HIV/AIDS patients, the most prevalent systemic fungal infections observed are cryptococcal meningitis, oesophageal candidiasis, PCP and aspergillosis. Consequently, the combination of the underlying immunocompromised state and superimposed fungal infection contributes to an increased mortality risk [490]. To treat these comorbidities, the co-administration of drugs is required, creating susceptibility to drug-drug interactions (DDIs). In some instances, in an effort to prevent potential adverse effects and treatment failure, the optimum therapy for fungal infections can be contraindicated in conjunction with medications used to treat co-morbid conditions [491, 492].

Amphotericin B or azole antifungals are the preferred treatments for these fungal infections. Studies on amphotericin B and tenofovir have suggested that renal function should be closely monitored as both drugs can cause nephrotoxicity when used concomitantly. Similarly, combination therapy of zidovudine and amphotericin B may cause anaemia and neutropenia [492, 493]. As DDIs are often unavoidable in HIV-infected patients, the potential effects of these DDIs cannot be overlooked [490], particularly in Africa, where very few drugs used have been evaluated for DDIs and pharmacogenetics [494]. The evaluation of the potential DDIs and pharmacogenetics could aid in managing patients with co-morbidities and broaden our understanding of these DDIs in various populations.

In addition to this, antifungal resistance is also a possibility [495]. *Candida auris*, an emerging fungus, has been reported to be resistant to all major classes of antifungal drugs, posing a serious threat to global health [496, 497]. Other studies have reported fluconazole resistance among *C. neoformans* complex isolates from Africa [498] and *Candida* spp. isolated from women with VVC [499].

Undoubtedly, there is need for further research into new treatment strategies [500] to address the issues of adverse effects, drug interactions, and antifungal resistance as these significantly impact the health and well-being of those affected. Moreover, it has been suggested that the susceptibility of mammals to fungal diseases is determined largely by the temperature gradient between mammalian temperatures and temperatures tolerated by most fungal species [501]. Hence, as the world warms with climate change, this may raise the possibility of new fungal diseases as many fungal species with the potential for pathogenicity in mammals will adapt to higher temperatures and consequently pose threats to human health [12]. Henceforth, it is of imperative importance that collaborative research on the global burden of fungal diseases and their impact on human health, coupled with capacity building, is implemented to combat these diseases.

The current study showed that fungal diseases are likely far more prevalent than are documented in clinical practice. This data serves as a starting point for gaining a better understanding of the fungal problem in the country, generating awareness, and proposing relevant studies and interventions to combat fungal diseases that pose a significant threat to public health. The study nonetheless had a few limitations. Sufficient local data for a precise estimate of the current burden was not obtained, as majority of the available studies were outdated. Consequently, most of the data used came from other countries, which may introduce some inaccuracies when calculating the burden in Zimbabwe due to socioeconomic and geographical differences [421]. Another significant limitation is the incomplete nature of the data. For instance, the burden of PCP in children or non-HIV patients was not calculated, nor could the burden of mycetoma, chromoblastomycosis, blastomycosis, and sporotrichosis be determined due to a lack of data.

3.6 Conclusion

The current study determined for the first time the burden of fungal diseases in Zimbabwe and provided its impact on public health. Superficial fungal infections were shown to be the most prevalent fungal diseases in the country. More importantly, it was demonstrated that fungal diseases are likely far more prevalent than is documented in clinical practice. The paucity of data on fungal infections in the country warrants further epidemiology studies and better diagnostics to aid patient management. Overall, the findings from this study contribute to the global fungal diseases estimation project and awareness campaign. The findings further provide a numerical tool for expanded advocacy. Importantly, specialised training of medical personnel and research geared towards improving diagnosis, treatment and management of patients with fungal diseases is crucial.

Chapter 4 Fungal allergic sensitisation in young rural Zimbabwean children: gut mycobiome and seroreactivity characteristics

Part of this work has been published in Current Research in Microbial Sciences journal [502]. A copy of the publication is included in **Appendix D.**

4.1 Introduction

Sensitisation to allergens throughout childhood is thought to be a key risk factor for developing allergic disorders [503]. These allergic disorders have complex etiologies involving genetic and environmental factors [504]. Changes in lifestyle and environmental exposures are thought to be the key contributors to global rises in allergies. Among these contributors, it has been proposed that increases in allergic disorders may be related to gut microbiome dysbiosis [505], as well as the immunological implications of decreased exposure to infectious diseases in more developed and metropolitan areas [224].

Recent research has revealed that, while the bacterial community remains relatively consistent over time, the fungus population (gut mycobiome) inhabiting the murine gut changes significantly throughout the rodent's life [506]. Consequently, the notion that gut fungal populations in humans are more variable than bacterial populations and that environmental fungi may alter their composition has been suggested [507]. Despite evidence that fungi inhabit the mammalian GI tract and interact with the host immune system [507, 508], the composition and characteristics of the mycobiota in humans have been poorly explored. In addition, only a limited number of microbiome research have included African populations, with the majority of these studies focusing on the influence of geographical and lifestyle variations on the bacterial component of the microbiome of African and Western populations [128, 509]. Furthermore, most of these studies have

focused on older individuals (i.e. 20–40 years old) whose microbiome structure is already established [510]; hence the factors integral to young African children are still to be fully elucidated.

To date, studies on allergic sensitisation and inflammation have found that an alteration in the microbiome [295, 296] is associated with the development or exacerbation of allergic conditions such as asthma [154, 156, 157, 296]. However, these studies have produced inconsistent results due to factors such as the heterogeneity of study populations, differences in profiles and study designs, varying definitions of allergy and varying sample sizes [511].

Data from previous studies suggest that microbial dysbiosis occurs early in life, preceding the onset of sensitisation. This dysbiosis results in differences in gut microbial composition, which have been associated with allergy SPT response [179], specific IgE levels [512], and allergy status [513]. However, much of this early research focused on the bacterial microbiome and relied on culture methods, excluding the large majority of organisms that cannot be cultured [514].

A few allergy studies have specifically investigated the interaction between gut mycobiome and allergen sensitisation [295], highlighting a need for more research in this area. Investigating the gut mycobiome in individuals with fungal sensitisation and/ or allergic disorders may identify associations between the gut mycobial composition and sensitivity or tolerance to fungal allergens. Additionally, the influence of a number of these unculturable fungal species on fungal-sensitised children is yet unknown and may be identified in these studies. This will provide insight into opportunities to design treatment regimens based on the microbiome to improve the overall health and development of children [515].

4.2 Study aims

This chapter aimed to characterise the abundance and diversity of the gut mycobiome and subsequently relate this to fungal sensitisation and fungal seroreactivity among PSAC (1–5 years old). In addition, the effect of host-related factors, including age, sex, *S. haematobium* infection status, growth and nutritional indices on fungal sensitisation and seroreactivity were determined.

4.3 Method

4.3.1 Study design, population and site

This cross-sectional study included stool samples from a subset of 116 PSAC (1–5 years) that met the inclusion criteria of the larger Paediatric schistosomiasis study (Study A) described in **Chapter 2**. As mentioned in **Chapter 2**, the 116 children included in the study were selected based on (i) consent from guardian/career, (ii) consent for stool samples to be used for microbiome characterisation, (iii) consent for serum samples to be used for serological assays, (iv) availability of socio-demographic data and; v) consent to perform skin prick testing (SPT) using allergen extracts.

4.3.2 Sample collection and processing

Blood

Blood samples were collected by local nurses from the Madziwa rural clinic. Up to 5 mL of venous blood was collected from each participant into serum separator tubes (BD Vacutainer®) and processed for serum for serological / immunoassays. The blood was transported on ice packs from the field to the Shamva (local district) hospital laboratory and stored at 4 °C overnight. After centrifugation (3000rpm) the following day, sera were extracted, and aliquots of the sera were frozen at -20 °C in the field. These aliquots were then transferred to a -80 °C freezer in the laboratory at the University of Zimbabwe before

being shipped on dry ice to the University of Edinburgh where immunological assays were conducted.

Stool and Urine

Sample processing for parasitological tests were performed by the research team, and microscopy was done by trained field technicians from the University of Zimbabwe. Approximately 50 mL of at least two separate urine samples were collected from each participant on three consecutive days, and a stool specimen was collected on a single day from each participant. Samples were collected between 10:00 and 14:00 hours and processed within two hours of collection. For very young children, urine bags (Hollister 7511 U-Bag Urine Specimen Collector, Hollister Inc., Chicago, IL, USA) and disposable diapers were used to collect urine and stool samples, respectively.

Following the standard urine filtration method, urine samples were examined microscopically for *S. haematobium* infection [301]. Briefly, each urine sample was mixed thoroughly, and 10 mL of urine was aspirated using a 10 mL plastic syringe. A nitrocellulose mesh filter was then attached, and the urine slowly passed through. If present, parasite eggs were left trapped in the filter, which was then observed under a light microscope and number of eggs/10 mL of urine enumerated.

Stool samples collected were processed using the Kato–Katz thick smear method [302]. Briefly, the stool samples were sieved to remove large particles, and duplicate slides were prepared using the standard 41.7 mg templates and to aid parasite egg identification, the prepared slides were stained with glycerol–malachite green which stains stool components but does not penetrate parasite eggs. Parasite eggs of intestinal helminths were enumerated under a light microscope per gram of stool. A child was diagnosed with helminth infection if at least one parasite egg was found in their urine or stool samples. Those positive for *S. mansoni* and STH were excluded from the study.

As mentioned in **Chapter 2**, stool samples were also collected to extract DNA for gut mycobiome characterisation.

Stool DNA isolation

Aliquots of fresh stool samples were collected and DNA was extracted stool using the QIAamp DNA Stool Mini Kit (QIAGEN, catalogue number 51504), according to the manufacturer's instructions. In brief, stool samples in 2 mL cryotubes were homogenized in buffer and heated at 95 °C to lyse cells. An InhibitEx tablet was added to remove potential inhibitors, and the lysates were treated with proteinase-K and buffer at 70 °C for 10 minutes to remove protein and polysaccharides. Isolated DNA was precipitated by ethanol, applied to a column and washed twice with buffers. The DNA was eluted and then dissolved in buffer. DNA was stored at -20 °C in the field and transferred to a -80 °C freezer in the laboratory at the University of Zimbabwe. The DNA was also shipped on ice to the University of Edinburgh and stored at -80 °C until use.

4.3.3 Skin prick tests (SPT)

To determine the prevalence of fungal sensitisation in the cohort, SPTs were conducted using six (*Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans* and *Saccharomyces cerevisiae* (Stallergenes Greer, France)) instead of seven different fungal allergen extracts. This was due to logistical challenges, as the *Aspergillus fumigatus* SPT extract was not shipped and delivered in time of the fieldwork and hence it was excluded in the SPT analysis.

SPT assay

Briefly, drops of each allergen extract were placed on the forearm 2 cm apart and pricked using a calibrated lancet that introduces approximately 1 µg/mL of allergen into the dermis (**Figure 4.1**). Histamine dihydrochloride (10 mg/mL) (Stallergenes Greer, France) was

used as a positive control and a saline solution (Stallergenes Greer, France) as a negative control. Reactions were considered valid if the histamine wheal diameter was greater than the negative control. Results were read at 15 minutes. The largest diameter of the wheal for each allergen extract was measured, and a wheal of ≥ 3 mm scored positive.

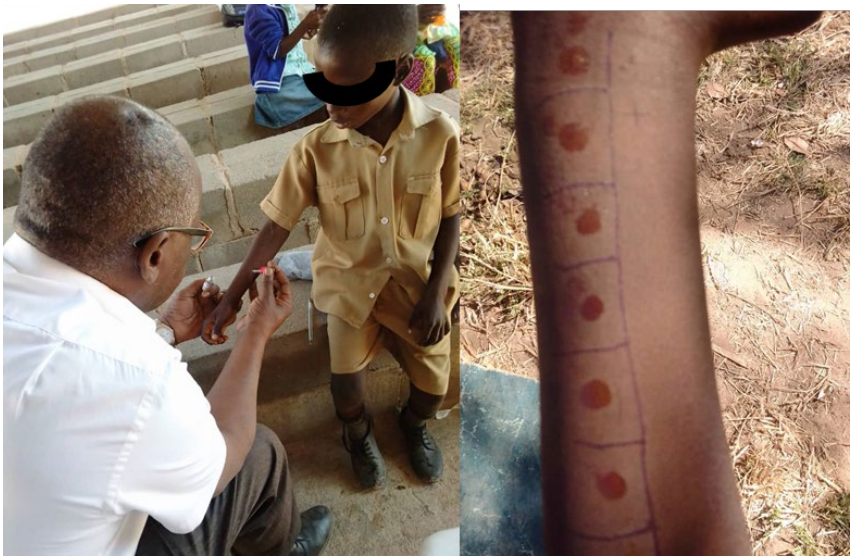

Figure 4.1: Clinician carrying out Skin prick tests (SPTs) in Shamva

Limitations of SPT

Although SPT was used in this study and is a valuable diagnostic tool [269], it has some limitations. For example, it is not practical in patients who have extensive eczema, dermographism or who are taking antihistamines or other medications which interfere with the proper interpretation of the test results [516]. Furthermore, there are inconsistencies in the preparation of fungal extracts; with other manufacturers using, for example, either fungal mycelia or spores for production [3, 216, 517]. Additionally as crude extracts (containing different proteins) are often used, sensitised/ allergic individuals, form IgE antibodies to different proteins in the extract. However, suppose the specific protein(s) to which IgE is directed in a specific individual is not represented within the allergen extract (due to manufacturing processes or protein lability). In that case, this may result in a false negative result even if the individual is allergic to the substance when encountered in nature.

Likewise, the clinical relevance of SPT results varies, depending on the allergen utilised, and the population tested due to the seasonal differences of various allergens based on geographic locations Likewise, the clinical relevance of SPT results varies, depending on the allergen utilized and the population tested due to the seasonal differences of various allergens based on geographic locations [516].

4.3.4 Serological assays

Antigens

All fungal antigens (*Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans* and *Saccharomyces cerevisiae*) were lyophilized crude extracts obtained from Stallergenes Greer, in the USA. These preparations were reconstituted in Phosphate buffered saline (PBS), and protein content was determined by Bradford protein assay. All antigens were stored at -20°C in concentrations >1 mg/mL.

Determination of protein concentration by Bradford Assay

Following the manufacturer's instructions, a set of standards were prepared using Bovine serum Albumin (BSA) from 0.1 – 2 mg/ mL. Fungal extracts were used undiluted and as doubling dilutions (i.e. 1:2- 1:256). Standards and extracts were mixed with 190 µL of Coomassie Brilliant Blue G-250 dye, and the absorbance was read at 595nm. The absorbance values of the BSA set of standards were plotted against their known protein concentrations to obtain a standard curve (example shown in **Supplementary Figure A.1 Appendix A**), which was used to calculate the protein concentrations of the fungal extracts.

Antibodies

Levels of IgA, IgG, IgM, IgE and IgG4 antibodies directed against fungi (*Aspergillus fumigatus, A. alternata, C. herbarum, E. nigrum, P. chrysogenum, R. nigricans* and *S. cerevisiae*) were measured by indirect ELISA.

Rationale for antibody assays

There is heterogeneity in the responses to allergens between different people, and to capture the diversity of the responses mounted, five antibody isotypes (IgA, IgE, IgG, IgG4 and IgM) were chosen for assays. These antibody isotypes were selected because they have been linked to allergic reactions [518], where IgE is involved in immediate response to allergens, and IgG4 antagonizes effector functions mediated by IgE [519]. IgG and IgM are involved in delayed immune responses against Type III allergens [520]. IgA and IgG may initiate some adverse food reactions [521, 522]. In addition, there is cross-regulation of immune responses, e.g. the IgG4 response may ameliorate the pathological effects of IgE, so the ratio of IgE/IgG4 may be more informative than each antibody isotype alone. All the antibodies used for ELISAs and Western Blotting, their catalogue numbers and suppliers are given in **Table 4.1**.

Table 4.1: Antibodies used for ELISA and Western blotting experiments with descriptions as supplied on product sheets at the time of purchase.

Antibody Isotype	Description	Supplier	Catalogue number
IgA	Detection	Dako	P0216
IgE	Detection	Sigma	A9667
IgG	Detection	Dako	P0124
IgG4	Detection	The Binding Site	AP009
IgM	Detection	Dako	P0215
CD23	Capture	R&D Systems	MAB1231
CD23	Detection	R&D Systems	BAF123
CD23	Standard	R&D Systems	123-FE

Optimizing ELISA protocol

To determine optimum dilutions of antigens, sera and secondary antibodies, as well as enzyme-substrate incubation times, were determined by several serial dilutions, following published procedures [523] and those recommended by manufacturers. Two different types of ELISA plates were tested: Thermo Scientific Nunc and Greiner Bio-One. No significant differences were observed between these microplates, and the latter was

selected for use in all assays as they were relatively cheaper. The method was developed using the following pools of sera:

(i) **Pool 1** Zimbabweans with the highest fungi SPT-reactivity

(ii) **Pool 2** Zimbabweans non-reactive to fungi (determined by SPT)

(iii) **Pool 3** Study participants (PSAC, aged 1-5 years)

Microtitre 96-well plates were coated overnight at 4 °C with 5 µg/mL and 10 µg/mL of fungal antigen diluted in PBS. Plates were washed once in PBS/tween20 (see **buffer recipes, Appendix C)** and blocked with BSA (Melford) for an 1 hour at room temperature so as to prevent non-specific binding. After three washes to remove the unbound blocking agent, pooled serum samples were added onto the wells (diluted from 1:10- 1:10240 in blocking buffer (see **buffer recipes, Appendix C**), and plates were incubated at 37 °C for two hours. After three washes to remove excess serum components, the HRP-conjugated secondary antibody was added (diluted at 1:1000 and 1:2000 in the blocking buffer to determine the most appropriate secondary antibody concentration), and the plates incubated again at 37 °C for 1 hour. Throughout, serum samples and secondary antibodies were diluted in blocking buffer to minimize the non-specific binding effect since this may occur on the plate at any time during the assay. Plates were washed six times, and the substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was added. The colourimetric reaction (expressed in optical densities) was detected and quantified by an EMax precision microplate reader (Molecular devices) at a wavelength of 450nm and processed using Softpro Max v5 software. Optimum concentrations were deduced from checker-board titration (see **Figure 4.2** below). The reaction time for the enzyme-substrate reaction was determined by reading plates every 10 minutes for an hour and plotting the results (absorbance against time) to detect the linear phase. The optimised conditions for each fungal ELISA assay are shown in **Supplementary Table A.1, Appendix A**.

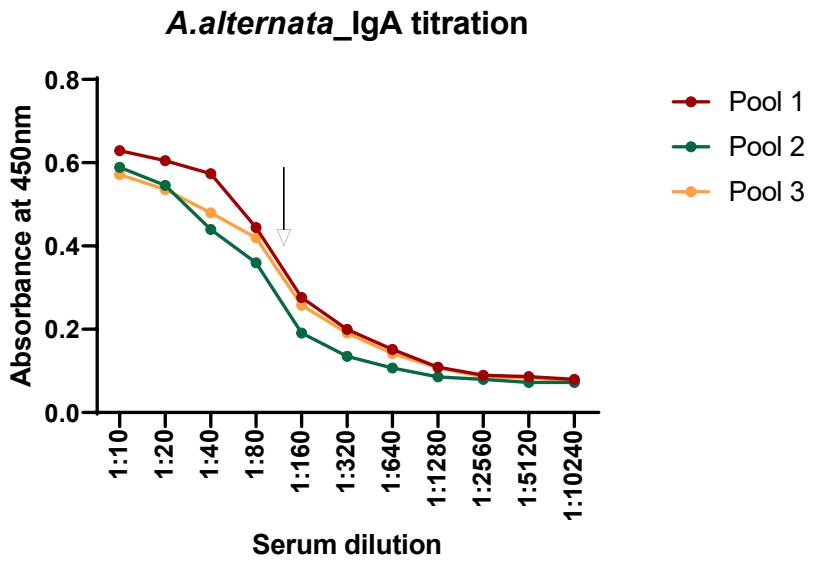

Figure 4.2: Checkerboard titration

Titration curve of pool sera against *A. alternata* antigen at 5 µg/mL (pool 1: Zimbabweans with the highest fungi SPT-reactivity; pool 2: Zimbabweans non-reactive to fungi (determined by SPT); pool 3: Study participants (PSAC, aged 1-5 years). The optimum serum dilution for subsequent assays was decided as 1:100 since this is the intermediate dilution where the linear phase of the reaction occurs for all sera (including sera with the largest and the smallest amount of *A. alternata*-IgA). The same procedure was done for all the fungal antigens

4.3.4.1 Antibody ELISAs

Following assay optimization, fungal-specific IgA, IgG, IgG4, IgE and IgM levels were then quantified for each participant using indirect ELISAs at the appropriate serum dilution, antigen concentration and secondary antibody dilution following the protocol detailed above and summarised in **Figure 4.3**. To minimize variations across plates, positive and negative controls were repeatedly run on each plate and plotted to detect any outliers (in which case the assay was repeated). The positive control was pooled sera of individuals with the highest fungi SPT-reactivity and the negative control was pooled sera of individuals who were non-reactive to fungi. To account for background variation, a blank well (containing no sera) was included on each plate and absorbance was subtracted from all plate readings. All the samples and blank were run in duplicate on each plate; reported values are the mean of duplicates. The mean of the negative pools plus two

standard deviations of the readings was used to determine the cut-off points for antibody levels.

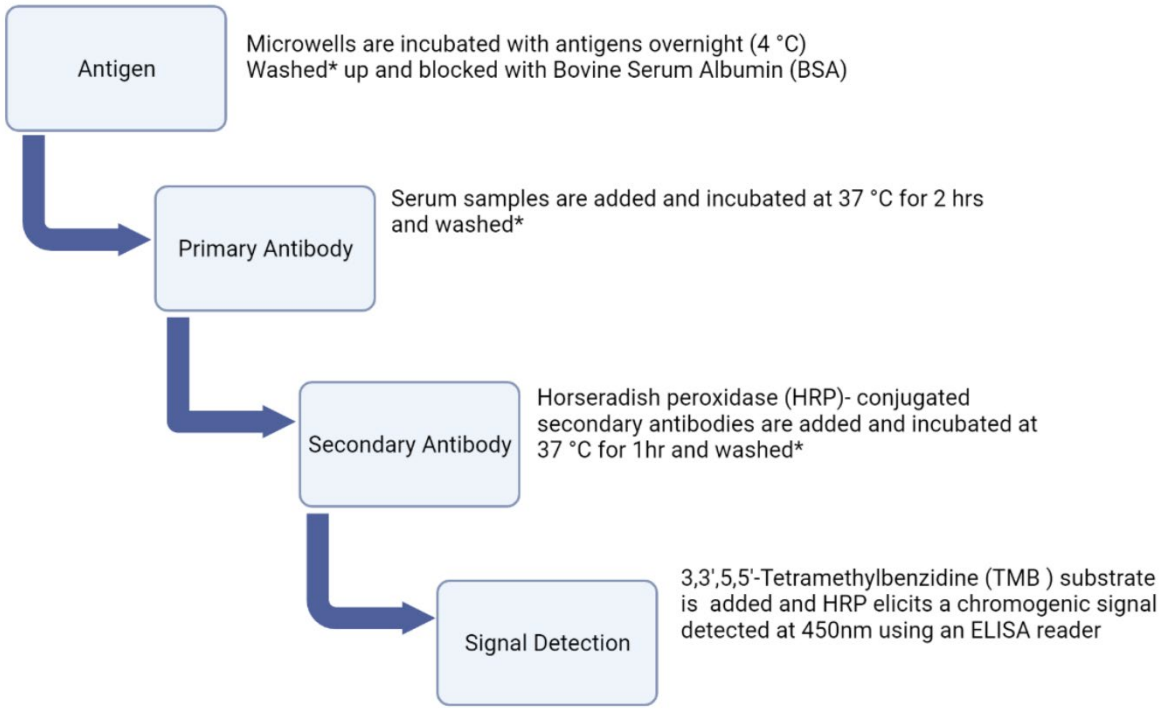

Figure 4.3: Summary of Indirect ELISA protocol
(*wash with Phosphate buffered Saline x3 following the incubation period). Chromogen is applied to visualise the antibody/antigen complex.

4.3.5 Next generation sequencing

Sequence library preparation and sequencing

To characterise the gut mycobiome of PSAC, the extracted stool DNA were shipped on dry ice for library preparation and sequencing at the Beijing Genomics Institute (BGI, Shenzhen, China). DNA was quantified using the Qubit fluorometer (ThermoFisher Scientific) and NanoDrop™ (ThermoFisher Scientific). A 1% agarose gel electrophoresis then assessed the integrity and purity of DNA. DNA passing quality control was sheared by ultrasonication into fragments (Covaris S/E210). Fragments were mixed with End Repair Mix (BGI), adaptors were ligated to the ends of the DNA fragments and then

purified using the QIAquick Polymerase Chain Reaction (PCR) Purification Kit (QIAGEN). Adapter-ligated DNA fragments were separated by electrophoresis through a 2% agarose gel to recover the target fragments and purified using the QIAquick Gel Extraction kit (QIAGEN).

Library preparation to enrich the adapter-ligated DNA was done via PCR amplification, size-separated by electrophoresis, and then purified using the QIAquick Gel Extraction Kit (QIAGEN). The final library was quantified using the Agilent 2100 bioanalyzer. The suitable DNA libraries were amplified using the cBOT system (Illumina) and sequenced on the Illumina Hiseq 4000 platform (Illumina) using paired-end 150-bp sequencing.
Direct mapping of sequence reads against reference sequence databases was done using a novel reference-based mapping and alignment tool known as k-mer alignment (KMA) [524].

4.3.5.1 Quality control and trimming

Raw FASTQ format (a text-based format for biological sequence outputs) sequences from each sample were quality assessed using FASTQC v0.10.0 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The output of the FASTQC analysis showed that the raw FASTQ sequences were of good quality, and the number of read pairs generated per sample ranged from 9,263,538 to 21,350,613. Subsequently, reads were trimmed, including removing adaptors, using BBduk2 (BBMap—Bushnell B.—https://sourceforge.net/projects/bbmap/) with an output quality Phred threshold score of ≥20 and a minimum read length of 50 bp. K-mer length for finding contaminants was k=19 to include shorter k-mers at read pairs down to k=11, and reads were trimmed at the right end.

4.3.5.2 Mapping of sequence reads

Direct mapping of sequence readings against reference sequence databases was done using k-mer alignment (KMA), a novel reference-based mapping and alignment tool [524]. This method was selected because it improves mapping against redundant databases and is faster, more precise, and more sensitive than existing methods[524]. KMA uses heuristic mapping to map k-mers between query sequences and selected template databases, including massive redundant databases, then scores matching k-mers. K-mers with positive scores are employed as alignment seeds; each matching seed is expanded, scored, and mismatch regions are found. KMA uses a special version of Needleman-Wunsch [525] to align mismatched k-mers. To provide the best match template for query reads, multi-mapping reads are resolved using ConClave. The technique adds the alignment scores for all initially accepted best matching templates to get the ConClave score and chooses the best matching sequence template. Uniquely mapped readings have a unique ConClave score, while multi-mapped reads with the same score favour the first template contributed to the database. In such cases, both templates will be equally suitable candidates, but favouring the parent template contributed to the database ensures reproducibility.

The steps described above allow reads to be assembled, resulting in a final consensus sequence for the reference or template sequence. To rule out bias associated with base calling across multiple sequencing platforms, the strength of each named nucleotide is assessed for overrepresentation using the McNemar test (= 0.05) [526]. **Figure 4.4** depicts a schematic diagram of the process.

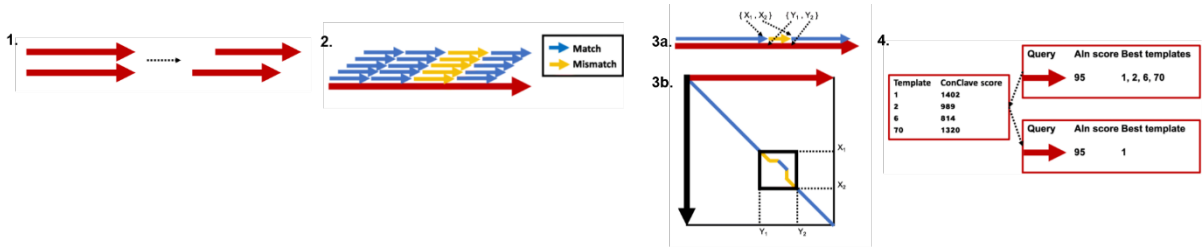

Figure 4.4: Overview of the k-mer alignment (KMA) algorithm steps
Read trimming (1), followed by k-mers matching between query and database (2). Matching k-mer seeds are extended, regions with mismatching k-mers are identified (3a) and using the Needleman- Wunsch algorithm, these mismatching k-mers are aligned (3b). Following that, Conclave scoring is used to select one best-aligning template per query sequence (4). Image adapted from [524].

To obtain the microbiome sequence component present in the samples, read pairs and singletons were aligned to a custom reference genomic database. Mapped reads were counted as one copy for both read pairs and singletons. Unless otherwise specified below, databases were downloaded via the National Center for Biotechnology Information (NCBI) GenBank clade-specific assembly_summary.txt files (ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank). The custom database consisted of the following: bacteria (closed genomes; downloaded 05.02.2019), archaea (downloaded 13.02.2019), MetaHitAssembly (PRJEB674–PRJEB1046; downloaded 01.07.2014), HumanMicrobiome (genome assemblies; downloaded 02.07.2014), bacteria_draft (downloaded 05.02.2019), plasmid (downloaded 05.02.2019), virus (https://bitbucket.org/genomicepidemiology/kvit_db (downloaded 05.02.2019); https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=IMGV (downloaded 28.01.2019)), fungi (downloaded 13.02.2019), protozoa (downloaded 13.02.2019), and parasites (downloaded 04.04.2019). The total read count for each microbial community of interest in a sample was calculated as the sum of read counts from each of the databases of interest; bacteria (bacteria, bacteria_draft, MetaHitAssembly, and HumanMicrobiome), fungi, protozoa, archaea and parasites.

The most similar alignment obtained for mapped sequences was used to assign taxonomy based on the taxonID obtained. TaxonIDs and associated taxonomy classifications were obtained from downloaded reference microbial genomes from NCBI

(ftp://ftp.ncbi.nih.gov/pub/taxonomy/taxdump.tar.gz), and assignment at all taxonomic levels was done. Sequences that had no similarities detected in the nucleotide (nt) database to assign a taxonomic classification were deemed to be unknown sequences and thus labelled “unknown”.

4.3.5.3 Data handling and processing

To account for probable sample-wise sequencing depth differences and the size-dependent probability of observing a reference, mapping counts from the custom genomic database were normalised to the total genome sizes for the genomic database.

The total number of observed mapping counts is relative and it may account for confounding effects in subsequent analyses [527]. This could be due to arbitrary total limitations imposed by different sequencing platforms, technical variations in sequencing libraries, or even random variation [528]. Thus, to obtain information about the abundances of features in the dataset relative to each other, datasets were treated as compositional [528]. Data were transformed using the log-ratio approach introduced by Aitchison to make the data symmetric, linear and in a log-ratio coordinate space [529]. However, compositional methods like this one do not account for the presence of zeros in abundance datasets. To address this, a small pseudo count of half the smallest non-zero abundance per feature was added to each feature for all the normalised abundance matrices prior to transformations [530]. Mycobiota abundance data tables with counts, x, and k number of populations (taxa members), were centred log-ratio (clr) transformed, defined as:

$$clr(x_1,\ldots.x_k) = \left(log\left(\frac{x_1}{g(x)}\right)\right),\ldots,\left(log\left(\frac{x_k}{g(x)}\right)\right),$$

Where $g(x) = (\Pi_{xi})^{1/k}$ is the geometric mean of the particular composition.

4.3.5.4 Data visualisation

Data visualization was performed within the R environment v 3.6.1 and GraphPad Prism v 7.02 (GraphPad Software, Inc.). Bar plots from normalised, zero-corrected abundance matrices were used to give an overview of the microbiota abundance across all samples. For cluster dendrograms, the Aitchison distance (Euclidean distance) was calculated using clr-transformed abundance data, and samples were clustered based on distances (Complete-linkage-clustering). To explore underlying variabilities in the microbiota across the data set, clr-transformed abundance data for each matrix, centred on the geometric sample mean and scaled by the total variance, was ordinated based on eigenvectors and eigenvalues using Principal Component Analysis (PCA) [530].

4.3.6 Statistical analyses

Data were analysed using various Bioconductor packages in R software v3.6.1 and SPSS v22 (IBM Corp.). Details of the statistical methods used here are described in **Chapter 2**.

To test whether sample-related metadata predicted within-group dispersion of the microbiome, the Euclidean distances were calculated using R/Bioconductor package vegan [531]. The effect of such metadata on sample dissimilarities was determined using permutational multivariate analysis of variance (PERMANOVA; adonis2 function in the vegan package) using $p < 0.05$ as the significance threshold. A false discovery rate (FDR (Benjamini–Hochberg FDR)) correction was applied to counteract multiple testing [347]. Bar plots from normalised, zero-corrected abundance matrices were used to give an overview of the microbiota gene abundances across all samples.

To investigate further how specific taxa composition varies across the statistically significant metadata (from PERMANOVA) while controlling for other variables, an analysis of composition of microbiomes (ANCOM) was used [532]. Analysis was based on a log-ratio transformation of raw count data (clr), where the normalizing reference value is the

abundance of all remaining taxa, taken one at a time. The FDR was set at 0.05 [347], and a taxa member was considered at a W-statistic cut-off of 0.80. The ANCOM test for the association of *S. haematobium* infection was controlled for age and sex. As ANCOM only provides a list of taxa that vary in composition, the magnitude and direction of associations of taxa that vary in composition across independent variables was further determined. Box plots stratified by specific independent variables, using the clr-transformed abundance data of significant taxa previously identified by ANCOM were used to highlight differences in groups.

To determine whether age, sex, and *S. haematobium* infection status affected SPT reactivity, binary logistic regression was performed. To test whether serological reactivity to fungi varied with age, sex, *S. haematobium* infection status, and gut mycobiome structure, analysis of variance (ANOVA) was used. Differences were considered to be significant at $P < 0.05$. The experimental data are presented as the mean ± standard error of the group means.

Due to the skewed nature of the fungus-specific antibody responses, values were square root-transformed. To characterise patterns of the different fungal-specific antibodies, all titres (IgM, IgA, IgG, IgG4 and IgE) were reduced into axes to facilitate the interpretation of patterns and differences among groups using non-metric multidimensional scaling (NMDS) as described in **Chapter 2**. NMDS was run in R using a Bray Curtis distance method, and 1- 6 dimensions were trialled. The stress scores were recorded to determine the number of axes needed. The stress score produced is a goodness of fit statistic based on the differences between the actual distances and their predicted values. A stress score >0.1 is 'poor, <0.1 is 'fair' and <0.05 is 'good' [533]. Pearson's correlations were used to determine the relationship between the original variables and the axis, and only antibodies with an r^2 <-0.5 or> 0.5 were considered adequately reflected by the axis.

NMDS scores were compared by sex, age group, SPT reactivity and *S. haematobium* infection.

4.3.7 Data availability

Raw sequence data files from all 116 samples and associated metadata used in the current study are deposited in the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI) database under the BioProject accession number PRJNA521455. All other data files, including QC, sequence, Taxon ID mapping Information, Antibody titres and SPT results (downloadable from Edinburgh datashare: https://doi.org/10.7488/ds/3188)

4.4 Results

4.4.1 Population characteristics

Of the 116 participants included in the study, 59 (50.9%) were female, and 57 (49.1%) were male; the mean age was 3.7 ± 1.1 years. *S. haematobium* infection prevalence was 15.5% (18/116).

Regarding the children's dietary habits and nutritional status, most were breastfed for six or more months. Children were introduced to solid foods between 1 and 24 months after birth. The main food component of the diet was traditional maize flour porridge. Anthropometric measurements, adjusted for age, were used to assess nutritional status [534, 535]. Based on the weight-for-height Z-scores (WHZ), 3.7% (4/107) of individuals were malnourished, and 14.7% (16/109) were stunted based on the height-for-age Z-scores (HAZ). **Table 4.2** details the demographic characteristics of the study population.

Table 4.2: Demographic characteristics of the study population

Demographic categories		Frequency	Percentage
Gender	Female	59	50.9
	Male	57	49.1
Age group (years)	≤3	52	44.8
	4	33	28.4
	5	31	26.7
S. haematobium infection status	Negative	98	84.5
	Positive	18	15.5
Nutritional and growth factors			
Breastfed (months)	<6	1	1.1
	≥ 6	89	98.9
Solid food introduction (months)	<6	79	77.5
	≥ 6	23	22.5
Malnourished (WHA)	Yes	4	3.7
	No	103	96.3
Stunted (HAZ)	Yes	16	14.7
	No	93	85.3
Total		**116**	**100**

4.4.2 Taxonomic composition of the microbiome

Using data recently published by our research group [536], the relative abundance was calculated for each microbial community in all samples.

The number of classified read pairs per sample ranged from 3,994,704 to 13,164,482. An average of 45.1% of read pairs were mapped to specific reference sequences in the genomic database [536], similar to other studies with the proportion of unmapped reads ranging from 42%–68% [537-539]. At any taxonomic level, a taxonomic classification could not be assigned to at least 33% of the mapped read pairs, which were thus classified as “unknown”.

The mycobiome made up less than 1% of the sequenced gut microbiome, as shown in **Figure 4.5**. However, it showed high diversity (**Figure 4.6**). In the 116 stool samples analysed, 228 fungal genera (from six unique phyla) were detected.

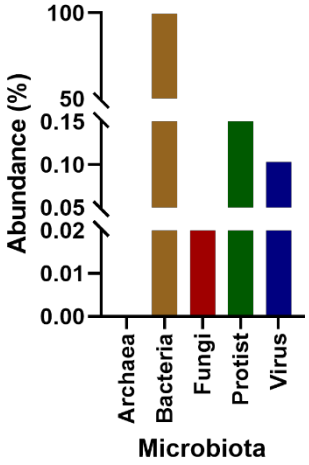

Figure 4.5: Composition of the gut microbial communities.

Relative abundance of fungal phyla and genera in the gut microbiome

Abundance was calculated for each microbial taxon across all samples. The most prevalent phyla were Ascomycota (genera: *Protomyces* (present in 100% of the samples), *Taphrina* (98%), *Aspergillus* (91%), *Saccharomyces* (91%)), Microsporidia (*Enterocytozoon* (100%)), and Zoopagomycota (*Entomophthora* (100%)) **Figure 4.6a-b**.The bar charts were generated using normalised, zero-corrected abundance matrices.

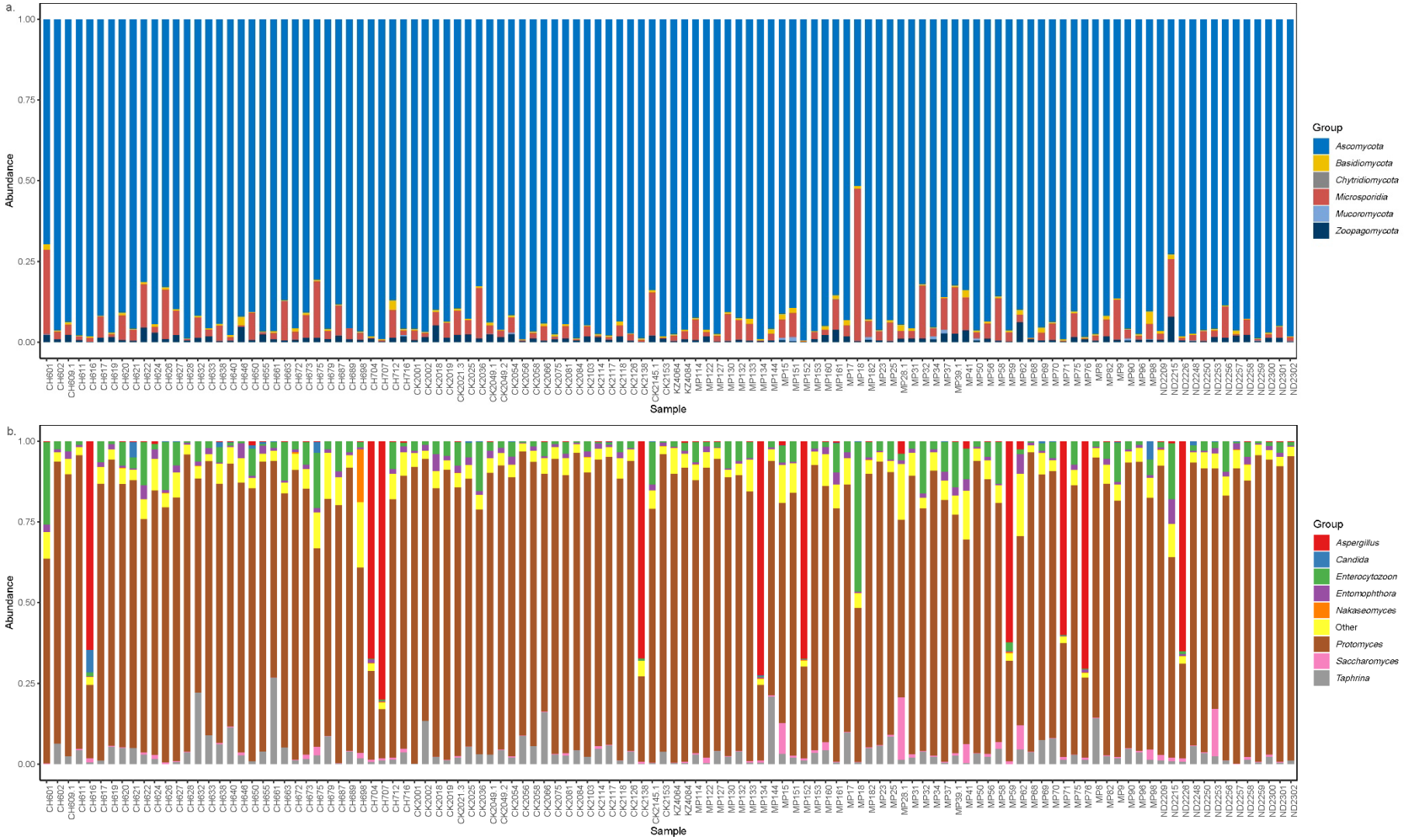

Figure 4.6: Overview of the fungal microbiota abundance and diversity
Stacked bar charts show the most abundant fungi (a) phyla and (b) genera per sample, proportional to the total microbiota within each sample (n=116 biologically independent samples). “-Other” represents abundance data for all other taxa in the abundance data set.

4.4.3 Variation in the mycobiome and association with participant metadata

To examine variability and patterns in the data set, PCA was used. At the phylum level, PCA explained 62% of the total variation in fungi. At the genus level, however, PCA explained 34% of the total variation in fungi. The model showed homogeneity in components with no distinct clustering according to metadata, which may reflect a high diversity in the cohort. PCA plots and cluster dendrograms for fungi content per sample are shown in **Figures 4.7** and **4.8,** respectively.

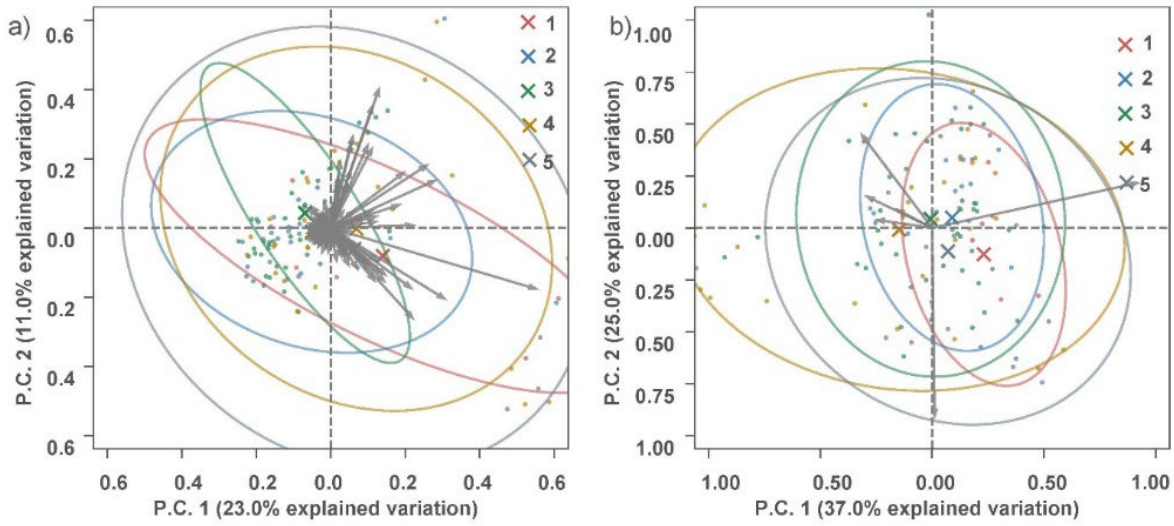

Figure 4.7: The plots of principal component analysis (PCA) for mycobiota across samples, annotated by age.
Figures (a) and (b) show PCA plots for fungi classified according to genus and phylum, respectively. Raw counts were clr-transformed, and the resulting matrices were ordinated using PCA. The projections of features are plotted as arrows, and the projection of samples are plotted as points. The eigenvalues associated with the eigenvectors are used to describe the amount of explained variation per axis.

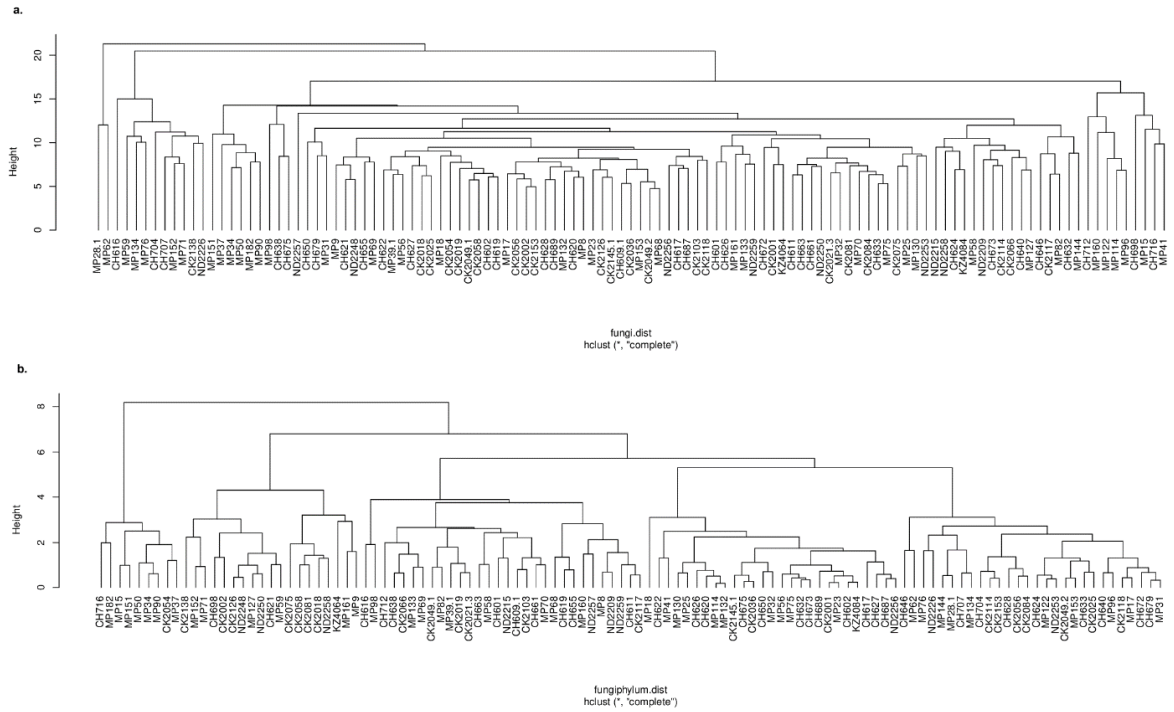

Figure 4.8: Fungal abundance and composition dendrograms.
Clustered dendrograms show fungal (a) genera and (b) phyla per sample. The vertical axis of the dendrogram represents the distance or dissimilarity between clusters. The horizontal axis represents the population and clusters

PERMANOVA analysis showed a significant effect of *S. haematobium* infection status (FDR= 0.007) on fungi genera across samples. However, no such effects were observed for age, sex, nutrition, growth, or feeding variables. The summary output for the analysis is shown in **Table 4.3**.

Table 4.3: Summary of sample metadata and association with the gut mycobiome

Variable	n	p-value	Explained sum of squares	Total sum of squares	FDR
Gender	116	0.370	85.8	9673.3	0.726
Age (years)	116	0.098	123.0	9636.1	0.343
Malnourished (WHA) yes/no	107	0.830	59.5	9148.0	0.830
Stunted (HAZ) yes/no	109	0.624	71.5	9229.7	0.728
Breast-fed (months)	90	0.415	75.2	6986.5	0.726
Solid food introduction (months)	102	0.602	73.6	8794.3	0.728
***S. haematobium* status**	116	0.001	339.1	9281.2	**0.007**

Classification of nutritional status was based on a cut off <−2 Z scores (MOH Malawi, 2016). WHA, weight-for height Z scores; HAZ, height-for-age Z scores; p-value- unadjusted p-value; FDR- adjusted p-value (FDR-corrected)

Microbiome analysis by schistosome infection status

From the PERMANOVA results, further analysis via ANCOM showed that the abundance of three specific fungal genera were associated with *S. haematobium* infection. *Aspergillus* (W= 75), *Tricholoma* (W= 72), and *Periglandula* (W= 70) showed variation with *S. haematobium* infection. The magnitude of the differences in abundance between *S. haematobium*-infected and uninfected children is shown in **Figure 4.9**. The abundance of all three genera in schistosome-positive children was higher than in schistosome-negative children.

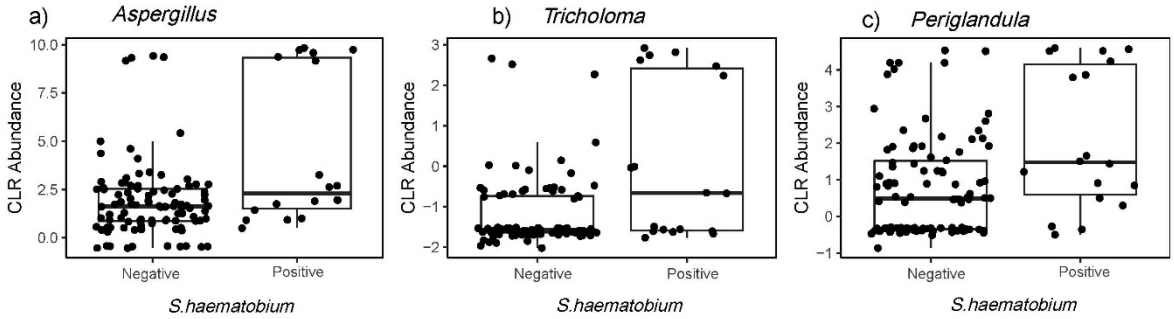

Figure 4.9: Fungi genera whose abundance varies significantly with schistosome infection
Figure a–c) Box plots showing the mean abundance of specific fungi genera grouped by *S. haematobium* infection status. The horizontal box lines represent the first quartile, the median and the third quartile. Whiskers denote the range of points within the first quartile − 1.5× the interquartile range and the third quartile + 1.5× the interquartile range. The clr transformed abundance data were used for all plots. The sample sizes for the *S. haematobium* infection status were: negative (n=95) and positive (n=18).

Of the 116 children with a characterised microbiome, 71 were included in the subsequent analysis. The decrease in sample size was due to loss of SPT follow-up or inadequate serum sample volumes. The age range for the final samples was 2-5 years, with a mean age of 3.98 years and a female/male ratio of 1.2.

4.4.4 Antibody profiles for fungi-specific immune responses

Overall, fungal seroprevalence was 100% (95% Confidence Interval (CI) 94.94-100). IgE antibodies were detected in 85.9% (95% CI 75.6-93.0) of the study population whereas

IgG4 antibodies were detected in 32.4% (95% CI 21.8- 44.6). **Figures 4.10 and 4.11** depict the frequency distribution of children producing each antibody (with 95% CI) and mean antibody levels (absorbance) against each fungal species, respectively.

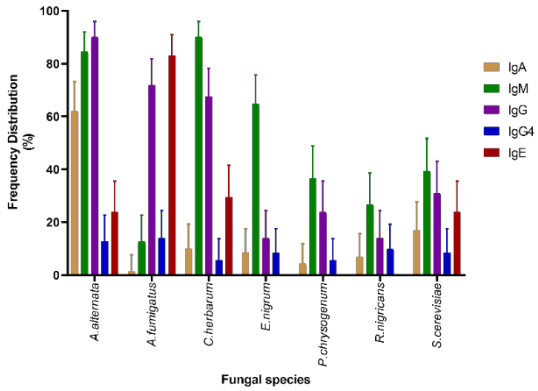

Figure 4.10: Frequency distribution of children producing each antibody against each fungal species with a 95% confidence interval (CI).

The immune response to allergens induces different levels of immunoglobulin classes, depending on the specific allergen. IgM and IgG, which are produced predominantly upon the first exposure to an allergen, [540] had higher mean antibody responses for all fungal species except *A. fumigatus,* where IgM titre was low. In all other cases, where IgM and IgG responses were highest compared to other antibody subclasses, IgE titres were low or not detected altogether (*R. nigricans, P. chrysogenum,* and *E. nigrum*), as shown in **Figure 4.11**.

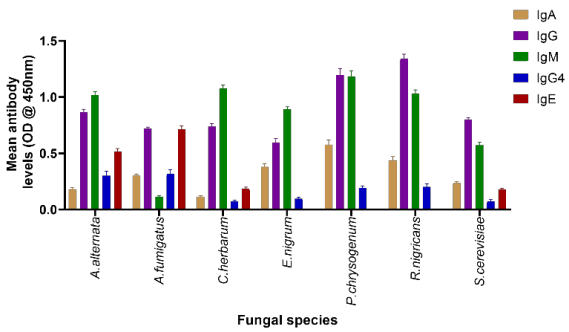

Figure 4.11: Mean antibody production with SE bars for each antibody for each fungal species.

4.4.4.1 Characterisation of fungi-specific antibodies

To characterise patterns of different fungi-specific antibodies, all the antibodies produced by the participants fit into NMDS axes, which follow the ordination with a rotation via PCA. This is useful because it ensures that NMDS axis 1 (NMDS1) reflects the principal source of variation [344].

NMDS1, which accounted for the most variation within the data, was positively correlated with *A. fumigatus (A.f)* –IgG4; *E. nigrum (E.n)*-IgG; *P. chrysogenum (P.c)* – IgG and IgG4, whilst negatively correlated with *A. alternata (A.a)*-IgG4 and IgE; *C. herbarum (C.h)* – IgM and IgG; *S. cerevisiae (S.c)*- IgM, IgA and IgG; *P. chrysogenum* (P.c)-IgM; *R. nigricans (R.n)*-IgA, IgG and IgG4. NMDS2 was positively correlated with *A. fumigatus* –IgA and IgE; *P. chrysogenum*- IgA; *S. cerevisiae*- IgE and IgG4, but negatively correlated with *C. herbarum*- IgG4; *E. nigrum*- IgM and IgA; *R. nigricans* –IgM. NMDS3 was positively correlated with *E. nigrum*-IgG4 and IgM; *P. chrysogenum*- IgA, *A. alternata*-IgG and negatively correlated with *A. fumigatus* –IgM and *S. cerevisiae*- IgA **Figure 4.12.** The NMDS scores are summarised in **Supplementary Table A.2, Appendix A**.

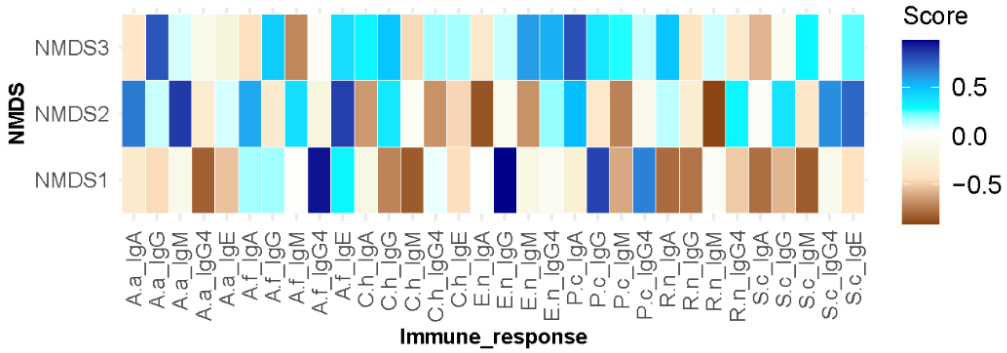

Figure 4.12: The NMDS scores of the original variables for each axis calculated after the NMDS of fungal-specific antibodies.
Variables which had a significantly positively correlated (score >0.5) on an NMDS axis are shown in dark blue, and variables which were significantly negatively correlated (score <-0.5) are shown in dark brown. The colour gradient reflects the loading score, whereby the darker the colour, the higher the score (if >0) or lower the loading score (if <0).

Given IgM and IgG are often associated with the first exposure to an allergen, *C. herbarum, P. chrysogenum* (IgM), *R. nigricans* and *S. cerevisiae* were negatively associated with allergen exposure. While *A. alternata, P. chrysogenum* (IgG) and *E. nigrum* were positively associated with allergen exposure. Given IgE is associated with allergic sensitisation, *A. fumigatus* and *S. cerevisiae* were associated with allergic sensitisation.

4.4.5 Skin Prick Test Reactivity

Of the seventy-one children included in the serological study, only fifty had SPTs performed. Ages ranged from 3 to 5 years, with a female/male ratio of 1.5. Forty-eight study participants (96%) were SPT-positive to at least one fungal source. **Table 4.4** summarises the prevalence of different patterns of fungal sensitisation. Most participants were sensitised to two (TWO-FS=18.6%) or multiple fungi (MFS= 66.7%).

Table 4.4: Prevalence of fungal sensitisation based on skin prick testing (SPT) of the study population

		ONE-FS*	TWO-FS*	MFS*
Total	**48(96.0)**	7(14.6)	9(18.6)	32(66.7)
A. alternata	16 (33.3)	0	0	16(50.0)
C. herbarum	28(58.3)	3 (42.9)	1(11.1)	24(75.0)
E. nigrum	27(56.25)	1(14.3)	5(55.6)	21(65.6)
P. chrysogenum	28(58.3)	0	4(44.4)	24(75.0)
R. nigricans	24(50.0)	0	4(44.4)	20(62.5)
S. cerevisiae	29(60.4)	2(28.6)	4(44.4)	23(71.9)

** ONE-FS: one positive SPT to a single fungal species; TWO-FS: two positive SPT to fungi; MFS: more than two positive SPT to fungi (multiple fungi sensitisation).*

S. cerevisiae, *C. herbarum*, and *E. nigrum* were positive in 85.7% of the participants. All participants sensitised to *A. alternata* were sensitive to more than one fungi (poly-sensitised). **Figure 4.13** illustrates the sensitisation pattern observed in the study population.

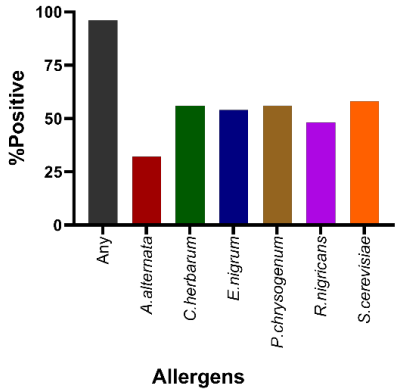

Figure 4.13: Prevalence of fungal sensitisation based on the study population's skin prick testing (SPT); Prevalence of positive SPT to different fungal species tested.

The prevalence of positive SPTs decreased with age (26% for the <3 years group, 40% for the 4 years group, and 30% for 5 years; **Figure 4.14.**

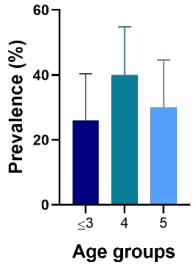

Figure 4.14: Prevalence (%) of positive SPTs for the different age groups with a 95% confidence interval (CI)

4.4.5.1 Effects of age, sex and *S. haematobium* infection on SPT reactivity

A binary logistic regression was performed to ascertain the effects of age, sex, and *S. haematobium* infection on SPT reactivity. In all cases, the predictor variables (age, sex, and *S. haematobium* infection) had no significant effect on SPT reactivity ($p>0.05$) (see Supplementary **Table A.3, Appendix A**).

4.4.5.2 Serological reactivity to fungi varies with sex, *S. haematobium* infection and SPT reactivity

To determine whether SPT reactivity influenced the antibody profiles of this study population and to identify whether age, sex and *S. haematobium* infection status affected the antibody profiles identified, three factorial ANOVA models were used. These models assessed the influence of age and SPT status on NMDS scores, sex and SPT status on NMDS scores, and *S. haematobium* infection status and SPT status on NMDS scores.

When examining the effects of age followed by SPT status, the results showed that age had no significant effect on NMDS scores for all the fungal species included in the analysis (**Figures 4.15- 4.18(a, d & g) and Supplementary Figures A.2- A.3, Appendix A**).

When examining the effects of sex and SPT status, the results showed that sex had a significant effect on NMDS score for NMDS1 (comprising of IgG) for *E. nigrum* and (IgG, IgG4 and IgM) for *P. chrysogenum,* with males having a significantly higher NMDS score compared to females (ANOVA, F (1, 46) =8.035, p=0.007; **Figure 4.15b** and F (1, 46) =4.398, p=0.042; **Figure 4.16b** respectively).

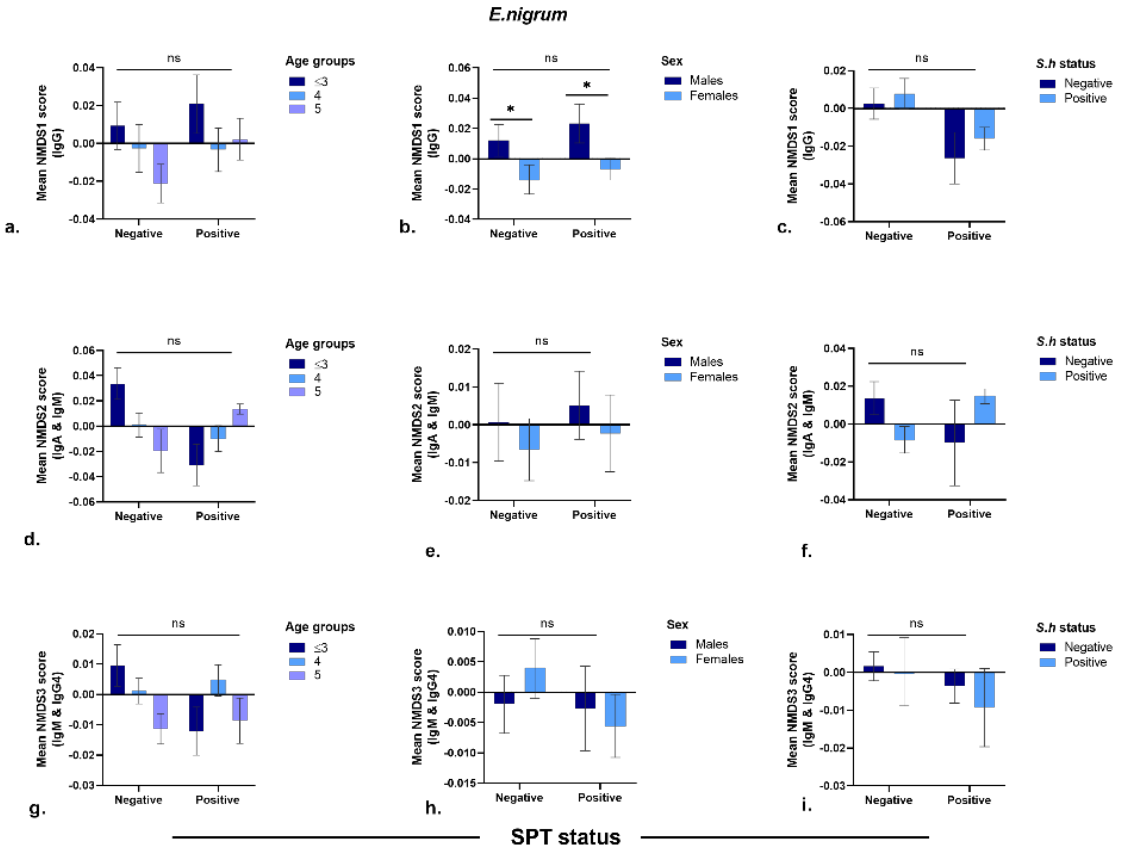

Figure 4.15: The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S. haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses.
Significance between the main effects, age and SPT reactivity, sex and SPT reactivity, as well as *S. haematobium* infection status and SPT reactivity, is indicated on the graph using *, p<0.05 =*, p>0.05=ns. The standard error of mean (SEM) is indicated on the graphs for each group by the vertical black lines. S.h; *S. haematobium*

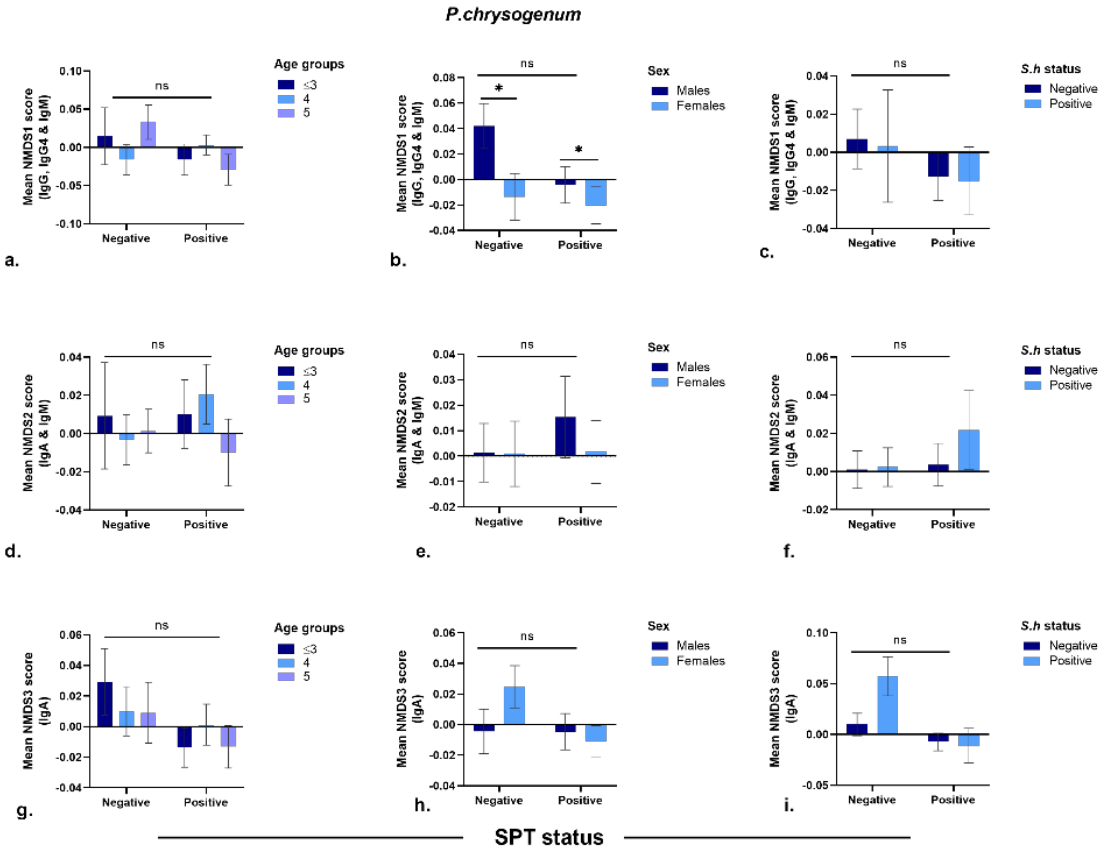

Figure 4.16: The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S. haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses.
Significance between the main effects, age and SPT reactivity, sex and SPT reactivity, as well as *S. haematobium* infection status and SPT reactivity, is indicated on the graph using *, p<0.05 =*, p>0.05=ns. The SEM is indicated on the graphs for each group by the vertical black lines. *S.h; S. haematobium*

For *C. herbarum*, SPT status had a significant effect on NMDS score for NMDS1 (IgG and IgM), with SPT-positive individuals having significantly higher NMDS scores compared to SPT-negative (ANOVA, F (1, 46) =4.727, p=0.035; **Figure 17b**). There was a statistically significant interaction between sex and SPT status on NMDS2 for *C. herbarum* (F (1, 46) = 5,245, p = 0.027). A pairwise comparison was conducted and revealed that there were a statistically significant difference in mean NMDS2 (IgG4) scores between males and females who were SPT positive (F (1, 46) = 4.305, p=0.044; **Figure 17e**).

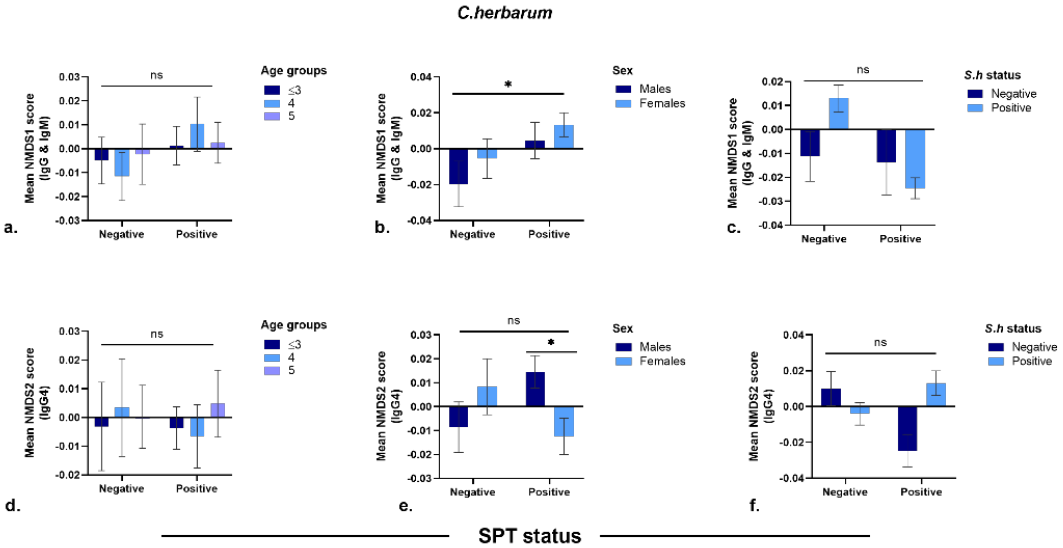

Figure 4.17: The mean NMDS scores for each axes, by age and SPT reactivity (a & d), by sex and SPT reactivity (b & e), and *S. haematobium* infection status and SPT reactivity (c & f) for the NMDS of antibody responses.
Significance between the main effects, age and SPT reactivity, sex and SPT reactivity, as well as *S. haematobium* infection status and SPT reactivity, are indicated on the graph using *, p<0.05 =*, p>0.05=ns. The SEM is indicated on the graphs for each group by the vertical black lines. *S.h; S. haematobium*

When examining the effects of *S. haematobium* infection status and SPT status, the results showed that SPT status had a significant effect on NMDS score for NMDS3 (IgA) for *S. cerevisiae* with SPT-negative individuals having significantly higher NMDS scores compared to SPT-positive (F (1, 46) =4.330, p=0.043; **Figure 4.18i**).

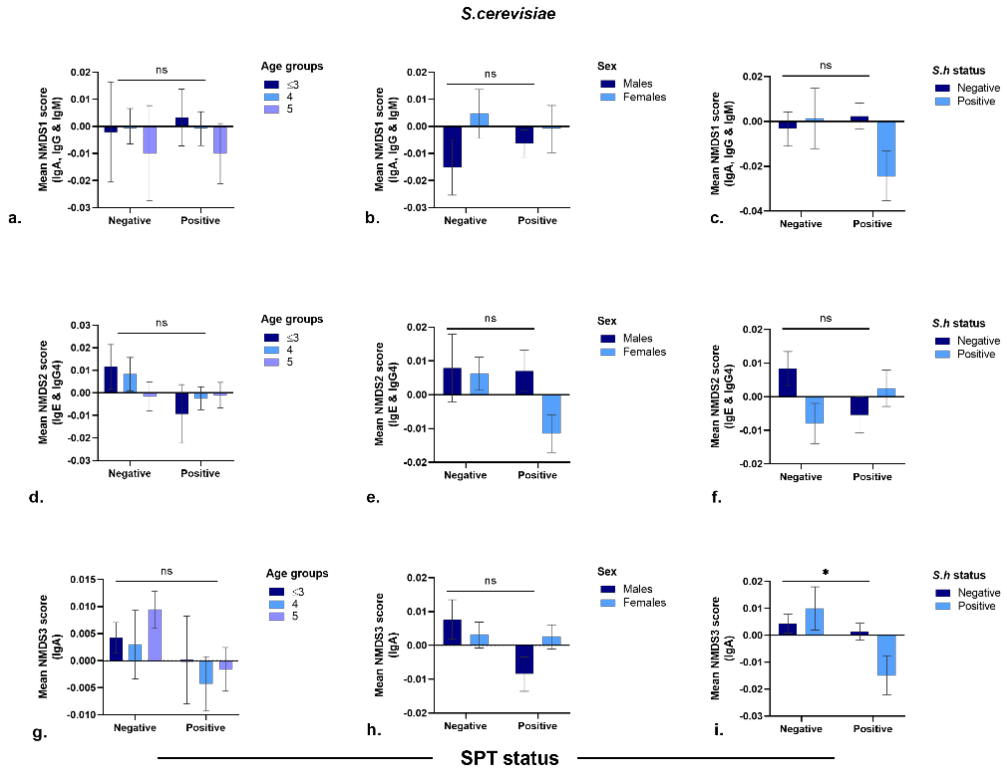

Figure 4.18: The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S. haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses.
Significance between the main effects, age and SPT reactivity, sex and SPT reactivity, as well as *S. haematobium* infection status and SPT reactivity, is indicated on the graph using *, p<0.05 =*, p>0.05=ns. The SEM is indicated on the graphs for each group by the vertical black lines. *S.h; S. haematobium*

4.4.5.3 Association between gut mycobial abundance with SPT reactivity and fungal-specific antibody responses

There was no association between mycobiome abundance and SPT reactivity or fungal-specific antibody responses as determined by PERMANOVA analysis, **Table 4.5** and **4.6,** respectively.

Table 4.5: Summary of SPT reactivity and association with gut mycobiome

Variable	n	p-value	Explained sum of squares	Total sum of squares	FDR
A. alternata	50	0.155	117.2	4323.4	0.458
C. herbarum	50	0.944	52.9	4387.8	0.944
S. cerevisiae	50	0.252	109.6	4331.0	0.458
P. chrysogenum	50	0.270	101.8	4338.9	0.458
R. nigricans	50	0.371	92.1	4348.5	0.458
E. nigrum	50	0.382	91.4	4349.2	0.458

p-value-unadjusted p-value; FDR- adjusted p-value (FDR-corrected)

Table 4.6: Summary of fungal antibody responses and their association with gut mycobiome

Variable		n	p-value	Explained sum of squares	Total sum of squares	FDR
A. alternata	IgA	71	0.507	72.6	5556.5	0.839
	IgG	71	0.956	47.3	5581.7	0.956
	IgM	71	0.881	54.2	5574.8	0.924
	IgG4	71	0.381	80.2	5548.9	0.839
	IgE	71	0.708	62.5	5566.6	0.888
A. fumigatus	IgA	71	0.471	76.5	5552.5	0.839
	IgG	71	0.740	61.6	5567.5	0.888
	IgM	71	0.830	56.6	5572.4	0.924
	IgG4	71	0.495	74.2	5554.9	0.839
	IgE	71	0.582	69.3	5559.7	0.839
C. herbarum	IgA	71	0.403	79.9	5549.1	0.839
	IgG	71	0.562	70.1	5558.9	0.839
	IgM	71	0.306	85.9	5543.2	0.839
	IgG4	71	0.242	94.3	5534.8	0.839
	IgE	71	0.898	50.7	5578.4	0.924
S. cerevisiae	IgA	71	0.545	71.7	5557.4	0.839
	IgG	71	0.739	61.3	5567.7	0.888
	IgM	71	0.192	97.3	5531.8	0.839
	IgG4	71	0.283	88.5	5540.6	0.839
	IgE	71	0.458	75.9	5553.2	0.839
E. nigrum	IgA	71	0.529	72.2	5556.9	0.839
	IgG	71	0.150	108.0	5521.1	0.839
	IgM	71	0.689	64.0	5565.0	0.888
	IgG4	71	0.168	101.2	5527.9	0.839
P. chrysogenum	IgA	71	0.868	54.6	5574.5	0.924
	IgG	71	0.678	64.5	5564.5	0.888
	IgM	71	0.273	90.3	5538.7	0.839
	IgG4	71	0.236	94.1	5535.0	0.839
R. nigricans	IgA	71	0.151	106.8	5522.3	0.839
	IgG	71	0.472	75.9	5553.2	0.839
	IgM	71	0.838	55.6	5573.5	0.924
	IgG4	71	0.537	70.9	5558.1	0.839

p-value-unadjusted p-value; FDR- adjusted p-value (FDR-corrected)

4.4.5.4 Association between fungal abundance and SPT reactivity with IgE response and IgE/ IgG4 ratio to specific fungal species

The association between the abundance of specific fungal genera with fungal SPT reactivity and antigen-specific IgE responses was further assessed by ANOVA and linear regression analysis, respectively.

No significant differences were found between species abundance in SPT-positive and SPT-negative children for *A. alternata* and *C. herbarum* (**Figure 4.19 [1a** and **b])**. In contrast, species abundance of *S. cerevisiae* was significantly higher in SPT-negative children (**Figure 4.19 [1c])**. No significant correlation was found between the species abundance and IgE reactivity or IgE/Ig4 ratios for *A. alternata*, *C. herbarum or S. cerevisiae* (**Figure 4.19 [2a-f]**)

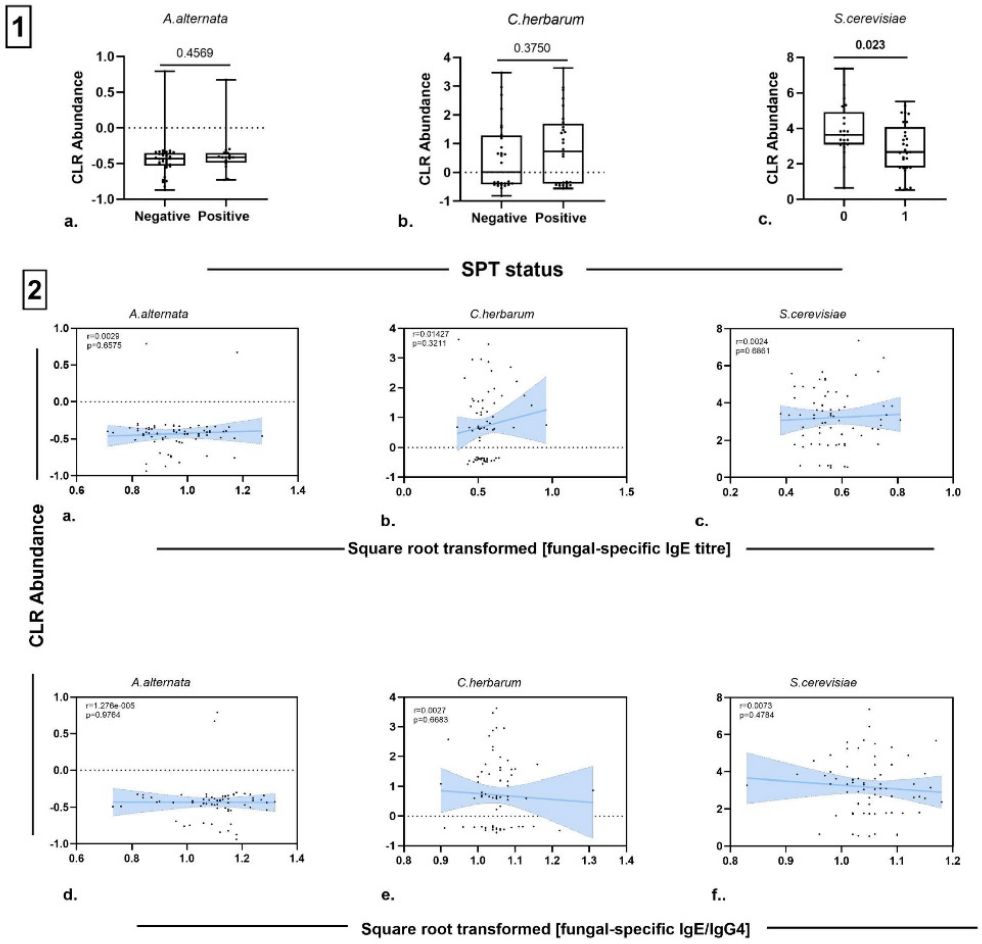

Figure 4.19: Relationship between fungal species abundance and [1] skin prick test reactivity, [2] antigen-specific IgE response and IgE/IgG4 ratios

Box plots show the mean abundance of specific fungal genera grouped by SPT reactivity [1a-c]. The horizontal box lines represent the first quartile, the median and the third quartile. Whiskers denote the range of data within the first quartile −1.5× the interquartile range and the third quartile +1.5× the interquartile range. [2a-c] Scatter plots show linear regression analysis of fungus-specific IgE antibody titres and fungal genera abundance. [2d-e] Scatter plots showing linear regression analysis of fungus-specific IgE/IgG4 ratios and fungal genera abundance Shaded areas indicate the 95% CI. The significant p-value is indicated in bold.

4.5 Discussion

The human gut mycobiome is vital in maintaining overall health [541]. Therefore, changes that occur, especially during the microbiota establishment in early life, have implications on health and disease risk throughout life [542, 543]. Understanding the changes that occur in the mycobiome during allergy sensitisation, particularly in PSAC, will provide insight into the factors underlying allergic disease progression, differences in disease patterns, as well as insight into the potential of influencing health through the mycobiome in young children. In this chapter, the structure (abundance and diversity) of the human gut mycobiome in rural pre-school aged (≤ 5 years old) Zimbabwean children was characterised. The relationship between the mycobiome and fungal sensitisation/ seroreactivity as well as the effects of age, sex*, S. haematobium* infection status and gut mycobiome composition on fungal sensitisation and seroreactivity was further described. The results showed that in this PSAC population, fungal sensitisation is common, and the mycobiome is heterogeneous and is associated with *S. haematobium* infection status.

In the current study, the mycobiome comprised less than 1% of the sequenced gut microbiota, which is comparable to previous studies [544, 545]. The dendrograms showed no distinct clustering, reflecting high inter-individual variability, which is similar to that reported in the Human Microbiome Project (HMP) cohort. Specifically, the HMP reported that the mycobiome has both high inter- and intra-individual variability over time [542].

Previous studies have shown that diet [546], age, environmental patterns [128], geography [509, 547] and sex [548] are determinants of the gut microbial community structure [549, 550]. Specifically, *Candida* has been associated with carbohydrate-rich diets [546], and this genus was among the most abundant fungi genera found in the current study population. The presence of this genus in the mycobiota reflects the dietary

lifestyle among populations in developing countries [128, 133], including infants [551], who subsisted largely on corn porridge in the current study.

There was a significant association between *S. haematobium* infection status and gut mycobial abundance. These findings are consistent with observations that schistosome infection is associated with alterations in the microbiome's diversity and abundance of specific taxonomic groups [552, 553].

However, no significant differences were observed with age, sex and nutritional and growth variables, possibly due to homogeneity across the study population for dietary and environmental exposure, as the children were born in and were permanent residents of the study area. Moreover, they were all ≤ 5 years. Therefore, it is not surprising that the participants had similar age- and sex-specific microbiome profiles because it has been suggested that these differences emerge after puberty [554, 555].

The most prevalent genera were *Protomyces*, *Aspergillus, Saccharomyces,* and *Taphrina*. In the Hoffmann study in the USA, which examined diet as a determinant of the gut mycobial community structure, the most abundant fungal genera recorded were *Saccharomyces*, *Candida* and *Cladosporium* [546]. In contrast *Pichia*, *Candida, Aspergillus* and *Cladosporium* dominated the South African gut mycobiome when geographical location was studied [547]. The current study found a mix of both USA and South African study's top genera, which show that *Aspergillus, Candida, Malassezia, Penicillium*, *Pichia,* and *Saccharomyce*s genera are among the most prevalent fungal genera [556]. The differences observed may be due to factors discussed in **Chapter 1,** such as diet, lifestyle or age [123, 509, 546, 547].

To date, the role of the gut mycobiota in health and disease is still poorly understood. However, studies in mice models have suggested that the gut mycobiome directly or

indirectly helps maintain healthy intestinal homeostasis and that dysbiosis has immunological consequences relevant to disease risk progression [154]. For example, as observed in the current study, increases in specific fungi populations such as *Aspergillus* have been associated with increased eosinophil levels [557] and an exaggerated Th2 response [154], both of which are characteristic of allergic responses. Additionally, the resulting Th2 response is characteristic of schistosome infection and is important in the downregulation of host immune responses and promoting parasite survival in the host [558, 559]. This may explain the increased abundance of specific fungal populations (*Aspergillus, Tricholoma* and *Periglandula*) in schistosome-infected children. However, it remains to be established if these observations were due to primary changes in the fungal population or due to secondary changes in other microbial communities such as bacteria [536]. Further exploration of the mycobiome using clinically defined allergy cases would be informative.

No significant correlation was found between species abundance and IgE reactivity or IgE/Ig4 ratios. Although no significant differences were observed between children with a negative or positive SPT with the abundance of *A. alternata* and *C. herbarum,* there were significant differences for *S. cerevisiae*. *S. cerevisiae* was significantly reduced in SPT-positive children. For SPT-negative children with higher species abundance but who failed to elicit an immune response, there is a possibility of tolerance arising from an active control mechanism or a state of non-responsiveness whereby IgG4 antibodies inhibit IgE receptor-facilitated allergen binding to B cells, thereby diminishing SPT reactivity. This has been demonstrated in immunotherapy studies [560, 561] which have also shown how this could diminish mast cell and basophil activation [562]. Alternatively, some studies have reported an inverse relationship between microbiome abundance and sensitisation/ allergy [563, 564], thus consistent with our findings.

Atopic diseases are common chronic childhood disorders, and immune sensitivity to allergens is recognised as the most important risk factor [565]. From this data, a 96% prevalence of SPT reactivity to at least one of the six selected fungal species was found. Grouping the fungi-sensitised individuals according to the pattern of SPT reactivity showed that the largest subgroup of fungi-sensitised individuals was reactive to multiple fungi species, either due to genuine sensitisation to a variety of fungi or due to cross-reactivity between fungal allergens. The high rate of SPT reactivity found in this study is in contrast to rates reported by other countries, ranging from 3% to 58% [217-222]. These results highlight the variations between populations and thus emphasize the need for studies to characterise sensitisation patterns in different regions of the world [223, 224]. Many studies have suggested that the occurrence and severity of atopic disease symptoms in later childhood are directly related to allergen sensitisation in infancy [566, 567]. Thus, knowledge of the sensitisation patterns may provide a means by which to establish allergic disease management strategies for allergists [568].

Empirical studies have indicated sex and age differences in the prevalence of allergy sensitisation and diseases [569, 570], with males being more atopic in childhood than females [569]. However, this trend changes after puberty, with allergies becoming more apparent in females [571]. In this study, there were no significant effects of sex and age on SPT reactivity; however, the prevalence of SPT reactivity decreased with age. There was no significant effect of *S. haematobium* infection status on SPT reactivity, which might have been due to the small sample size of *S. haematobium*-infected people.

IgE antibodies to *E. nigrum, P. chrysogenum,* and *R. nigricans* were not detected in all participants, including those who were SPT-positive to these fungal species. This was not surprising because a positive SPT without detectable IgE in the ELISA could indicate a non-IgE-mediated response [572], and the presence of high titres of allergen-specific IgG antibodies can interfere with IgE reactivity through competition with IgE for binding to the

solid phase-bound allergens [573]. Seroprevalence of antibodies to fungal allergens was high in the population, and this could have been due to cross-reactivity as some of these fungal allergens are known to cross-react with each other resulting in false positives [242, 574].

Age had no significant effect on NMDS scores for all fungal species investigated. As mentioned above, the absence of an association with age is not surprising since the age range studied is narrow. However, when examining the effects of sex and SPT status, sex significantly affected NMDS score for NMDS1 (comprising of IgG) for *E. nigrum* and (IgG, IgM and IgG4) for *P. chrysogenum,* with males having significantly higher responses compared to females. It has been suggested that the risk of allergic diseases is greater for males in childhood [575], which may explain the observation of significantly higher antibody responses in males. This gender difference seems to be less pronounced after puberty as girls become more likely to be atopic throughout the reproductive years [569]. This has been suggested to be due to differences in sex hormones during the onset of puberty [570, 576]. However, this cannot explain the differences observed in the current study population; therefore, the exact pathophysiologic mechanism of gender differences in atopy remains unclear [577].

SPT status had a significant effect on NMDS score for NMDS1 (IgG and IgM) for *C. herbarum*, with SPT-positive individuals having significantly higher responses compared to SPT-negative. The findings are consistent with the suggestion that IgM followed by IgG responses are produced primarily in response to the first exposure to an allergen and are associated with allergic diseases [540]. Therefore, a higher response in SPT-positive individuals would be expected, as observed with *C. herbarum*.

Several epidemiological studies have shown inverse associations of chronic parasitic worm infections with allergy and atopy in regions with a high prevalence of such diseases

[224, 578-581]. However, other studies on schoolchildren have produced conflicting results [91, 579, 582-584]. In this study, there were no significant effects of *S. haematobium* infection on SPT reactivity, which might have been due to the small sample size of *S.haematobium*-infected people. However, when the effects of *S. haematobium* infection status and SPT status were examined on the NMDS scores, the results showed that SPT status had a significant effect on NMDS scores for NMDS3 (IgA) for *S. cerevisiae* with SPT-negative individuals having significantly higher IgA response compared to SPT-positive individuals. This observation might be due to the protective role of IgA, which is suggested to contribute to the maintenance of mucosal tolerance by dampening immune responses and is thought to prevent the development of hyper-inflammatory responses towards environmental allergens that would otherwise cause allergic inflammation [585].

The strength of the current study lies in the fact that metagenomics sequencing was used to characterise the mycobiome, which enabled the identification of more fungal species which otherwise would not have been identified using the culture method. It also determined the relationship between fungal sensitisation, seroreactivity and the mycobiome independent of host-related factors, including sociodemographic factors and growth and nutritional indices. The current study nevertheless also had a few limitations. Not all study participants with the characterised mycobiome could be included for the subsequent analysis due to loss of follow-up, inadequate sample volumes or participants who withdrew due to aversion to the SPT procedure. The descriptive study design allowed the characterisation of the gut mycobiome and its relationship with SPT and seroreactivity to fungi, as well as the determination of the effects of host factors on seroreactivity and SPT reactivity of PSAC at a single time point only. A longitudinal study would be more useful for evaluating the relationship between the host factors and the development of fungal sensitivity over time and assessing its clinical relevance. Furthermore, the lack of participants with clinical symptoms meant the clinical relevance of the SPT could not be ascertained.

A further study evaluating the mycobiome in healthy and confirmed fungal-allergic individuals would be interesting to see whether variations in the mycobiome will be observed depending on allergy status. This study did not evaluate for possible interactions between other biomes such as bacteria, viruses and fungi. Investigating this would help evaluate which interactions occur between these biomes and the effect they may have on the host [586]. Furthermore, future studies should assess the profile of fungal sensitisation among atopic children in other parts of Zimbabwe as the area studied was homogenous, and thus, generalisations to other parts of the country or low and middle income countries (LMICs) are very limited. Hence, larger epidemiological studies are needed to enhance applicability and confirm the results obtained in this study. These studies will aid in improving awareness of fungal sensitisation, early diagnosis of atopic diseases and implementation of preventative measures in the country.

4.6 Conclusions

In the current study, I characterised the gut mycobiome and related it to fungal sensitisation and seroreactivity among a cohort of PSAC (≤5 years old) from Zimbabwe. The results showed that fungal sensitisation is common and that gut mycobial abundance and diversity are not associated with SPT reactivity or seroreactivity. This study provided the first comprehensive characterisation of the gut mycobiome and fungal allergic sensitisation of rural PSAC in Zimbabwe. The findings in this chapter suggest that fungal sensitisation is high but reported allergic disease is low. Further mechanistic studies with a larger number of well-characterised patients and controls will contribute to more understanding of the role of mycobiome in allergic diseases.

Chapter 5 The identification and characterisation of fungal proteins recognised by sera from Zimbabweans sensitised to fungi

Part of this work has been published in the International Archives of Allergy and Immunology journal [587]. A copy of the publication is included in **Appendix D**.

5.1 Introduction

As discussed in **Chapter 1**, fungi have long been recognised as a significant threat to human health, causing both primary and secondary infections of various tissues and body cavities. They also produce mycotoxins that may contaminate foods, as well as cause allergic disease through the production of spores and filamentous structures [3]. Allergic diseases can result from exposure to different developmental stages of fungi with distinctive morphological features [128] and produce diverse proteins with diverse IgE-binding abilities [588, 589]. Consequently, commercially available fungal extracts are heterogeneous in allergen content and vary between suppliers, batches and strains. This has resulted in inconsistent results in standard skin tests and serologic assays, resulting in diagnostic and therapeutic challenges.

The current standard diagnostic tests for allergy diagnosis are SPTs utilising crude extract as well as the detection of allergen-specific serum IgE antibodies. However, species-specific IgE reactivity is difficult to confirm due to cross-reactivity between crude allergen extracts from different fungi, which poses a significant problem for evaluating IgE tests in clinical practice [242, 574]. Identifying species-specific allergens responsible for confirmed sensitisation is essential for optimising the management of allergic conditions [272].

The characterisation of the allergenic molecules that an individual is sensitised to can help discriminate between the likelihood of local versus systemic reactions and the persistence of clinical symptoms [590]. Likewise, the characterisation of allergenic molecules can resolve genuine versus cross-reactive sensitisation in poly-sensitised patients, thereby improving the understanding of triggering allergens [590].

As the prevalence of fungal allergies increases worldwide [591], so do poor health consequences associated with allergies, thus representing a growing public health concern. However, there is a lack of data on allergies and their pathogenesis due to a lack of immunological and allergy research facilities in most African countries [70]. This is further confounded by the presence of endemic parasitic and infectious diseases in Africa, which can influence allergy outcomes. Although documentation is poor, a wide range of aeroallergens have been observed across Africa [224, 292, 592-597]. Westritschnig and colleagues [598] have suggested that there are differences in sensitisation patterns between African populations and Europeans; hence there is a need for population-relevant studies to improve the diagnosis and management of allergic individuals in Africa.

Fungi present a unique challenge in terms of allergen discovery as the magnitude and diversity of species present creates heterogeneity of allergen structure and cross-reactivity among various species. To date, more than 100 allergens in fungi have been officially registered in the WHO/IUIS Allergen nomenclature subcommittee (http://www.allergen.org). However, most of these were reported from western countries, and the fungal allergen repertoire in an African population is yet to be characterised.

Allergen characterisation is a crucial underlying factor for developing novel hypoallergenic-based immunotherapy to induce tolerance in allergic individuals. Furthermore, no studies have been conducted characterising fungal allergic sensitisation

in an African population. Hence, the present study was conducted to identify allergenic proteins from seven fungal species (*Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans* and *Saccharomyces cerevisiae*) in Zimbabwe.

5.2 Study aims

1. To detect specific IgE/ IgG reactive proteins in the seven fungal species (*Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans and Saccharomyces cerevisiae*)
2. Identify all IgE/ IgG reactive proteins in the seven fungal species
3. Analyse all identified proteins for their potential allergenicity

5.3 Method

5.3.1 Study Population

This study was a descriptive study conducted in Shamva (see **Chapter 2**). This area was selected for the current study because the prevalence of fungal sensitisation was high, as determined by our previous study [502] (**Chapter 4**). To determine the prevalence of fungal sensitisation in the previous study (**Chapter 4**), 50 PSAC (aged 3-5 years) were assessed. Of these 50 PSAC, only 8 were included in this study because they had (i) ELISA results showing IgE-specific binding to different fungal species and (ii) were sensitive to fungi (i.e. IgE positive as determined by SPT). This included 3 males and 5 females.

5.3.2 SDS –PAGE Analysis of Protein

Pre-cast SDS-PAGE NuPAGE™ Bis-Tris electrophoresis system was used according to the manufacturer's instructions. The fungal extracts were diluted 4X in NuPAGE™ LDS Sample Buffer and heated at 80°C for 5 minutes. Running buffer was made by diluting 50

mL 20X NuPAGE™ MES SDS Running Buffer in 950mL of dH_2O. Using a Pasteur pipette, diluted running buffer was used to sufficiently wash a NuPAGE™ Bis-Tris 1.0mm thick gel. Gels with a polyacrylamide percentage of 12% pre-cast were used. Gels were placed in the inner chamber of an XCell SureLock™ Mini-Cell, and ~400 mL of running buffer was used to fill the inner chamber. The sample was loaded using 20 µL alongside 10 µL of the SeeBlue® Plus2 Pre-stained Protein Standard (4-250kDa) (Life technologies). Electrophoresis was run at 190 volts constant for 30 minutes. Once the run was finished, the power supply was stopped, and the gel was removed from the chamber and disassembled from its plastic cassette.

The gel was either stained or used for western blot analysis.

Protein staining (Coomassie blue)

Coomassie dye was used for the in-gel detection. Prior to staining, the gel was washed to remove residual SDS, which would interfere with dye binding. Then, the staining reagent was added, and then water used to wash away excess unbound dye from the gel matrix.

5.3.3 IgE and IgG-specific western blot analysis

Specific IgE and IgG immunoblotting was performed to detect allergenic proteins in the crude fungal extracts.

Semi-dry transfer method was used. The transfer buffer was made with 6.05g of 25mM Tris, 28.8g of 192mM glycine and 400 mL of methanol (20% v/v) diluted in 2 litres of dH_2O. After removing the gel from its glass slides, it was equilibrated in a 10 mL transfer buffer. A 2 µm Whatman nitrocellulose membrane and 2 pieces of blotting paper were cut to the gel's size and equilibrated in transfer buffer for 5 minutes. In a Trans-Blot® SD Semi-Dry Transfer Cell, a gel-membrane sandwich (as shown in **Figure 5.1**) was set up. The

transfer was run at 30V for 1 hour. To confirm the transfer of the proteins onto the membrane, it was stained for 10 minutes with enough 1% Ponceau Stain to cover the gel.

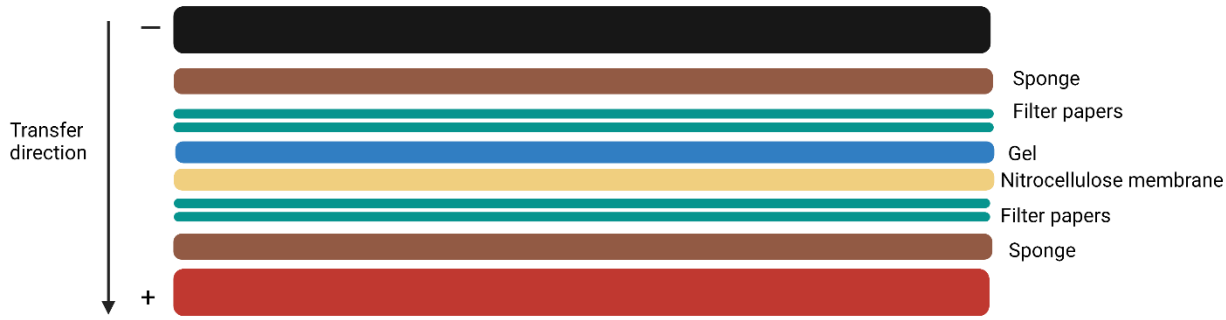

Figure 5.1: Western blot sandwich
During the transfer process, the negatively charged proteins will migrate out of the gel, towards the positively charged electrode, and onto the membrane. Created with BioRender.com

Membrane blocking

Proteins transferred to nitrocellulose membranes were then blocked at room temperature for 1 hour in 3% BSA-Tris-buffered saline (TBS) blocking buffer and 0.05% Tween 20. After blocking, the membrane was subjected to 2 separate 10-minute washes with TBS, 0.05% Tween, and 0.5% Triton-X 100 (TBS/TT).

Antibody incubations

A pool of serum samples (diluted 1:100 in blocking buffer) was added to the membrane, and the membrane was incubated overnight at 4 °C and then washed 3 times the next day for 10 minutes each time in TBS/TT. Secondary antibody (Horseradish peroxidase-conjugated rabbit antihuman IgE and IgG were diluted 1:1000 and 1:4000 respectively in TBS blocking buffer) was added to the membrane. The membrane was incubated at room temperature for 1 hour and then washed 4 times for 10 minutes in TBS/TT and 1 time for 10 minutes in TBS alone.

Detection

An enhanced chemiluminescent (ECL) HRP substrate (Amersham) was used to achieve a more sensitive detection method of serum-specific IgE and IgG binding proteins. The membrane was imaged using a ChemiDoc™ Imaging System (Bio-Rad Image Lab Software), which captured luminescence emitted from the substrate binding to the conjugate antibody. Proteins binding to serum specific-IgE/ IgG, as detected on the membrane by ECL, were matched to proteins detected on Coomassie-stained polyacrylamide gels. These specific IgE/ IgG-binding regions were selected for protein identification by mass spectrometry (MS).

5.3.4 Sample Preparation for mass spectrometry (MS)

Protein identification via MS was carried out using the "bottom-up" approach whereby the proteins in the fungal extract were separated prior to enzymatic digestion, followed by further peptide separation online coupled to tandem mass spectrometry.

In-gel digest of Coomassie-stained proteins

Protein bands from the Coomassie-stained 1-D gel corresponding to 1-D immunoblot were excised. The excised gel bands were separately placed in 1.5 mL centrifuge tubes and washed in 500 µL of 100mM ammonium bicarbonate for 30 minutes to 1 hour on the shaker. The wash was discarded, and the gel bands were further washed in 500 µL of 50% acetonitrile/100mM ammonium bicarbonate on the shaker for 30 minutes to 1 hour. To reduce the proteins, 150 µL of 100mM ammonium bicarbonate and 10 µL of 45mM Dithiothreitol (DTT) were added, and the mixture was incubated at 60 °C for 30 minutes on the heating block. To alkylate the proteins, the mixture was cooled to room temperature (10 minutes), and 10 µL of 100mM of iodoacetamide was added. The mixture was incubated in the dark for 30 minutes, and the solvent was discarded. The gel pieces were washed in 500 µL of 50% acetonitrile/100mM ammonium bicarbonate for 30 minutes to 1 hour on the shaker, and 50 µL of acetonitrile was added to shrink gel pieces. After 10

minutes, the solvent was removed, and the gel pieces were dried completely in a vacuum centrifuge. A vial of trypsin was re-suspended in 1 mL of 25mM ammonium bicarbonate, and a sufficient amount of trypsin to rehydrate the gel pieces (around 20 µL for an average band) was added. If trypsin was completely absorbed, an additional trypsin solution (10 µL at a time) was added until the gel band appeared fully rehydrated and had swollen to the previous size. The protein was digested overnight at 37 °C.

The tubes were briefly centrifuged to pellet the gel pieces, and all liquid was transferred to a fresh 96 well plate, taking care to avoid transferring any gel pieces. The plate was labelled, and a table listing plate position and sample name was made. To the gel pieces in the Eppendorfs, 20 µL of 5% formic acid was added, and the Eppendorfs were incubated for 20 minutes on the shaker. 40 uL of acetonitrile was added into the Formic acid and incubated with shaking for a further 20 minutes. The tubes were again briefly centrifuged to pellet gel pieces, and all liquid was transferred to the same 96-well plate used for the first extract (pooling the liquid for each sample), taking care to avoid transferring any gel pieces. The samples were dried in the vacuum centrifuge and stored in the freezer, awaiting MS analysis.

5.3.5 Nanoflow HPLC Electrospray Tandem Mass Spectrometry (nLC-ESI-MS/MS)

Dry peptide residues were solubilized in 20 µL 5 % acetonitrile with 0.5 % formic acid using the auto-sampler of a nanoflow HPLC system (Thermo Scientific RSLCnano). Online detection of peptide ions was by electrospray ionisation (ESI) mass spectrometry MS/MS with an Orbitrap Elite MS (Thermo Scientific). The ionisation of LC eluent was performed by interfacing the stainless steel nanopore needle with an electrospray voltage of 2.0 kV. An injection volume of 5 µL of the reconstituted protein digest was desalted and concentrated for 10 minutes on a trap column (0.3 × 5 mm) using a flow rate of 25 µL / minutes with 1 % acetonitrile with 0.1 % formic acid. Peptide separation was performed on a Pepmap C18 reversed-phase column (50 cm × 75 µm, particle size 3 µm, pore size

100 Å, Thermo Scientific) using a solvent gradient at a fixed solvent flow rate of 0.3 µL / minute for the analytical column. The solvent composition was (i) 0.1 % formic acid in water and (ii) 0.08 % formic acid in 80% acetonitrile in 20% water. The solvent gradient was 4 % B for 10 minutes, 4 to 60 % for 42 minutes, 60 to 99 % for 3 minutes, and held at 99 % for 5 minutes. A further 8 minutes at initial conditions for column re-equilibration was used before the next injection (see **Supplementary Figure B.1 in Appendix B**).

The Orbitrap Elite acquired full-scan MS in the range of 300 to 2000 m/z for a high-resolution precursor scan at 60,000 RP (at 400 m/z) while simultaneously acquiring up to the top 15 precursors isolated at 0.7 m/z width and subjected to collision-induced dissociation (CID) fragmentation (35 % normalised collision energy (NCE)) in the linear ion trap using rapid scan mode. Singly charged ions are excluded from selection, while selected precursors are added to a dynamic exclusion list for 30 seconds.

5.3.6 Database search and protein identification

For protein identification, the MS/MS data was uploaded on the Mascot search engine (v2.6.2, Matrix Science) and compared against protein sequences in the NCBIprot database using taxonomies *A. alternata, A. fumigatus, Cladosporiaceae, E. nigrum, P. chrysogenum, R. stolonifera (nigricans)* and *S. cerevisiae*. Searches were performed using the following parameters: (i) trypsin as the proteolytic enzyme, allowing for one missed cleavage; (ii) for fixed and variable modifications, carbamidomethylation of cysteine and methionine oxidation were used, respectively. The precursor mass tolerance was set at 10 ppm and 0.3 Da for MS/MS matching. Mascot software compares the observed spectra to a database of known proteins and determines the most likely matches based on protein scores [60]. For each protein match, Mascot calculates an overall protein score which reflects the combined scores of all observed mass spectra that can be matched to amino acid sequences within that protein. A higher score indicates a more confident match, and Mascot calculates a threshold for identity or extensive

homology (P< 0.05) based on ions score [60]. Hence in this study, only proteins identified with a Mascot score greater than 200 (significant at 95% confidence interval) are reported. **Figure 5.2** shows the workflow for the identification of the reactive proteins.

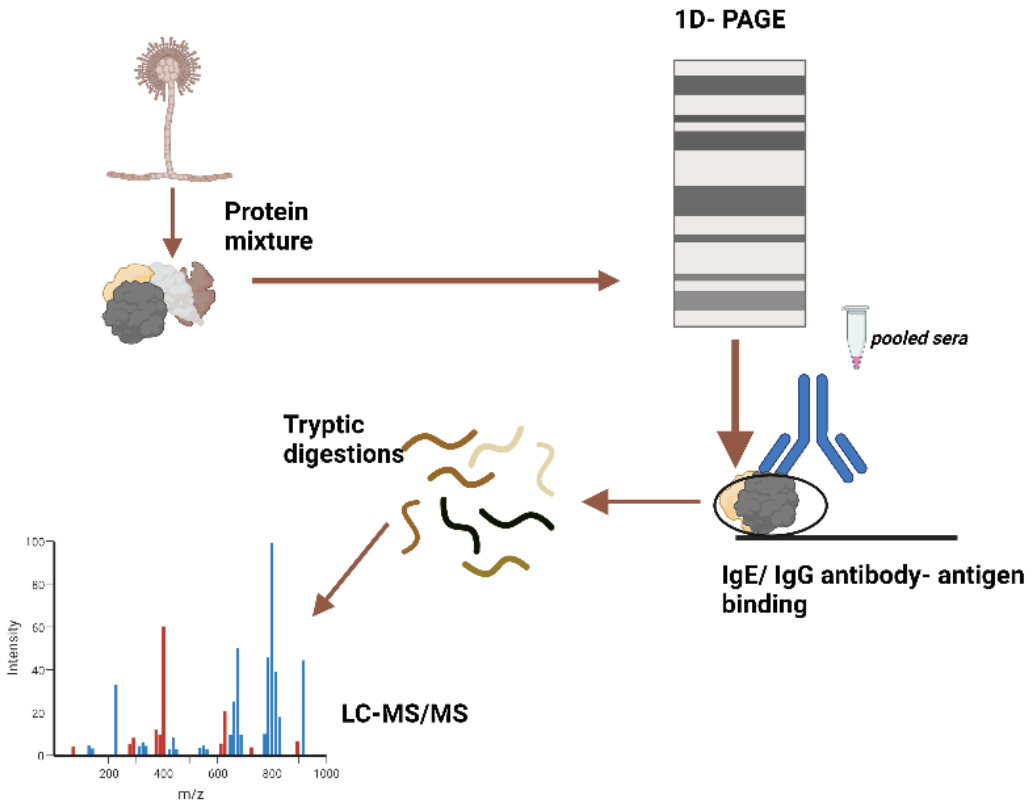

Figure 5.2: Workflow for identifying IgE/ IgG-reactive proteins from the fungal extracts.
IgE/ IgG-reactive proteins were identified by 1D-immunoblotting. Figure adapted from De Angelis *et al*., [599, 600]. Created with BioRender.com

Characteristics of the identified proteins

The identified proteins were characterised according to their structure, function, protein family and amino acid identity to the human analogue. These criteria were selected based on literature reports on the potential characteristics of allergenic proteins [601-604]. The protein structure was classified using the Structural Classification Proteins (SCOP) database (https://scop.mrc-lmb.cam.ac.uk/) [605, 606], and the proteins were classified according to their function using the links to Gene Ontology (GO) Annotation

(https://www.ebi.ac.uk/QuickGO/annotations) [607] embedded in the Universal Protein Resource [UniProt] database.

Allergen identification and prediction

To identify the likely allergen proteins present in the fungal species, the recognised proteins were searched through the structural database of allergenic proteins (SDAP) (http://fermi.utmb.edu/SDAP/index.html), a web server that provides rapid, cross-referenced access to the sequences, structures and IgE epitopes of allergenic proteins. The proteins were predicted under the following conditions: i) sequence similarity >35% between obtained proteins and reported allergen proteins and ii) a minimum of 80 amino acid overlap length [608, 609]. By these criteria, some of the fungal proteins identified by the sera were classified as likely corresponding to an allergenic protein.

Furthermore, the potential antigenic peptides of the predicted allergens were obtained using an online software (http://imed.med.ucm.es/Tools/antigenic.pl) based on the algorithm of Kolaskar and Tongaonkar [610], where the predictions are based on a table that reflects the occurrence of amino acid residues in experimentally known segmental epitopes. Segments are only reported if they are at least eight residues. This was done to identify the epitopes capable of stimulating an immune response.

5.4 Results

5.4.1 One-dimensional gel electrophoresis analysis

One-dimensional gel electrophoresis of crude extracts from fungal cells resulted in the separation of the fungal preparation into 28 protein bands that were visible after staining (shown in **Figure 5.3**). Of these, 20 bands reacted with the human serum samples collected from fungal-sensitised individuals as determined by anti-human IgE/IgG immunoblotting (shown in **Figure 5.4** and **Table 5.1**).

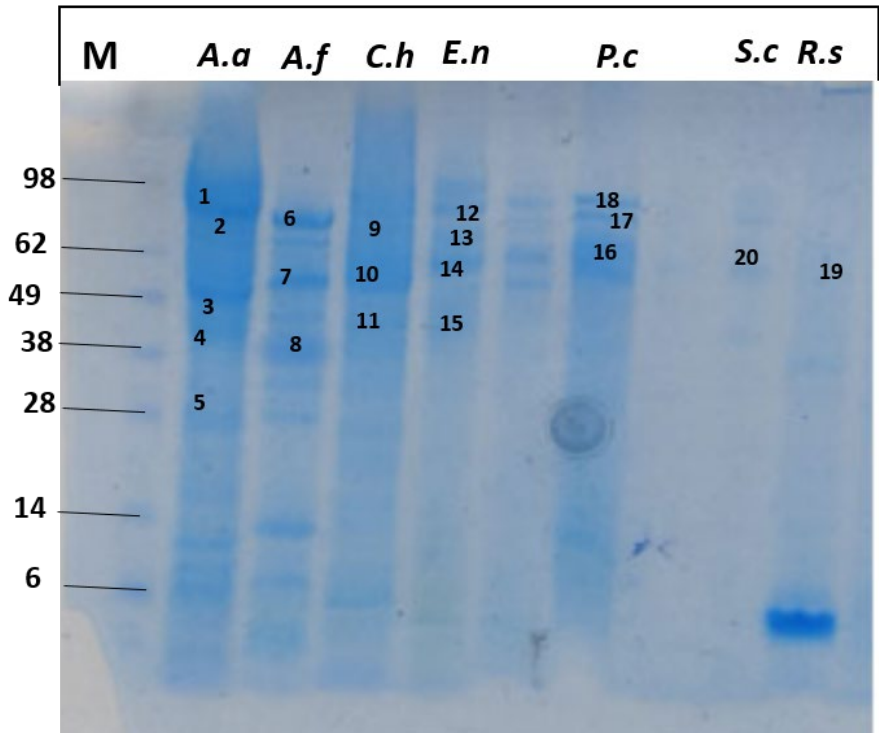

Figure 5.3: Coomassie blue-stained one dimensional gel showing bands matched to the Western blots.

Bands on the gel were excised and identified. Molecular weight markers (in kilodaltons) are given on the left. M, Marker; *A.a, Alternaria alternata; A.f, Aspergillus fumigatus; C.h, Cladosporium herbarum; En, Epicoccum nigrum; P.c, Penicillium chrysogenum; R.s, Rhizopus stolonifer (nigricans) and S.c, Saccharomyces cerevisiae*

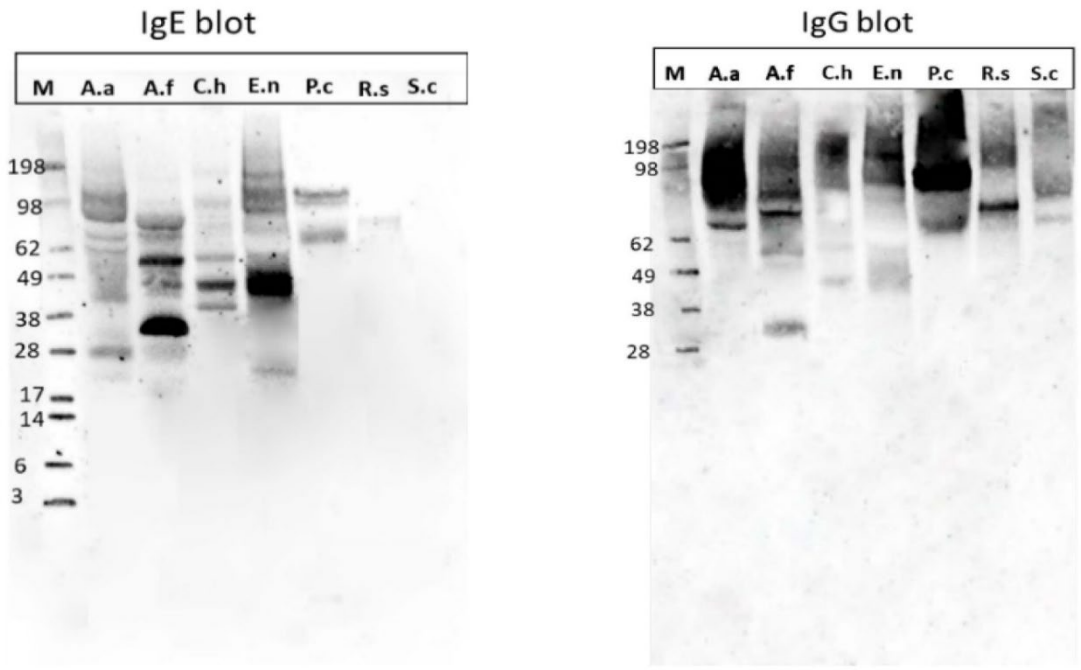

Figure 5.4: Western blot analyses of serological reactivity of serum samples
M, Marker; *A.a, Alternaria alternata; A.f, Aspergillus fumigatus; C.h, Cladosporium herbarum; En, Epicoccum nigrum; P.c, Penicillium chrysogenum; R.s, Rhizopus stolonifer (nigricans)* and *S.c, Saccharomyces cerevisiae*

5.4.2 Identification of immunogenic proteins/ or seroreactive proteins

The 20 sero-reactive protein bands were excised from the Coomassie blue-stained gel and were subjected to in-gel trypsin digestion. The bands were analysed for peptide composition by MS/MS and the peptide data obtained was used to search the NCBIprot databases to identify the parent protein. The majority of the proteins identified had a high mascot score, indicating that there is a high probability that the proteins had been correctly identified. The identity given for each band corresponds to the top hit score (the Mascot output statistic) which was >200 (significant at a 95% confidence interval).

Overall a total of 34 proteins were identified from the 20 bands from the seven fungal species. The majority of the bands corresponded to *A. alternata*. The predicted molecular weight (MW) as well as the associated fungal species are also provided in **Table 5.1**. The 34 proteins were characterised according to their molecular function, structure, sequence homology to human proteins, and protein family (**Table 5.2**). Of these 34 proteins, three

were hypothetical proteins, and eleven had no known function. The remaining 20 proteins included enzymes, most of which had not been previously shown to be immunogenic.

Table 5.1: ***Fungal proteins identified by mass spectrometry***

Band[a]	Protein name	Species	Accession number[b]	Hit score[c]	MW[d]	Reactivity	
						IgE	IgG
1	Glycoside hydrolase	*Alternaria alternata*	XP_018381330.1	2128	87237	Yes	No
1	Trehalose	*Alternaria alternata*	XP_018389336.1	2326	77106	Yes	No
1	Peptidase s41 family protein	*Alternaria alternata*	OWY41590.1	4458	83904	Yes	No
2	M6 metalloprotease	*Alternaria alternata*	OWY56860.1	2911	74095	Yes	No
2	FAS1 domain-containing protein	*Alternaria alternata*	XP_018380929.1	2217	50925	Yes	No
2	Cyclohexanone 1,2-monooxygenase	*Alternaria alternata*	OWY42352.1	1663	128346	Yes	No
2	Meiotically up-regulated 157 protein	*Alternaria gaisen*	KAB2110334.1	1290	57040	Yes	No
3	Vanadium chloroperoxidase	*Alternaria tenuissima*	RYN52497.1	7156	67460	Yes	Yes
3	Meiotically up-regulated 157 protein	*Alternaria gaisen*	KAB2110334.1	5868	57040	Yes	Yes
3	FAS1 domain-containing protein	*Alternaria alternata*	XP_018380929.1	897	50925	Yes	Yes
4	Subtilisin-like serine protease-like protein PR1A	*Alternaria alternata*	XP_018384475.1	2893	40384	Yes	No
4	Concanavalin A-like lectin/glucanase	*Alternaria alternata*	XP_018390955.1	1408	46193	Yes	No
5	GroES-like protein	*Alternaria alternata*	XP_018385554.1	1675	38120	Yes	No
5	Glycoside hydrolase	*Alternaria alternata*	XP_018382523.1	1426	44564	Yes	No
6	Dipeptidyl-peptidase 5	*Aspergillus lentulus*	GFF50131.1	5526	79688	Yes	Yes
6	Secreted dipeptidyl peptidase	*Aspergillus fischeri NRRL 181*	XP_001260402.1	5477	79675	Yes	Yes
7	Secreted dipeptidyl peptidase	*Aspergillus fischeri NRRL 181*	XP_001260402.1	2304	79675	Yes	Yes
7	Catalase B	*Aspergillus minisclerotigenes*	KAB8269428.1	1472	79856	Yes	Yes
7	Dipeptidyl-peptidase 5	*Aspergillus lentulus*	GFF50131.1	1366	79688	Yes	Yes
8	Chitinase	*Aspergillus fumigatus*	AAP23218.1	6271	47708	Yes	Yes
9	GPI anchored cell wall beta 1,3 endoglucanase EglC	*Aspergillus fumigatus var. RP-2014*	KEY82708.1	2198	44923	Yes	No

10	Hypothetical protein CNMCM8714_006228	*Aspergillus fumigatus*	KAF4253478.1	1319	105137	Yes	No
11	Hypothetical protein CDV57_00056	*Aspergillus fumigatus*	OXN30505.1	1063	16162	Yes	No
12	Catalase	*Aspergillus clavatus NRRL 1*	XP_001273665.1	521	80097	Yes	No
13	Alpha-glucosidase	*Rachicladosporium antarcticum*	OQO11764.1	625	67385	Yes	Yes
14	Hypothetical protein B5807_10540	*Epicoccum nigrum*	OSS44738.1	7238	112128	Yes	No
15	S-adenosyl-L-homocysteine hydrolase	*Aspergillus homomorphus CBS 101889*	XP_025550664.1	3028	49516	Yes	No
16	Hypothetical protein B5807_10540	*Epicoccum nigrum*	OSS44738.1	1485	112128	Yes	Yes
17	Glycoside hydrolase family 31	*Aspergillus oryzae*	OOO09042.1	2609	106688	Yes	Yes
17	Putative dipeptidyl-peptidase	*Penicillium chrysogenum*	KZN92610.1	4136	85215	Yes	Yes
18	Putative dipeptidyl-peptidase	*Penicillium chrysogenum*	KZN92610.1	15819	85215	Yes	No
18	Glucose oxidase	*Penicillium chrysogenum*	AFA42947.1	669	66471	Yes	No
19	RecName: Full=Alpha-(1-6)-linked fucose-specific lectin; AltName: Full=RSL	*Rhizopus stolonifer*	P83973.1	7343	3199	No	Yes
20	Glr1p	*Saccharomyces cerevisiae YJM320*	AJV98761.1	1540	53790	No	Yes

a.Band numbers indicated in Figure 5.2, b. Accession numbers according to NCBInr database, c. Mascot score reported after database search; score>200 indicates identity or extensive homology at p<0.05, d. Theoretical mass retrieved from NCBInr database.

5.4.3 Characterisation of the identified proteins

When the 34 proteins were classified according to protein families, it was observed that only six belonged to the known allergen protein families with 5 reported as allergens and registered with IUIS or allergome databases (see **Table 5.2**). **Table 5.2** below summarises the identified proteins, their amino acid sequence homology to the human analogue, molecular structure, function and if the protein was previously reported as allergenic.

Table 5.2: Characterisation of all the identified proteins

Protein name	Sequence Identity to humans	Protein family	Molecular function	Structure (SCOP)	Reported allergen Fungal/ other
Glycoside hydrolase	*2-13*	*Glycoside hydrolase family 3*	Hydrolase activity Xylan 1,4-beta-xylosidase activity	α and β protein	no
Trehalose	*3-29*	*Trehalase*	Alpha, alpha trehalase activity Hydrolase activity	α protein	no
Peptidase s41 family protein	*~12*	*Peptidase S41*	Serine-type peptidase activity	α and β protein	no
M6 metalloprotease	*~12*	unknown	Metallopeptidase activity Peptidase activity	Unknown	no
FAS1 domain-containing protein	*~3*	*Fasciclin (FAS1 domain)*	Unknown	β protein	no
Cyclohexanone 1,2-monooxygenase	*No human analogue*	*Pyridine nucleotide-disulphide oxidoreductase*	Oxidoreductase activity Monooxygenase activity	α and β protein	No
Meiotically up-regulated 157 protein	*No human analogue*	*Metal-independent alpha-mannosidase (GH125)*	Unknown	α protein	No
Vanadium chloroperoxidase	*No human analogue*	*Phosphatidylglycerophosphatase (PAP2)*	Peroxidase activity	α protein	No
Meiotically up-regulated 157 protein	*No human analogue*	*Metal-independent alpha-mannosidase (GH125)*	Unknown	α protein	No

FAS1 domain-containing protein	*~3*	*Fasciclin*	Unknown	β protein	No
Subtilisin-like serine protease-like protein PR1A	*No human analogue*	*Subtilases*	Serine-type endopeptidase activity Peptidase activity Hydrolase activity	α and β protein	Yes
Concanavalin A-like lectin/glucanase	*No human analogue*	*Glycoside hydrolase family 16*	Hydrolase activity Carbohydrate binding	β protein	No
GroES-like protein	*No human analogue*	*Zn-containing dehydrogenase*	Oxidoreductase activity Metal ion binding	β protein	No
Glycoside hydrolase	*No human analogue*	*cellulase*	Hydrolase activity	α and β protein	No
Dipeptidyl-peptidase 5	*2-57*	*Prolyl oligopeptidase family*	Serine-type peptidase activity Peptidase activity	α and β protein	No
Secreted dipeptidyl peptidase	*4-15*	*Prolyl oligopeptidase family*	Serine-type peptidase activity Peptidase activity Hydrolase activity	α and β protein	No
Secreted dipeptidyl peptidase	*4-15*	*Prolyl oligopeptidase family*	Serine-type peptidase activity Peptidase activity Hydrolase activity	α and β protein	No
Catalase B	*4-26*	*Catalase*	Catalase activity Heme binding Oxidoreductase activity Peroxidase activity	α and β protein	Yes
Dipeptidyl-peptidase 5	*2-57*	*Prolyl oligopeptidase family*	Serine-type peptidase activity Peptidase activity	α and β protein	Yes
Chitinase	*17-24*	*Glycosyl hydrolases family 18*	Chitinase activity Hydrolase activity Chitin binding	α and β protein	Yes
GPI anchored cell wall beta 1,3 endoglucanase EglC	Unknown	Unknown	Unknown	α and β protein	No
Hypothetical protein CNMCM8714_006228	Unknown	Unknown	Unknown	Unknown	No
Hypothetical protein CDV57_00056	Unknown	Unknown	Unknown	Unknown	No
Catalase	*9-26*	*Catalase*	Catalase activity Heme binding Oxidoreductase activity Peroxidase activity Metal ion binding	α and β protein	Yes

Alpha-glucosidase	*3-19*	*Glycosyl hydrolase 31 family*	Hydrolase activity	α and β protein	No
Hypothetical protein B5807_10540	*Unknown*	*Glycosyl hydrolase 31 family*	Beta-glucosidase activity Hydrolase activity Alpha-1,4-glucosidase activity Alpha-glucosidase activity	α and β protein	No
S-adenosyl-L-homocysteine hydrolase	*No human analogue*	*Adenosylhomocysteinase*	Adenosylhomocysteinase activity Hydrolase activity	α and β protein	No
Hypothetical protein B5807_10540	*Unknown*	*Glycosyl hydrolase 31 family*	Beta-glucosidase activity Hydrolase activity Alpha-1,4-glucosidase activity Alpha-glucosidase activity	α and β protein	No
Glycoside hydrolase family 31	*10-26*	*Glycosyl hydrolase 31 family*	Beta glucosidase activity maltose alpha-glucosidase activity Hydrolase activity Alpha-glucosidase activity	α and β protein	No
Putative dipeptidyl-peptidase	*No human analogue*	*Prolyl oligopeptidase family*	Serine-type peptidase activity	α and β protein	No
Putative dipeptidyl-peptidase	*No human analogue*	*Prolyl oligopeptidase family*	Serine-type peptidase activity	α and β protein	No
Glucose oxidase	*No human analogue*	*Glucose-methanol-choline (GMC) oxidoreductase*	Glucose oxidase activity Oxidoreductase activity	α and β protein	No
RecName: Full=Alpha-(1-6)-linked fucose-specific lectin; AltName: Full=RSL	Unknown	Unknown	Unknown	Unknown	No
Glr1p	Unknown	Unknown	Unknown	Unknown	No

5.4.4 Prediction of allergenic fungal proteins

Fungal species are known to contain several proteins that act as allergens [611]. Using structural and sequence predictive tools, eight fungal proteins were predicted to be allergens (**Table 5.3)**. Four of these corresponded with serine proteases from various fungal species, sharing 39.9%- 58% sequence homology to known fungal allergenic proteases.

The predicted antigenic peptides are shown in **Appendix B**, **Supplementary Figures B.2-B.5.** The remaining proteins were not identified as allergens using these tools.

Table 5.3: Predicted allergen-related proteins in the fungal species investigated

Predicted allergens				Corresponding known allergens				
Band[a]	**Accession no[b]**	**Description**	**AA[c]**	**Allergen[d]**	**Accession no[b]**	**AA[c]**	**Bit score[d]**	**E score[e]**
1	XP_018381330.1	Glycoside hydrolase (*Alternaria alternata*)	798	Asp n 14	CAB06417	804	500.0	2.8e-142
				Asp n 14	AAD13106	804	498.7	6.5e-142
4	XP_018384475.1	Subtilisin-like serine protease-like protein PR1A (*Alternaria alternata*)		Asp f 13	P28296	403	212.5	2.2e-56
				Asp v 13.0101	ADE74975	403	203.2	1.4e-53
				Pen c 13.0101	AAD25926	397	203.0	1.6e-53
				Pen ch 13	AAF23726	397	200.2	1.1e-52
				Tri r 2.0101	AAD52013	412	197.1	1.0e-51
				Asp fl protease	AAD47202	403	196.8	1.1e-51
				Asp o 13	CAA35594	403	196.8	1.1e-51
				Pen ch 18	AAF71379	494	186.1	2.4e-48
				Cur l 4.0101	ACF19589	506	113.2	2.2e-26
				Asp f 18.0101	Y13338	495	108.9	4.2e-25
				Pen o 18	AAG44478	503	107.2	1.4e-24
5	XP_018385554.1	GroES-like protein (*Alternaria alternata*)	352	Cand a 1	AAA53300	350	307.0	6.3e-85
				Cand a 1	P43067	350	304.3	4.0e-84
6	GFF50131.1	Dipeptidyl-peptidase 5 (*Aspergillus lentulus*)	721	Tri r 4.0101	AAD52012	726	666.6	1.5e-192
7	XP_001260402.1	Secreted dipeptidyl peptidase (*Aspergillus fischeri NRRL 181*)		Tri r 4.0101	AAD52012	726	677.2	9.5e-196
7	KAB8269428.1	Catalase B (*Aspergillus minisclerotigenes*)		Pen c 30.0101	ABB89950	733	930.0	0.0e+00
12	XP_001273665.1	Catalase (*Aspergillus clavatus NRRL 1*)	728	Asp f 15	O60022	152	224.0	1.4e-60
18	KZN92610.1	Putative dipeptidyl-peptidase (*Penicillium chrysogenum*)	772	Tri r 4.0101	AAD52012	726	530.8	1.2e-151

*a. Band numbers indicated in **Figure 5.2**, b. Accession numbers according to NCBInr database, c. Amino acid sequence, d. Bitscore- sequence similarity e. E score- homology*

5.5 Discussion

This chapter presents a comprehensive identification of the unreported allergens from fungi using immune-biochemical and proteomic approaches, which included fungal proteome profiling by SDS-PAGE, IgE /IgG antibody-specific reactive protein detection by immunoblots and finally identification of immunoreactive proteins by mass spectrometry (MS). In addition, additional potential 'novel' fungal allergens were identified.

Among the identified proteins, some were known allergens from other fungal, plant and insect species e.g., catalase, chitinase, subtilisin-like serine protease, beta-glucosidase and dipeptidyl-peptidase 5 [612-614]. However, novel IgE-binding proteins, i.e., possibly allergens were also identified. For example, M6 metalloprotease and cyclohexanone 1,2-monooxygenase from *A. alternata* as well as Exo-beta-1,3-glucanase and GPI anchored cell wall beta 1,3 endoglucanase from *A. fumigatus.*

The theoretical molecular weight values of some of the identified proteins were different from that of the observed values in the gel. These discrepancies may have been due to post-transcriptional modification such as phosphorylation and deamination or due to the structural subunits required for appropriate functioning [615]. The 34 proteins identified were characterised using the structure and the protein families according to Jenkins *et al.,* [601, 602] and Raduer *et al*., [616]. Only six identified proteins belonged to 17 main protein families described as including more allergens than all other protein families [616]. When the proteins were analysed for their function using the GO Annotation database, the majority of the proteins had hydrolase activity (i.e catalytic activity) as has been reported in most allergenic proteins. Additionally, using the allergen predicting software (SDAP), eight of the 34 sero-reactive proteins were classified as putative allergens.

The pooled sera of the study patients reacted with Catalase B, which is consistent with other studies that have identified other fungal catalases as allergens including, *A. fumigatus* [617], *Aspergillus versicolor* [618], and *Penicillium citrinum* [619]. In the present study, Catalase B exhibited high sequence identity (74.5%) with catalase from *P. citrinum* (Pen c 30.0101) [619]. The high sequence homology observed between these enzymes may represent conserved allergenicity of the catalase protein. Catalases are ubiquitous iron-containing enzymes that protect cells from oxidative damage through hydrogen peroxide hydrolysis [620, 621]. However, in fungi, catalases have been suggested to play additional roles in conidial germination [622], sporulation [623], and pathogenesis [624]; implying that fungal catalases contain some unique epitopes that may be immunogenic [625].

Of the eight putative allergens identified , only glycoside hydrolase, corresponded to an occupational (workplace-related) allergen (xylanase (Asp n 14)) from *Aspergillus niger* [626], which is often associated with baking, farming, and cereal handling [627]. The allergen Asp n 14, registered in the WHO/IUIS Allergen nomenclature, was initially identified by Sander *et al.*, using serum from 171 German bakers with workplace-related symptoms [626]. As the study population is from a rural village comprised predominantly of subsistence farmers, the observation of this allergen could be associated with para-occupational exposure. Previous studies have shown that occupational allergens can be transported home, on contaminated clothing and skin; with subsequent sensitisation of other household residents, including children, leading to severe allergic diseases in atopic patients if not diagnosed and treated [628].

Cross-reactivity among fungal allergens results in patients having allergic sensitisation to many physiologically similar fungi and therefore complicates the diagnosis and management of fungal allergies. In this study, several immunoreactive protein bands of the crude extracts belonged to two main fungi, *Alternaria* and *Aspergillus,* which is likely

associated with cross-reactivity amongst the fungal species, as this has been demonstrated between phylogenetically close and even distant species [242]. Although molecular diagnostics have improved the ability to identify clinically relevant cross-reactivity, there is still a need to understand the fungal and patient-specific factors that influence cross-reactivity's clinical relevance, including the degree of homology, exposure and immune response [603, 629]. Knowledge regarding the source of primary sensitisation could play a vital role in prevention and patient treatment.

Future epidemiological studies should analyse the samples individually for each patient to provide additional information on the status and frequency of the specific recognition of these candidate allergens. For instance, case-control studies of IgE-binding frequencies have been carried out to evaluate allergenic activity and clinical relevance of IgE antibody binding molecules [630].

Additionally, validation of these allergens is key and this could be achieved by investigating the potential recognition of these allergens in larger cohort populations. Furthermore, studies such as ELISA inhibition [631] assays should be conducted to evaluate the cross-reactivity of these candidate allergens so as to determine their clinical relevance.

The fact that numerous immunogenic proteins identified in this study were novel allergens emphasises a need to expand the reference database for the allergen prediction software and it further highlights the possibility of population differences in genetic variants in the major histocompatibility complex (MHC) specificity [587]. Therefore, there is need for genetic studies to determine any heterogeneity in fungal related pathology, clinical symptoms and accuracy of diagnostics.

The presence of cross-reactive allergens amongst related fungal species gives the potential for developing cross species therapeutics. By using a conserved epitope in developing immunotherapy, tolerance may be rendered towards not only the main epitope source, but also towards other cross reactive and co-sensitised species bearing the same epitope. So far, this technique has proved to be successful in treating birch allergies [632]. Such a method using a 'super epitope' that renders tolerance to several species simultaneously may prove to be medically effective. By validating and identifying the cross-reactive proteins in this study, a similar approach could be employed to manage the fungal-allergic patients in the African population.

5.6 Conclusion

In this chapter, 34 fungal proteins reactive with serum from a population of Zimbabweans sensitised to fungi were identified. Based on the structural and sequence predictive tools, eight of these were identified as putative allergens. This study was the first in Zimbabwe to identify immunogenic fungal proteins. Detailed studies of the allergenic activity and clinical importance of these allergens are now needed to better understand these proteins, and subsequently design immunotherapeutic strategies for fungal allergy management.

Chapter 6 Utility of *A. fumigatus* peptide in the differential diagnosis of fungal allergy

6.1 Introduction

Fungi are one of the most important inducers of allergy worldwide [3, 572]. The clinical manifestations of fungal allergies range from rhinitis and conjunctivitis to severe asthma [164, 633, 634]. Among the allergenic fungi, *Aspergillus* species are highly associated with severe consequences dependent on the host's immune system [6]. For example, immunodeficient patients develop invasive diseases, and immunoreactive patients develop respiratory allergic disorders [572, 635].

A. fumigatus is of greater clinical relevance among *Aspergillus* species, causing over 80% of *Aspergillus*-related conditions [636]. It is thermotolerant and has a small spore size (approximately 2–3 μm). These properties enable it to grow at human body temperatures and allow the spore deposition in terminal airways and alveoli [637-639]. Resulting in severe allergic diseases such as allergic bronchopulmonary aspergillosis (ABPA) [164, 640].

Furthermore, this fungi produces several immunoreactive proteins recognised by IgE or IgG. These proteins are variably expressed depending on the growth stage and the culture conditions used and are essential in diagnosing different presentations of allergic diseases [641] and aspergillosis [642]. Currently, crude extracts of *A. fumigatus* are used to diagnose *Aspergillus* sensitisation. However, these crude extracts cross-react with other fungal allergens [574, 643], resulting in possible over-diagnosis of *A. fumigatus* sensitisation [644]. The presence of cross-reactivity among these allergens necessitates the characterisation of their immunodominant regions.

To date, 23 *A. fumigatus* allergens have been identified and reported on the WHO/IUIS allergen database (www.allergen.org). Of these 23 allergens, only 5 recombinant *A. fumigatus* antigens (rAsp f 1, f 2, f 3, f 4, and f 6) are commercially available with rAsp f 1, f 2 and f 4) thought to be species-specific and possible present true sensitisation [574]. The availability of single *A. fumigatus* allergens allows the generation of patients-specific reactivity patterns against single allergens enabling the correlation of these reactivity patterns with different diseases related to the fungus [645], as has been observed in the diagnosis of ABPA [646] using rAsp f 2, 4 and 6. While many studies have focused on the clinical relevance of sensitisation to Asp f 1-4 and f 6 as diagnostic markers of ABPA [588, 647-649], the clinical value of their peptides in general fungal allergy disorders has not been adequately examined.

The diagnostic relevance of fibrinogen binding protein (Asp f 2) has been reported in previous studies, and some of the Asp f 2 epitopes have been identified [650] but have not been evaluated for their diagnostic relevance. Identifying clinically relevant epitopes may facilitate the development of specific and standardised peptide-based diagnostics.

Currently, the evaluation of serum-specific IgE and IgG against *A. fumigatus* is considered the main criterion for diagnosing allergic diseases due to *Aspergillus* [651]. However, studies relating titres of the antibodies against recombinant peptides to clinically relevant outcomes are lacking [650]. Furthermore, the current evidence base is from high-income regions, and no studies have been conducted in resource-poor areas like Africa.

6.2 Study aim

This study aimed to evaluate IgE, IgG, IgG4 and sCD23 responses to an Asp f 2 peptide in fungal allergic and asymptomatic individuals to determine the peptide's diagnostic value.

6.3 Methods

6.3.1 Study population

This descriptive study utilised sera from PSAC who were sensitised to fungi in **Chapter 4** and those from the allergy specialist clinic (see **Chapter 2**).

To be included in this study, participants from the allergy specialist clinic had to be sensitised to common fungal allergens as determined by SPTs and allergen-specific IgE > 0.35 kU/l. Furthermore, these participants had to have complete clinical and metadata as well as clinical symptoms. Following these inclusion criteria, 30 participants were included and these were identified as the fungal allergic participants. For comparison purposes, 32 asymptomatic participants were also included in the study (these were sensitised to fungi but had no confirmed clinical symptoms (**Chapter 4**), totalling 62 participants.

These 62 participants were grouped into 2 groups:

(i) Fungal allergic: elevated IgE levels against *A. fumigatus* and confirmed fungal allergy with clinical symptoms

(ii) Asymptomatic: sensitised to fungi but with no known allergy/ clinical symptoms

A general description of the study participants is shown in **Table 6.1**.

Table 6.1: Description of the study participants

	Fungal allergic (Specialist clinic)	Asymptomatic (Shamva)
Total N	30	32
Mean age(age-range)	29.3 (5-55 years)	4.16(2.6-57 years)
Sex ration (m/f)	0.304(7/23)	0.406(13/19)
Fungal sensitisation prevalence % (CI_{95})	100 (88.43-100)	96.88(83.78-99.92)

6.3.2 Sample collection

Blood samples from the allergic participants were collected from the participants during their routine consultations at the specialist clinic, and serum was extracted as previously described (**Chapter 4**). The samples were also shipped on dry ice to the University of Edinburgh. All sera were stored at -80 °C upon arrival to Edinburgh until use. For the asymptomatic patients, sera collected in **Chapter 4** was used.

6.3.3 Peptide synthesis

Rationale for selecting one peptide

Due to the high cost of synthesising pure peptides, the current study focused on only one peptide (Asp f 2). As previously mentioned, this peptide was selected because it is species-specific [574] and has been shown to be clinically relevant among ABPA patients [646] .

Synthesis

One peptide (IDVPSNCHTH EGGQLHCT) (hereinafter referred to as A1) was selected for synthesis and was manufactured off-site by PEPperPRINT (https://www.pepperprint.com/) as per specifications. Briefly, A1 was synthesised using Fmoc (9-fluorenylmethoxycarbonyl) solid-phase technology [652]. During Fmoc solid-phase peptide synthesis, the peptide chain was constructed amino acid by amino acid while attached to an insoluble resin support. This enabled the removal of reaction byproducts at each step through simple washing. Following carboxylic acid terminus activation, amino acids were protected at their amino terminus by the Fmoc group and added to the developing chain. Using piperidine, the Fmoc group was subsequently eliminated and the procedure repeated. After the peptide had been constructed, trifluoroacetic acid (TFA) was used to extract the peptide from the resin. Furthermore, the protective groups on the amino acid side chains were removed, resulting in a crude linear peptide that was purified by reverse-phase High-performance liquid chromatography

(HPLC). The sample was received as a dry powder with a purity of >95%, as measured by mass spectroscopy. The peptide was re-assembled on site (at the University of Edinburgh) by reconstituting in distilled water at a concentration of 2 mg/ mL and stored in frozen aliquots (-20 °C) until use.

6.3.4 Serological assays

Optimizing ELISA protocol

IgE, IgG and IgG4 ELISA

Similarly to **Chapter 4**, optimum dilutions of Asp f 2 peptide, sera and secondary antibodies (IgE, IgG and IgG4), as well as enzyme-substrate incubation times, were determined by several serial dilutions, following published procedures [523] and those recommended by manufacturers. The method was developed using the following pools of sera:

(i) **Pool 1** IgM and IgG reactive to A1 peptide on microarray chip

(ii) **Pool 2** Non-reactive to A1 (microarray chip results)

(iii) **Pool 3** Exclusively *A. fumigatus* reactive (as determined by Euroimmune blot)

(iv) **Pool 4** Non-reactive to *A. fumigatus* (as determined by Euroimmune blot)

Microtitre 96-well plates (Greiner-Bio One) were coated overnight at 4 °C with 1 µg/mL and 2 µg/mL of A1 diluted in PBS. Plates were washed once in PBS/tween20 and blocked with 3% BSA (Melford) for an 1 hour at room temperature. After three washes to remove the unbound blocking agent, pooled serum samples were added onto the wells (diluted from 1:10- 1:12800 in blocking buffer), and plates were incubated at 37 °C for two hours. After three washes to remove excess serum components, the HRP-conjugated secondary antibody was added (diluted at 1:1000 and 1:2000 in the blocking buffer to determine the most appropriate secondary antibody concentration), and the plates incubated again at 37 °C for 1 hour. Plates were washed six times, and the TMB substrate was added. The

colourimetric reaction was detected and quantified by an EMax precision microplate reader (Molecular devices) at a wavelength of 450nm and processed using Softpro Max v5 software. Optimum concentrations were deduced from checker-board titration.

CD23 ELISA

To determine optimal assay conditions to quantify soluble CD23 (sCD23), pooled serum samples were titrated against CD23 using a sandwich ELISA (described in **Chapter 2**). The following pools of sera were used in the optimisation assays:

(i) **Pool 1** Allergic to *A. fumigatus*

(ii) **Pool 2** Non-allergic to fungi

(iii) **Pool 3** Sensitised to fungi

Microtitre 96-well plates (Greiner-Bio One) were coated overnight at 4 °C with 1 µg/mL and 2 µg/mL of capture antibody (anti-CD23, R&D) diluted in carbonate buffer (see **buffer recipes**, **Appendix C**). Plates were washed once in PBS/tween20 and blocked with 3% BSA (Melford) for an 1 hour at room temperature. After three washes to remove the unbound blocking agent, pooled serum samples were added onto the wells (diluted from 1:2- 1:1024 in blocking buffer), and plates were incubated at 37 °C for two hours. After three washes to remove excess serum components, the Biotinylated Anti-human CD23 (R&D) secondary antibody was added (diluted at 0.1µg and 0.2µg /mL) and the plates incubated again at 37 °C for 1 hour. Plates were washed four times, and streptavidin at 1:6000 was added and plates were incubated for 2 hours at 37°C. Finally the plates were washed three times and TMB substrate was added. The colourimetric reaction was detected and quantified by an EMax precision microplate reader (Molecular devices) at a wavelength of 450nm and processed using Softpro Max v5 software. Optimum concentrations were deduced from checker-board titration.

ELISA assay

Following assay optimisation, A1-specific IgE, IgG and IgG4 as well as sCD23 levels were then quantified for each participant following the protocols detailed above. The optimum conditions used were as follows (**Table 6.2**):

Table 6.2: Optimised ELISA conditions for each assay.

	Antigen/ Capture antibody	Antigen/ Capture antibody concentration (µg/mL)	Serum dilution	Secondary antibody	Reaction time (minutes)
IgE	A1	1	1:50	1:1000	30
IgG	A1	1	1:100	1:1000	15
IgG4	A1	1	1:10	1:1000	30
sCD23	CD23	1	1:4	0.2 µg/mL	10

Similar to **Chapter 4**, positive and negative controls were repeatedly run on each plate to minimise variations across plates and plotted to detect any outliers. The positive control was pooled sera of *A. fumigatus* –allergic individuals and the negative control was pooled sera of non-allergic. To account for background variation, a blank well (containing no sera) was included on each plate and absorbance was subtracted from all plate readings. All the samples and blank were run in duplicate on each plate; reported values are the mean of duplicates.

6.3.5 Statistical analyses

Due to the skewed nature of the fungus-specific antibody responses values were square root-transformed. Binary logistic regression was performed to determine whether fungal allergic and asymptomatic individuals exhibited differences in antibody levels. To test whether serological reactivity to fungi varied with fungal allergy status, an Independent Samples t-Test was used. Linear regression analysis was used to determine the relationship between sCD23 and A1-specific IgE. Fisher's Exact test for association was conducted between clinical symptoms and antibody reactivity.

Differences were considered to be significant at $p < 0.05$. The experimental data are presented as the mean ± standard error of the group means.

6.4 Results

The IgE, IgG, IgG4 and sCD23 reactivity frequency of A1 was determined in sera from 62 individuals (**Table 6.3**). Thirty-two percent of these individuals showed IgE reactivity, whereas the IgG, IgG4, and sCD23 reactivity frequencies were 24%, 26% and 42% respectively.

Table 6.3: Antibody reactivity of A1

	Reactivity N (%)	Fungal-allergic %	Asymptomatic %
IgE	20 (32.26)	25.81 (15.53-38.50)	6.45(1.79-15.70)
sCD23	26(41.94)	20.97(11.66-33.18)	20.97(11.66-33.18)
IgG	15(24.19)	14.52(6.86-25.78)	9.68(3.63-19.88)
IgG4	16(25.81)	17.74(9.20-29.53)	8.06(2.67-17.83)

6.4.1 Allergen-specific antibody levels between allergic and asymptomatic individuals

Overall, the levels of all antibody isotypes tested tended to be higher in the allergic population than the asymptomatic. However, as shown in **Figure 6.1**, only IgE levels were significantly higher (t=2.946, p<0.0049).

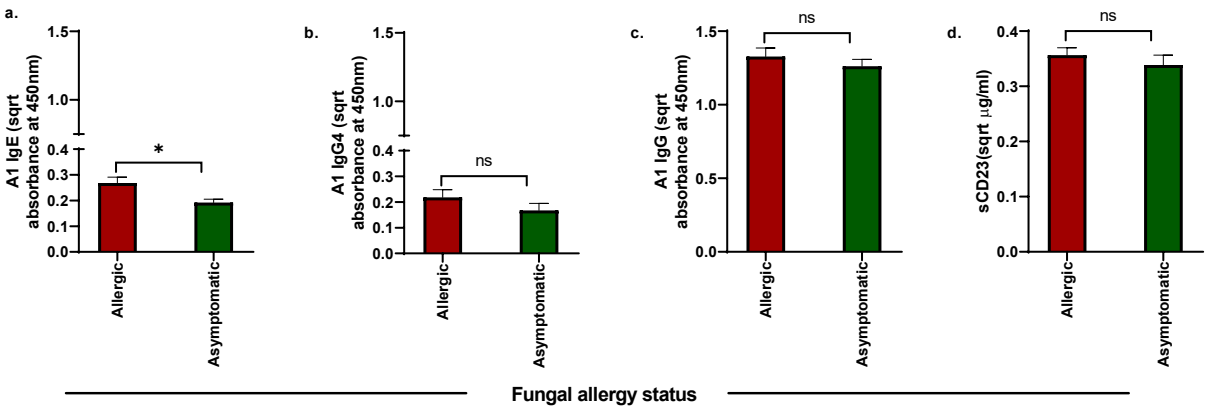

Figure 6.1: Comparison of antibody levels between allergic and asymptomatic individuals
The mean of the square root transformed concentrations of specific IgE (a), specific IgG4 (b), specific IgG (c) and soluble CD23 (d) are plotted for the allergenic and asymptomatic individuals. Bars represent SEM and asterisks represent significant pvalues at * p<0.05 and ns= non-significant and represents p>0.05

6.4.2 Association between allergy and antibody levels

A binomial logistic regression was performed to determine whether fungal allergic and asymptomatic individuals exhibited differences in antibody levels (IgE, IgG, IgG4 and sCD23). The logistic regression model was statistically significant, $x^2(5) = 14.173$, $p < 0.015$. The model explained 27.3% (Nagelkerke R^2) of the variance in allergy status and correctly classified 72.6% of cases. Increasing IgE antibody levels were associated with an increased likelihood of being allergic (B= 9.004, Wald= 4.753, p=0.029; fig 1a). However, the levels of sCD23, IgG and IgG4 did not differ between the allergic and asymptomatic individuals (B=1.013, Wald= 0.083, p=0.774, B=1.497, Wald= 1.437, p=0.231 and B=2.529, Wald=1.470, p=0.225 respectively; **Figure 6.1b-d**).

6.4.3 Association between sCD23 and IgE

To determine the relationship between sCD23 and A1-specific IgE, linear regression analysis was conducted, adjusting for host age and sex. As illustrated in **Figure 6.2**, anti-A1 IgE responses were significantly positively associated with levels of sCD23 (r=0.2615, p=0.04), with increasing sCD23 explaining 6.8% of the variability of the IgE levels.

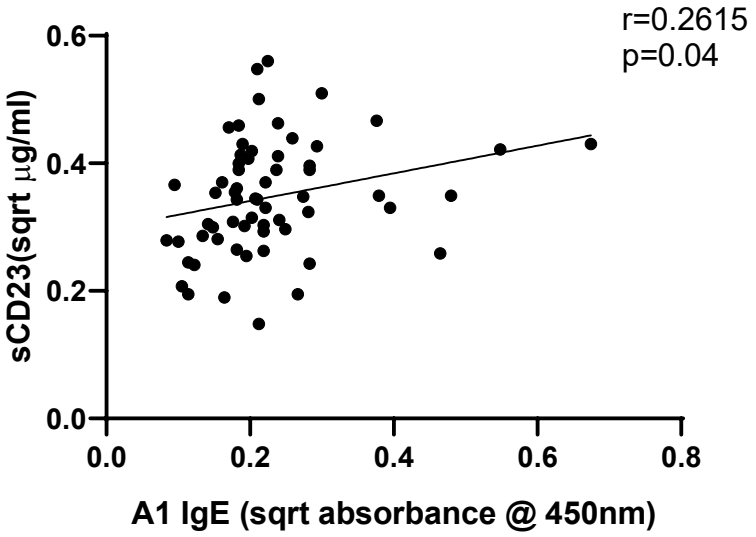

Figure 6.2: Linear regression analyses of IgE and sCD23
Anti-A1 IgE responses (square root transformed absorbencies) are plotted against sCD23

6.4.4 Association between antibody reactivity to A1 with clinical symptoms

Of the 26 individuals that were reactive to A1 and had confirmed allergy, 62% had respiratory –related symptoms (which included, asthma, alveolitis and rhinitis) and 38% had skin-related symptoms (which included eczema, dermatitis, urticaria and pruritus). The majority (46%) of patients with respiratory-related symptoms were IgE reactive (**Figure 6.3**).

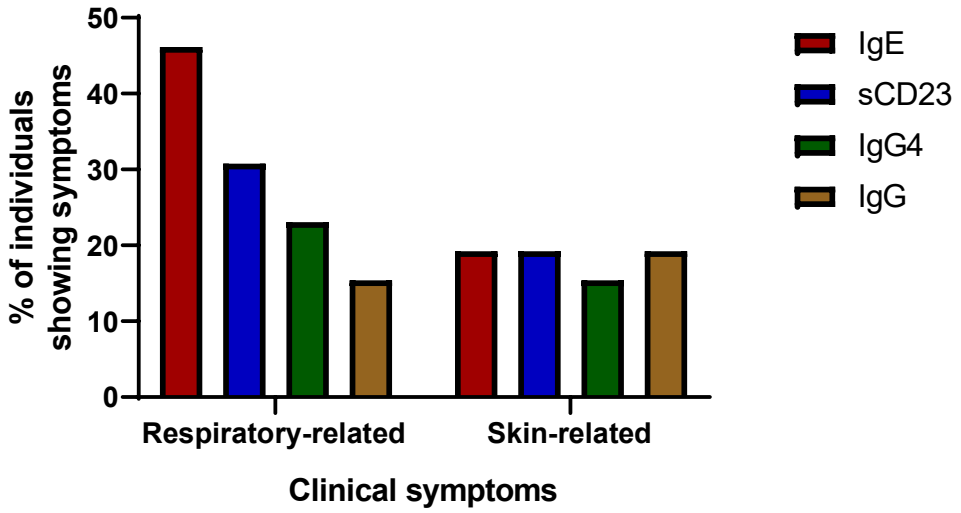

Figure 6.3: Percentages of patient showing fungal-related symptoms

A Fisher's Exact test for association was conducted between clinical symptoms and antibody reactivity. There was no statistically significant association between clinical symptoms and all the antibody isotypes tested as shown in **Table 6.4** below.

Table 6.4: Fischer's exact test results

Antibody isotype		Respiratory-related symptoms	Skin-related symptoms	P value
IgE	Reactive	11	5	0.293
	Non-reactive	5	5	
IgG4	Reactive	6	5	0.412
	Non-reactive	10	5	
IgG	Reactive	4	5	0.189
	Non-reactive	12	5	
sCD23	Reactive	8	5	O.656
	Non-reactive	8	5	

6.5 Discussion

The knowledge of the primary structure of allergens from different sources results in an improved understanding of allergy reactivity. Furthermore, the production of recombinant allergens has been useful for diagnostic purposes, particularly for organisms such as fungi, whose extracts are difficult to standardise [228]. The use of recombinant allergens to establish the exact proteins to which a person is sensitised has increased the specificity of allergens as biomarkers of disease severity, particularly food allergies [653]. However, a similar approach to fungal allergy is still in its infancy.

To date, there have been numerous reports of diagnostically relevant native and recombinant antigens for the serodiagnosis of aspergillosis [588, 647, 654-658]. However, the reported diagnostic sensitivity and specificity vary significantly among the studies [657]. This current study evaluated the diagnostic relevance of Asp f 2 peptide (A1). It was found that approximately 32% of the individuals exhibited IgE reactivity to A1. Of this 32%, 5% were asymptomatic. Although purely speculative, a potential explanation could be that these asymptomatic individuals have an undiagnosed fungal allergy. Alternatively, since there is a probability of heterogeneous recognition of an IgE epitope by sensitive patients from various populations, the results could suggest that the use of the specific A1 is not diagnostically relevant in this population. However, it is plausible that measurement of IgE antibodies to additional epitopes other than A1 are required to identify genuine *A. fumigatus* allergy [659].

The low-affinity IgE receptor CD23 is expressed in various cells, but its function in the context of allergic responses is not yet fully understood [660, 661]. However, it has been shown to negatively regulate IgE levels [35-37], but other atopy and food allergy studies have yielded conflicting results [662-664]. It has been suggested that allergen(s) can cleave CD23 resulting in the production of soluble CD23 (sCD23) and elevated IgE levels

[665]. Furthermore, previous studies have shown that the expression of CD23 is increased on B cells and monocytes and that the concentration of sCD23 is elevated in allergic patients [666, 667]. Similarly, in the current study, sCD23 was higher in allergic compared to asymptomatic individuals. As the difference in concentration levels was insignificant, it is conceivable that sCD23 is a marker for the diagnosis of atopy.

The results presented here show that A1 is unlikely to be a useful indicator for the diagnosis of fungal allergy. Since Asp f 2 has other known allergenic epitopes that elicits highly variable IgE reaction patterns, it is evident that using one single epitope is insufficient in differentiating fungal allergy. Antibodies may be formed to one or more of the allergenic epitopes in a specific allergenic protein [668, 669], and as each epitope has different characteristics, these may contribute to additional information concerning the management of the allergic patient [670]. Testing more recombinant or synthetic peptides could result in high diagnostic sensitivity as well as the identification of both clinically relevant as well as cross-reactive allergens in a specific population.

The current study was limited by its small sample size and sampling frame, which limits its generalisation to the general population. Therefore, to obtain conclusive results, an enrolment of much larger groups of patients, including fungal allergic patients (i.e. asthma, ABPA, SAFS), sensitised patients, and non-atopic individuals as a control group, should be performed. Likewise, more epitopes need to be evaluated in such a study to increase diagnostic sensitivity.

6.6 Conclusion

This study evaluated the utility of an Asp f 2 peptide in the differential diagnosis of fungal allergy. While IgE was significantly higher in fungal allergic patients and serum levels of sCD23 appeared to be a marker of atopy, this peptide was not diagnostically relevant.

However, these findings require further validation in multicentre studies due to the heterogeneous recognition of IgE epitopes in various populations. Further serology characterisation of this peptide is required to obtain a thorough diagnostic profile regarding its sensitivity, which would impact its use in diagnosis.

Chapter 7 General discussion

7.1 Introduction

Infectious diseases are a daily reality globally, and significant advances have been made to mitigate their transmission and effect on humans. To date, diseases such as smallpox, influenza, bacterial infections and, more recently, dengue, Zika, Chikungunya, and COVID-19 have affected millions of people in both developed and developing countries [671-674]. The impact of these conditions accelerated the generation of knowledge, resulting in the eradication of smallpox [675], the availability of effective vaccines, and the development of diagnostic as well as preventive tools [674, 676, 677]. Likewise, the ongoing development of vaccines, new drugs, and diagnostic tests for NTDs was urged by the negative impact these diseases have on public health [678].

However, unlike all these diseases, fungal infections have been neglected as a significant public health threat until recently. Although they are now recognised as a growing problem globally, there continues to be a lack of epidemiological data on these diseases. This is due to the ubiquitous nature of their clinical symptoms, which can be easily mistaken for other diseases. Furthermore, symptoms of other diseases such as asthma, TB, cancer and HIV infection can obscure the accurate diagnosis of fungal diseases. Therefore, public health efforts to increase awareness and facilitate earlier diagnosis and treatment are of utmost importance. This PhD aimed to characterise the epidemiology, immunology, and aetiology of fungal allergic diseases in Zimbabwe, with the ultimate goal of improving the recognition, prevention and diagnosis of fungal infections.

7.2 Burden of fungal diseases: Scale of the problem in Zimbabwe

To date, the LIFE portal has made it possible to estimate the global burden of serious fungal infections for more than 80% of the world's population [350]. These studies have shown differences in the global burden between countries and at-risk populations [20]. Although there is a lack of data in most African countries, studies from Nigeria, South Africa, Senegal, Namibia, Mozambique, and Ghana [408, 410, 425, 440, 485] have shown that the burden of fungal diseases is high, despite the limited awareness and inadequate facilities to diagnose and treat these diseases.

Consistent with the findings from the African countries, the current study has shown that the burden of fungal diseases in Zimbabwe is high (14%) (**Chapter 3**). Similar to Kenya and Ethiopia [679, 680], the burden of tinea capitis in children was markedly higher in this study. The high burden could be due to poor sanitation, lack of water supply, limited health facilities, poor handwashing behaviour, close contact with animals, and the tropical region with high humidity [681-683]. Therefore, to prevent or control this infection, improved water, sanitation and hygiene (WASH) practices are needed within the affected communities. The use of international data to determine the burden of fungal infections in the at-risk population may have overestimated or underestimated the burden of some diseases.

Epidemiological studies from Africa show that allergies are rising in this continent, which has been linked to lifestyle changes and less exposure to pathogens in childhood [684]. Several studies have examined the relationship between reduced exposure to parasitic worm infections and allergy outcomes in recent years, with contradictory findings [91, 224, 578-584]. To date, it has been shown that there are parallels in the immune responses to helminths and allergies. Both conditions are associated with elevated levels of IgE, high

numbers of Th2 cells, eosinophils, and mast cells. These immune parallels have resulted in the possible hampering of the diagnosis of allergies in helminth-endemic regions [684].

In Zimbabwe, the current allergy prevalence is unknown but is thought to be increasing [292, 685-687]. However, these studies suffer from a pro-urban, and potentially an economic bias being derived from patients who visited hospitals or private clinics with suspected allergic symptoms [688].

Thus, in **Chapter 4** the prevalence of fungal sensitisation in Zimbabwean PSAC without known atopic disease and from a schistosome endemic area was determined by SPTs. The prevalence of fungal sensitisation in this study was high, consistent with findings reported by Kwizera *et al*., [689]. This finding would suggest that these children have undiagnosed fungal atopy. Alternatively, the high prevalence could be due to cross-reactivity among the fungal allergens or an association between helminth infection and allergy. However, the latter was shown not to be the case in this study as there was no association between *S. haematobium* infection and SPT reactivity. This could have been due to the small sample size of infected people due to the regular mass drug administrations (MDAs) to help control and eliminate these helminth infections [690].

Though it is thought that allergy prevalence is lower in rural areas due to lifestyle and environmental factors, the findings in this study and those of Ndlovu *et al*., [688] suggest that the situation could be changing [28, 691]. Although the generalizability of both study findings are limited. The findings could reasonably represent sensitisation patterns in areas with similar environmental exposures.

As the optimal cut-off wheal size to define fungal sensitisation in our population is unknown, the generally accepted 3 mm cut-off point was used. However, this may have overestimated the fungal sensitisation in the study population. For example, in a Ugandan

study, when the 3mm cut-off point was used, 60% of the population was found to be sensitised. Yet, when the cut-off was increased to 5mm, the prevalence was reduced to 8%, which is more likely to be an accurate value [689]. Therefore, to avoid atopy misclassification or overestimation of SPT prevalence, there is a need to define a suitable cut-off wheal size relevant to the population being investigated. Likewise, in a Zimbabwean population with a high prevalence of helminths, IgE antibodies to the carbohydrate epitope galactose-a-Gal, induced by parasite infection, impede routine allergy diagnosis [692]. As a result, available diagnostics must be evaluated and validated in all affected populations rather than extrapolated from different populations or environments in order to accurately quantify disease burden, inform policy, and create health systems tailored to the needs of the affected people. This is especially important in African countries due to the high genetic diversity and exposure to infectious agents [693], which results in different disease dynamics and diagnostics that do not work optimally for these countries.

7.3 The role of gut mycobiome in fungal sensitisation/ reactivity

The human gut microbiome is increasingly considered a crucial factor in the development of allergy, with a strong interrelation between the human gut microbiota, environmental factors, human genetics and gastrointestinal atopy [694, 695]. In particular, gut microbiota composition and metabolic activity are intimately linked with the development of oral tolerance [696]. Therefore, disturbed microbial homeostasis appears to influence allergic disease susceptibility, especially early in life. For example, Mahdavinia *et al*., [697, 698] compared the stool microbiome of 38 South African children, 29 of whom had atopic dermatitis, and 17 of these were sensitised to at least one food. The authors found that *Blautia* and *Bifidobacterium* genera were higher in cases with food sensitisation than cases without food sensitisation [697, 698]. Likewise, studies in Sweden and Denmark

showed that reduced gut microbiota diversity in infants is associated with an increased risk of allergic disease in childhood [134, 699, 700].

Recently, alterations in the mycobiome have been reported to predict subsequent susceptibility to allergic diseases in children [295, 296]. Resulting in the mycobiome being recognised and granted a patent for its potential as a probiotic, diagnostic and treatment tool for Ulcerative colitis [701]. Therefore, developing a clear understanding of the role played by the mycobiome in allergic sensitisation will provide researchers with the opportunity for targeted and rational means to manipulate the mycobiome for the benefit of the host.

In line with this, the mycobiome in Zimbabwean children was characterised and related to fungal reactivity and other host factors **(Chapter 4)**. In contrast to other mycobiome and allergy studies [702-705], the current study showed that there was no association between the mycobiome and fungal reactivity or any of the host factors investigated. This could, possibly be due to the homogeneity across the study population for dietary and environmental exposure since the children were born in and were permanent residents of the same area. Moreover, they were all ≤ 5 years old, with 96% of them being sensitised to fungi. As sensitisation does not always translate to allergic diseases, it is conceivable that the observed sensitisation was clinically irrelevant resulting in no significant differences between the sensitised and non-sensitised children. Therefore, the use of well-characterised individuals with confirmed allergy vs non-allergic as well as broader age ranges might produce results, which are significantly different.

Interestingly, *S. haematobium* infection independent of host-related factors, including socio-demography, fungal sensitisation and growth and nutritional indices was shown to be associated with significant alterations in the abundance of specific gut mycobiota populations. There was predominantly an increased abundance of *Aspergillus,*

Tricholoma and *Periglandula* in schistosome-infected children compared to uninfected children. These findings are consistent with observations that schistosome infection is associated with alterations in the microbiome's diversity and abundance of specific taxonomic groups [552, 553]. As *S. haematobium* worms mostly reside in the pelvic venous plexus, though some have occasionally been detected in the intestine in Egyptian autopsies [706], the observed effect of infection on the diversity of the mycobiome is most likely through a more indirect or systemic route than through direct interactions. For example it has been suggested that the upregulation of IL-22 during helminth infection may favour the abundance of specific microbial taxa [707].

Aspergillus and *Saccharomyces* have been reported in both healthy and disease states. These genera seem to represent a core mycobiome that is unaffected by disease states [708]. Consistent with other studies, this current study shows a predominance of these genera [542, 546, 547], possibly suggesting that they are part of the core mycobiome of the study participants. However, the DNA extraction method and the specific reference databases used differ between studies which may affect yield, diversity and abundance calculations so that the fungal profile observed may not have been accurately represented, thus presenting challenges in mycobiome characterisation.

Although this study included a small number of study participants, insufficient to understand the gut mycobiome fully, the data presented provide useful insights for future investigations. Furthermore, many of the fungal species detected in gut mycobiome studies are known or speculated to be allochthonous due to the high level of exposure to the environment or food-associated fungi [709]. Hence, prospective studies are needed to fully investigate causality and assess the stability of the mycobiome over time in fungal sensitisation and allergy. Additionally, research on the mycobiome should include the interactions between the mycobiome and other microbial groups such as bacteria, viruses

and archaea as alterations in one part of the microbiome may have implications for the other parts of the microbiome.

As discussed in **Chapter 1**, the diversity and composition of the gut microbiome have implications for human health [710]. Multiple factors influence the diversity and overall composition of the gut microbiome, including geography, mode of infant delivery and feeding, age, diet composition, and medications [711]. Therefore to understand the factors underlying disease progression, differences in disease patterns, and the development of new strategies aimed at reducing disease burden, it is critical to understand the relationship between mycobiome and clinical diseases in populations with different diets, environments, and associated co-infections. Understanding the relationship, for example, will provide a foundation for understanding the role of interventions such as nutraceuticals in influencing health via the mycobiome.

7.4 Fungal allergens

Although it is possible to partially manage allergic diseases through antihistamines or avoidance , there is no cure yet. Immunotherapies with purified allergens have shown some promise but are often complicated by adverse side effects. Mapping IgE-binding epitopes on the tertiary structure help in the rational design of allergens with reduced allergenicity. To aid this, identifying allergens is a prerequisite from any allergenic source. However, no studies have documented or identified the fungal allergen repertoire in an African population. This has left a knowledge gap in diagnosing and managing fungal allergies in African populations.

In this study, most participants were sensitised to more than one fungal allergen (**Chapter 4**). Based on previous reports [242], it is indeed possible that such multiple co-sensitisations are, in fact, cross-reactivity. However, without IgE binding studies to

specific and discriminatory proteins from each species, it is difficult to be sure of this hypothesis. Hence in **Chapter 5**, the specific allergenic proteins were identified using proteomics which has proven to be an excellent approach to identifying allergens [712]. Similar to other studies [238, 257, 713, 714], this technique enabled the identification of putative novel fungal allergens. The IgE-binding proteins detected in this study were relatively different from those reported in previous studies, probably due to the region where the study was carried out as it is well known that fungal exposure is affected by climate and other environmental factors conditions [216]. These conditions affect allergenicity, resulting in different sensitisations patterns [217, 715]. This finding was consistent with those of Muddaluru and colleagues[716], who observed that sensitisation profiles to house dust mite allergens differed considerably between geographical locations. The authors, reported that Der p 7 and Der p 23 were major sensitisers in South Africa, which was different from what was observed in Europe and USA [716]. Likewise, research from SSA has shown that novel allergens exist that are relevant to this region [717].

Although the approach used in this study was old [608, 610], it has proven to still harbour novel allergens, especially for well-defined sub-populations, as shown in previous studies [718]. The current study used pooled sera, which does not provide individual reactivity profiles, which can vary within individuals according to atopy status [719]. Furthermore, validation of the allergen candidates was not performed, which limits the reliability of the findings. However, future work will evaluate the cross-reactivity of the identified allergen candidates as well as investigate their potential recognition in larger well-characterised cohorts. Despite these limitations, the identification of putative allergens from **Chapter 5,** open up further avenues to purify and characterise individual allergenic components of these fungi to improve component resolved diagnosis (CRD) and immunotherapy in fungal allergy. Thus, forming a potential basis for diagnostics specific to the African population.

To date, several studies have demonstrated the diagnostic benefit of component testing over extract testing, leading to increased CRD in routine allergy care [590]**.** For example, although not related to fungi, Wollmann and colleagues used CRD to investigate IgE reactivity to peanut allergen components (Ara h 1–3, 6, 8, 9) among allergic patients in Zimbabwe who were IgE-sensitised to whole peanut extract but had no symptoms of peanut allergy [720]. While 46% of these Zimbabwean patients had IgE to at least one of the highly allergenic peanut components, half of this patient cohort had elevated levels of IgE to CCDs [720]. This study demonstrates how CRD can be useful in improving the specificity of in vitro allergy diagnosis in SSA.

Hence, **Chapter 6** evaluated the utility of Asp f 2 in differential diagnosis of fungal allergy. With fungal spores being ubiquitous, it may be challenging to obtain a completely healthy control that has never been exposed to fungi, but it may be attempted to recruit people who maximally match the criteria. However, in this study all the participants recruited were sensitised to fungi which could have limited our findings. The fungal allergic individuals were recruited based on allergy symptoms; individuals sensitised to fungi but with no confirmed clinical diagnosis were included in the asymptomatic group. Hence IgE sensitisation in both groups was not unexpected. Previous studies on immune disorders have reported the immuno-regulatory effect of CD23 [660]. For example, Singh and colleagues reported lower levels of sCD23 in children presenting with juvenile arthritis compared to age-matched healthy controls [721].

Furthermore, correlation between IgE and soluble low-affinity IgE receptor (sCD23) has been demonstrated in individuals with allergic disease [722]. However, studies on a role for CD23 in atopy and food allergy have yielded conflicting results [662-664]. In the current study, there was a positive correlation between IgE and sCD23, suggesting that the CD23 receptor is involved in the regulation of IgE synthesis by B cells [173]. It is suggested that

the allergen(s) can cleave the CD23 and the allergen-cleaved CD23 loses the ability to suppress IgE synthesis, leading to elevated IgE levels [723].

Since *A. fumigatus* can produce more than 40 IgE-binding components and elicits a highly variable pattern of IgE reactivity depending on the status of sensitisation of the investigated individuals, the use of one single epitope of Asp f 2 **(Chapter 6)** is evidently not sufficient to identify or differentiate all individuals reactive/ sensitised to the fungi. Hence, it would be more informative to include more recombinant allergens in the evaluation of differential diagnosis. Furthermore, the study only focused on humoral immune responses; however, the T-cell-mediated immune responses should also be determined to understand the mechanisms underlying allergic diseases.

7.5 Future directions

This study has raised questions relevant to the management of atopy and fungal diseases. For instance, it is imperative to investigate whether the sensitisation observed in this study population is clinically relevant. These studies would inform the management and diagnosis of allergy/atopy in sensitised Africans. Furthermore, strategies that improve medication access, including initiatives like the WHO Essential Medicines list, low-cost equipment and implementation of culturally appropriate educational programmes for healthcare workers and the public, are essential [724].

Under recognition and under-diagnosis of fungal diseases in Zimbabwe are major challenges attributable to the lack of diagnostic facilities and skilled personnel. Furthermore, most of these diseases are diagnosed using equipment that requires electricity and trained personnel to perform them. This is a challenge in Africa and Zimbabwe, where electricity supply is sporadic. Hence, for improved diagnosis and management of patients, there is a need to develop point-of-care tests like; cryptococcal

antigen (CrAg) test, 'COVID-19 rapid antigen tests' or 'pregnancy test strip'. These tests are easy to use and do not require electricity, bridging the diagnostic gap. The development of these diagnostic tests will allow quicker implementation of appropriate therapeutics, immediately impact mortality rates, and facilitate gathering more accurate epidemiological data.

Furthermore, these diagnostics should be readily accessible to all patients. This could be achieved by developing a standardised diagnostic algorithm based on clinical symptoms that can be easily identified by primary healthcare professionals [725]. The algorithm will help in the early diagnosis and treatment of fungal diseases and ensure the availability of information on fungal infections affecting the African population. This will enable the implementation of interventions in the primary care setting, potentially reducing morbidity and significantly enhancing quality of life.

Observational studies such as this one give a snapshot of the gut mycobiome and are essential in generating hypotheses. However, it would be important to establish how the mycobiome changes over time, what interactions occur with other constituents of the microbiome and what changes occur in host immunity in response to a change in the constituents of the mycobiome. Most study participants were sensitised to fungi, were of similar age and were born and raised in a similar environment. This homogeneity might have resulted in the study lacking the power to determine whether the mycobiome does differ in fungal sensitised vs non-sensitised individuals. Therefore, larger longitudinal studies with well-characterised study participants will be more informative.

The identified putative allergens were not validated in this study. Hence, it will be imperative to characterise their allergenicity, including cross-reactivity [726] *in vitro* and *in vivo* in more extensive studies to obtain reliable results. Utilising these allergens for CRD requires information on the clinical relevance of each allergen. Therefore, future research

evaluating the clinical reactivity of these allergens in fungal-allergic and non-allergic patients is needed. Purification followed up by Ig Ereactivity assay to each allergen should be performed, and the findings might provide insight, confirming the allergenicity of these proteins. Furthermore, epitope mapping of the identified putative allergens can be used as potential candidates for developing hypoallergenic derivatives [727].

To date there is limited awareness of fungal diseases in health care settings and the community. Hence, to raise awareness about morbidity, mortality, diagnostics and treatments associated with fungal diseases, the communities need to be educated. To achieve this, the medical mycology community should work closely with local organisations and community health workers to disseminate fungal-related information. This will enable the delivery of health messages in a culturally appropriate manner, which is also easily understood and will result in community-based surveillance. Overall, awareness of these diseases will improve the likelihood of early case detection [88] and reduce the stigma that is associated with some fungal infections.

As previously stated, Africa has greater genetic diversity among its populations [728] and a high prevalence of infectious diseases compared to other populations. Using data from non-African populations for diagnosis may lead to African individuals mistakenly thought to have a disease risk variant [729] that is relatively common in these populations and not clinically relevant. Hence it is important that studies are carried out in different populations to generate context-specific knowledge so that context-relevant interventions are developed in terms of diagnostics and therapeutics.

Extending the findings presented in this thesis to conduct further studies on different African populations will contribute evidence appropriate for improving the health of all people (SDG3). Likewise, by promoting the integration of fungal disease diagnosis and care into health systems, other SDGs will be directly addressed, including reducing long-

term morbidity for a productive future life and reducing poverty (SDG1). Furthermore, the provision of robust infrastructure (SDG9) will improve health care delivery.

7.6 Conclusion

The results presented in this thesis provide additional evidence to the ongoing global discussions on the burden of fungal diseases. Additionally, this thesis provides novel data on several important aspects of fungal allergy, which will influence the care of allergic individuals. Furthermore, the identification of putative allergens suggests that the African population might be sensitised to different fungal allergens yet to be identified and fully characterised**.** This finding requires further study, but it emphasises the need for improved diagnostics in this continent. Furthermore, there is a need for epidemiological surveillance of fungal diseases. The integrated implementation of improved surveillance and diagnostic capabilities, along with continued capacity building for African-based researchers and relevant medical research in the diagnosis and management of fungal disease as part of the universal health coverage package, can substantially improve patient outcomes.

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Appendices

Appendix A Supplementary information-Chapter 4

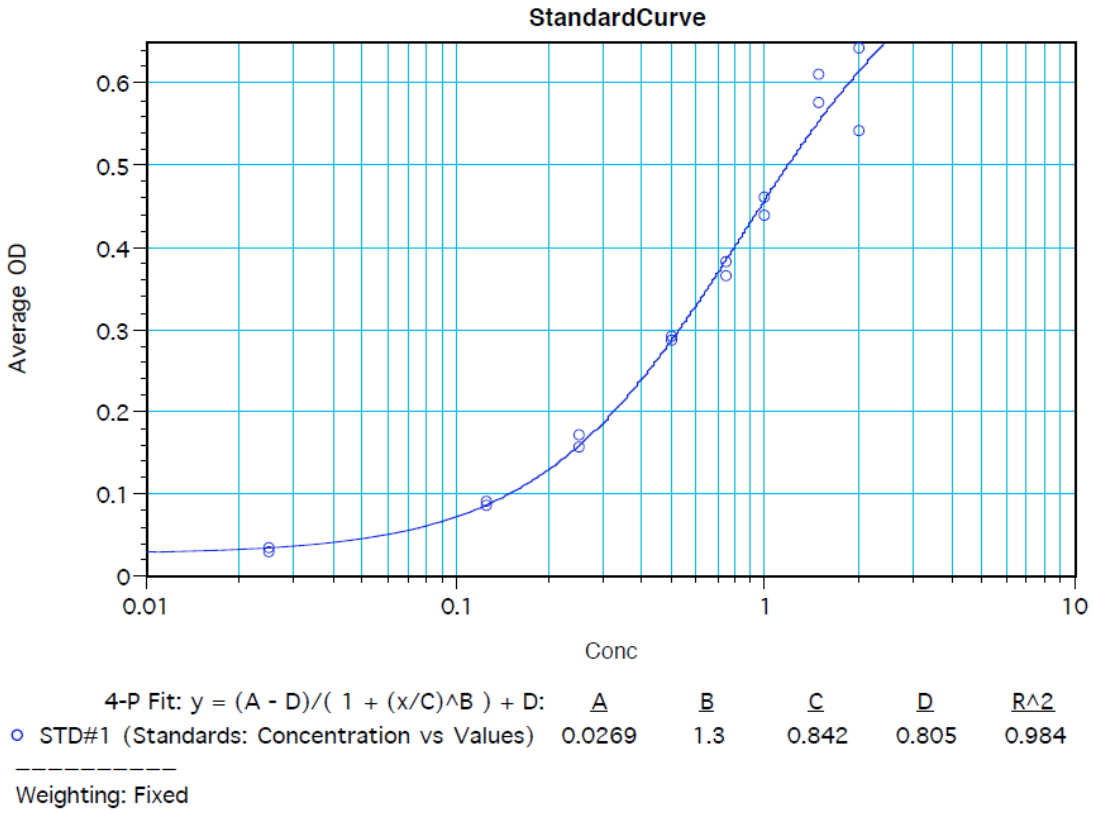


Figure A.1: Bradford Assay standard curve example
Standard curve of BSA absorbance at 595 nm against BSA concentrations

Table A.1: Optimal conditions for human anti-fungi ELISA

Antigen		IgA	IgE	IgM	IgG	IgG4
Alternaria alternata	**Antigen**	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL
	Sera dilution	1:100	1:10	1:200	1:500	1:10
	Secondary Antibody	1:1000	1:500	1:1000	1:1000	1:500
	Reaction time	30 minutes	1 hour	30 minutes	10 minutes	1 hour
Aspergillus fumigatus	**Antigen**	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL
	Sera dilution	1:100	1:10	1:200	1:200	1:10
	Secondary Antibody	1:1000	1:500	1:1000	1:1000	1:500
	Reaction time	30 minutes	1 hour	10 minutes	30 minutes	1 hour
Cladosporium herbarum	**Antigen**	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL
	Sera dilution	1:100	1:20	1:200	1:200	1:20
	Secondary Antibody	1:1000	1:500	1:1000	1:1000	1:500
	Reaction time	30 minutes	1 hour	10 minutes	30 minutes	1 hour
Saccharomyces cerevisiae	**Antigen**	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL	5 µg/mL
	Sera dilution	1:50	1:10	1:200	1:500	1:20
	Secondary Antibody	1:1000	1:500	1:1000	1:1000	1:500
	Reaction time	30 minutes	1 hour	15 minutes	30 minutes	1 hour
Epicoccum nigrum	**Antigen**	5 µg/mL	-	5 µg/mL	5 µg/mL	5 µg/mL
	Sera dilution	1:100	-	1:200	1:200	1:10
	Secondary Antibody	1:1000	-	1:1000	1:1000	1:500
	Reaction time	30 minutes	-	10 minutes	30 minutes	1 hour
Penicillium chrysogenum	**Antigen**	5 µg/mL	-	5 µg/mL	5 µg/mL	5 µg/mL
	Sera dilution	1:100	-	1:200	1:200	1:10
	Secondary Antibody	1:1000	-	1:1000	1:1000	1:500

	Reaction time	30 minutes	-	10 minutes	30 minutes	1 hour
Rhizopus nigricans	**Antigen**	5 µg/mL	-	5 µg/mL	5 µg/mL	5 µg/mL
	Sera dilution	1:50	-	1:200	1:500	1:10
	Secondary Antibody	1:1000	-	1:1000	1:1000	1:500
	Reaction time	30 minutes	-	15 minutes	30 minutes	1 hour

Table A.2: NMDS scores for fungal-specific antibody response

Factor	NMDS1	NMDS2	NMDS3
***A. fumigatus*_IgA**	0.170325	**0.562969**	-0.44955
***A. fumigatus*_IgE**	0.304744	**0.840148**	0.391543
***A. fumigatus*_IgG**	0.172853	-0.34234	0.459281
***A. fumigatus*_IgG4**	**0.949327**	-0.1889	-0.03587
***A. fumigatus*_IgM**	0.008885	0.388458	**-0.7034**
***A. alternata*_ IgA**	-0.34827	**0.688937**	-0.40293
***A. alternata*_IgE**	**-0.53535**	0.101638	-0.20004
***A. alternata*_IgG**	-0.46208	0.12269	**0.780319**
***A. alternata*_IgG4**	**-0.82464**	-0.3259	-0.1119
***A. alternata*_IgM**	-0.11218	**0.860026**	0.103412
***C. herbarum*_IgA**	-0.15681	-0.45528	0.307098
***C. herbarum*_IgE**	-0.45188	-0.47669	0.168303
***C. herbarum*_IgG**	**-0.71602**	0.354474	0.485397
***C. herbarum*_IgG4**	0.066936	**-0.6739**	0.177745
***C. herbarum*_IgM**	**-0.84197**	-0.02237	-0.46723
***E. nigrum*_IgA**	0.04168	**-0.86458**	-0.36527
***E. nigrum*_IgG**	**0.981253**	-0.07596	0.124666
***E. nigrum*_IgG4**	-0.04757	0.191025	**0.549954**
***E. nigrum*_IgM**	-0.15482	**-0.6742**	**0.598203**
***P. chrysogenum*_IgA**	-0.26169	**0.512291**	**0.795985**
***P. chrysogenum*_IgG**	**0.834782**	-0.37076	0.355029
***P. chrysogenum*_IgG4**	**0.669056**	-0.0964	0.123496
***P. chrysogenum*_IgM**	**-0.60789**	**-0.72236**	0.266903
***R. nigricans*_IgA**	**-0.79074**	0.1376	0.484033
***R. nigricans*_IgG**	**-0.76625**	-0.30415	-0.43294
***R. nigricans*_IgG4**	**-0.50955**	0.303398	-0.34809
***R. nigricans*_IgM**	-0.01324	**-0.91075**	0.116071
***S. cerevisiae*_IgA**	**-0.78133**	-0.01078	**-0.57287**
***S. cerevisiae*_IgE**	-0.43998	**0.73912**	0.240422
***S. cerevisiae*_IgG**	**-0.56965**	0.371898	-0.05216
***S. cerevisiae*_IgG4**	-0.10094	**0.633083**	-0.00641
***S. cerevisiae*_IgM**	**-0.83598**	-0.39198	0.296792

The NMDS scores of the original variables for each Component calculated after NMDS of fungal specific antibodies. Those variables which were significantly positively or negatively correlated (score >±0.5) onto a component are shown highlighted in bold.

Table A.3: Odds ratios for SPT reactivity for different fungal species

Variable		OR (95% CI)	P-value
A. alternata			
	Age group 2 vs 1	2.272(0.465-11.110)	0.311
	Age group 3 vs 1	1.858(0.339-10.171)	0.475
	Male vs Female	0.628(0.180-2.188)	0.465
	S. haematobium negative vs Positive	0.283(0.030-2.640)	0.268
C. herbarum			
	Age group 2 vs 1	0.811(0.184-3.571)	0.781
	Age group 3 vs 1	0.479(0.101-2.270)	0.354
	Male vs Female	1.855(0.547-6.295)	0.322
	S. haematobium negative vs Positive	0.195(0.033-1.155)	0.072
E. nigrum			
	Age group 2 vs 1	1.353(0.338-5.420)	0.669
	Age group 3 vs 1	3.144(0.683-14.470)	0.141
	Male vs Female	1.373(0.421-4.477)	0.600
	S. haematobium negative vs Positive	0.632(0.126-3.182)	0.578
P. chrysogenum			
	Age group 2 vs 1	0.541(0.129-2.270)	0.401
	Age group 3 vs 1	0.621(0.135-2.847)	0.540
	Male vs Female	0.649(0.196-2.145)	0.479
	S. haematobium negative vs Positive	3.006(0.510-17.718)	0.224
R. nigricans			
	Age group 2 vs 1	1.743(0.424-7.168)	0.441
	Age group 3 vs 1	2.294(0.516-10.191)	0.275
	Male vs Female	0.623(0.194-2.002)	0.427
	S. haematobium negative vs Positive	1.112(0.231-5.367)	0.894
S. cerevisiae	Age group 2 vs 1	0.751(0.186-3.028)	0.687
	Age group 3 vs 1	1.524(0.333-6.967)	0.587
	Male vs Female	0.77(0.234-2.535)	0.667
	S. haematobium negative vs Positive	2.304(0.397-13.387)	0.352

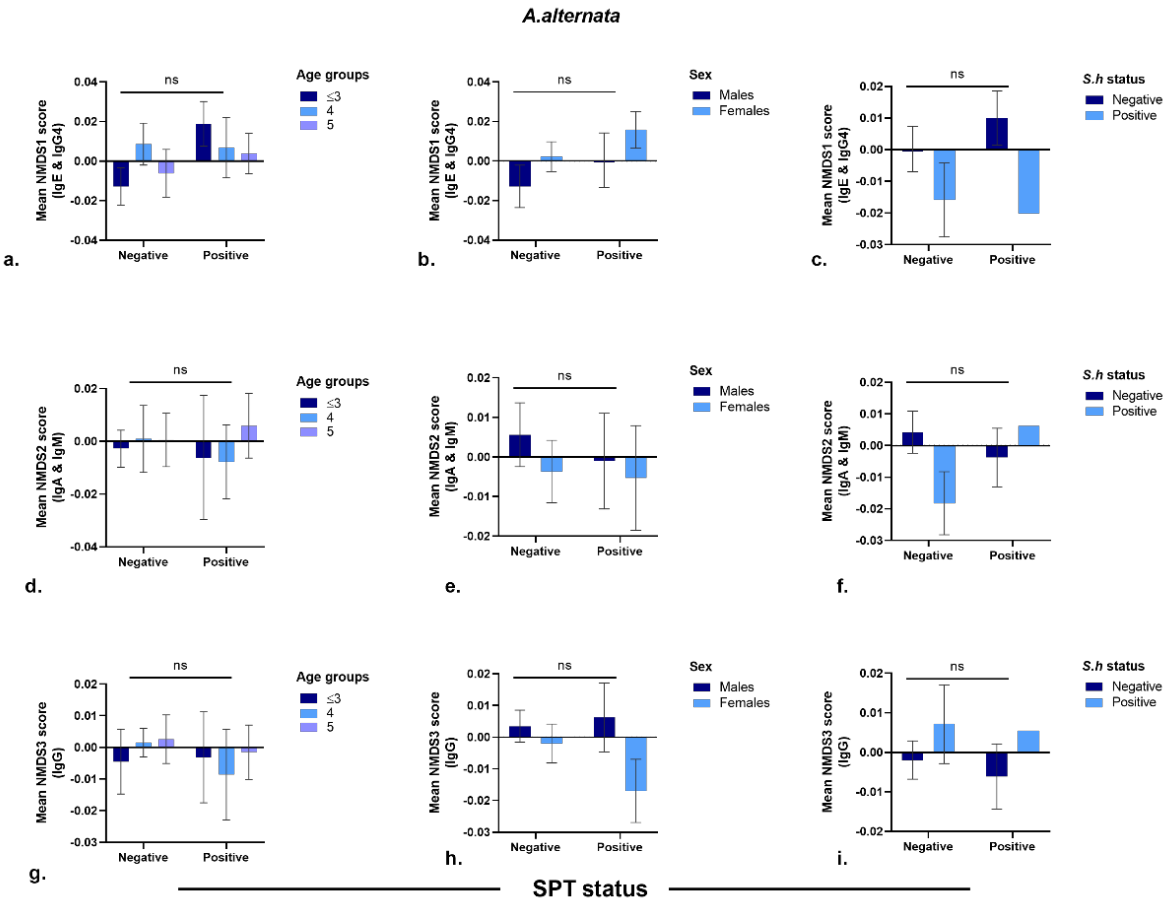


Figure A.2: The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h) and *S. haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses.
Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S. haematobium* infection status and SPT reactivity, are indicated on the graph using *, p<0.05 =*, p>0.05=ns. Standard error of mean is indicated on the graphs for each group.

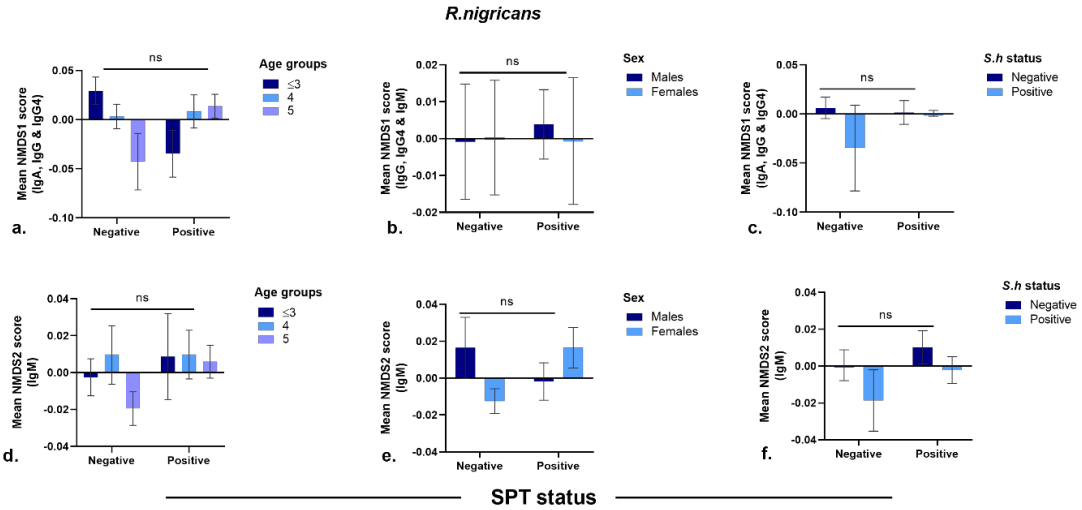
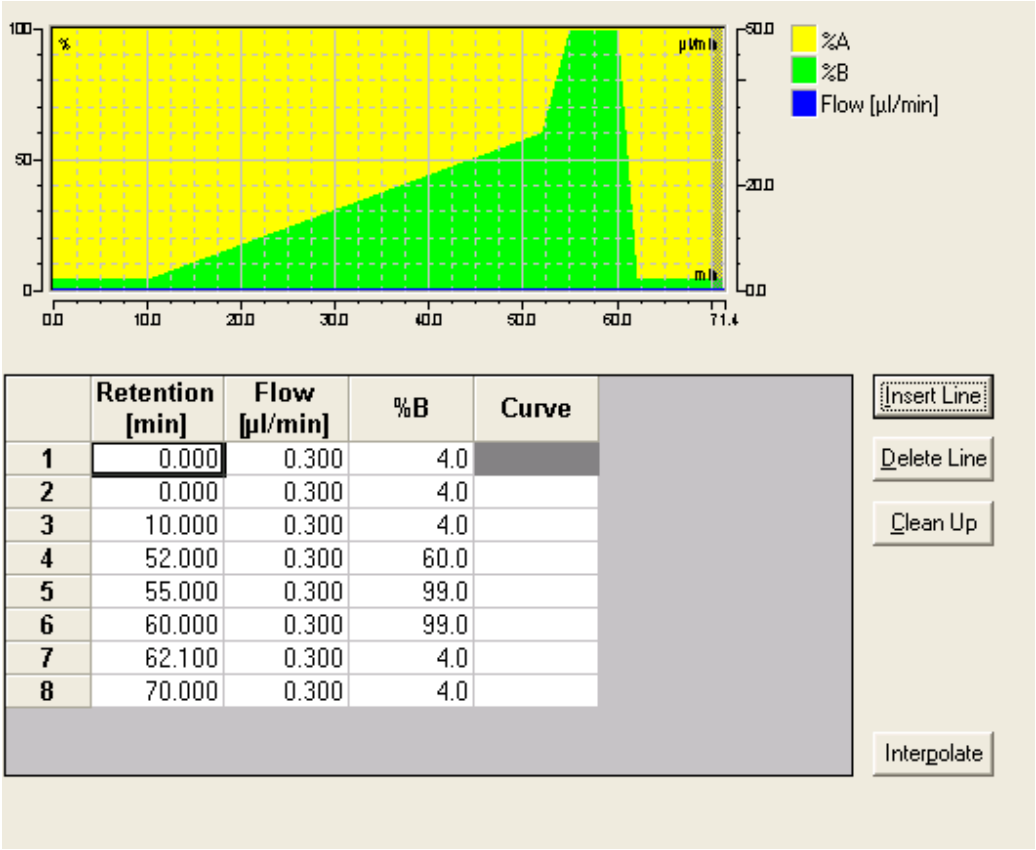


Figure A.3: The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h) and S. haematobium infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses.

Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S. haematobium* infection status and SPT reactivity, are indicated on the graph using *, p<0.05 =*, p>0.05=ns. Standard error of mean is indicated on the graphs for each group.

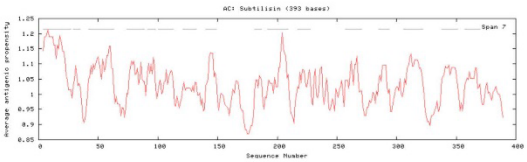
Appendix B Supplementary information Chapter 5



	Retention [min]	Flow [µl/min]	%B	Curve
1	0.000	0.300	4.0	
2	0.000	0.300	4.0	
3	10.000	0.300	4.0	
4	52.000	0.300	60.0	
5	55.000	0.300	99.0	
6	60.000	0.300	99.0	
7	62.100	0.300	4.0	
8	70.000	0.300	4.0	

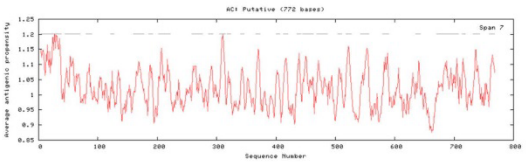
Figure B.1: Solvent gradient conditions for the peptide separation

NCBI Accession no. XP_018384475.1
(subtilisin-like serine protease-like protein PR1A (*Alternaria alternata*)



n	Start Position	Sequence	End Position
1	4	SLLLALLPVALALPAPVIVPRAGS	27
2	29	IPGRYIV	35
3	42	LQALIDAALKVLKKDPVHVYKF	63
4	74	ADDIVELLQNLPGVDY	89
5	91	EQDAVVKA	98
6	100	LGEIHLEKKAYVTQ	113
7	121	GRISHQAKGISS	132
8	140	GAGTCAYVID	149
9	181	HGTHCAG	187
10	192	KTYGVAKKTKLYAVKVLD	209
11	217	SGVIAGINYVAN	228
12	257	AANAVTAGVFMAVAA	271
13	285	SEPTVYTVGA	294
14	306	NYGSIVDILAPGVSILS	322
15	338	ATPHVAGLGAYILG	351
16	357	TPAALSSRLTALSL	370

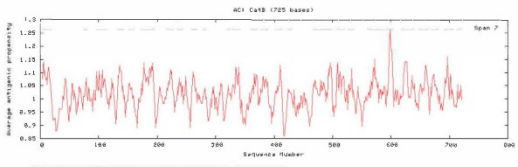
NCBI Accession no. KZN92610.1
Putative dipeptidyl-peptidase (*Penicillium chrysogenum*)



n	Start Position	Sequence	End Position
1	4	SSLCVWSPSIVISVTVRSAVRLLFCAFLLTCVAGY	38
2	44	RVFQLTTALGSIIVSATALTPEQLIAA	70
3	80	PSGEVALFSTS	90
4	121	NVSELTW	127
5	160	ANSAYKAASLPAPLSGLKT	178
6	185	DINFLVYG	192
7	202	NKELVAAPLSSARIHDSIYVR	222
8	258	SLKNLVAGVKNLESPYPPF	276
9	286	SNGKLVAFK	294
10	305	HTASYIYVVPH	315
11	318	SKKAVAI	324
12	340	SSSPVFS	346
13	366	RRTVYVYT	373
14	400	GKSLVLN	406
15	412	RSRLFLLPA	420
16	435	GSVAAYYG	442
17	445	NGEYLVSSSAIHTSRTVYT	463
18	469	GVTGVIFSANEVDPALKN	486
19	504	IHSWIIYP	511
20	518	KSYPLLFYIH	527
21	543	NYKVFADQGYVVIA	556
22	579	SYPYDDLVKCWE	590
23	635	DGTFVADAK	643
24	671	DPSAPEHIKQFATPQLIIHSDLDYRLPISEGLALFNI	707
25	713	VPSRLLS	719
26	732	NSLVWHQQVLGW	743
27	760	DTTIPVVDY	768

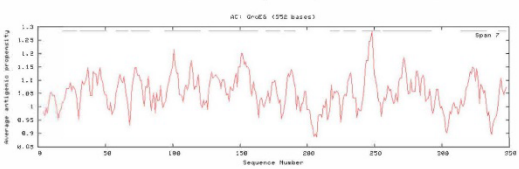
Figure B.2: Subtilisin-like serine protease-like protein PR1A *(A. alternata)* and Putative dipeptidyl-peptidase *(P. chrysogenum)* determined antigenic peptides. Predictions are based on a table that reflects the occurrence of amino acid residues in experimentally known segmental epitopes [608, 610]

NCBI Accession no. KAB8269428.1
Catalase B (*Aspergillus minisclerotigenes*)



n	Start Position	Sequence	End Position
1	4	LSLASLIGIASAACPY	19
2	74	GPTLLED	80
3	96	PERAVHARGVGAHGVFTS	113
4	132	ETPVFVRFSTV	142
5	151	LARDVHGFAT	160
6	173	GNNIPVFFIQDAILFPDLIHAVK	195
7	216	FSQQFSSLHTLLWA	229
8	235	IPRSLRHVDG	244
9	258	DSKLVKFHWK	267
10	275	EEAGGVS	284
11	320	LKFGFDL	326
12	328	DPTKVPEEYVPIT	341
13	361	QVMFQPGHIVRG	372
14	377	EDPLLQGRLFSYID	390
15	401	FEQLPNQPRVPV	413
16	423	QMFIPLNP	430
17	466	FTRAVSPSFEDWSQPRLFYNS	487
18	489	TPAEQQKVVDA	499
19	507	VKSSVVRNNVIIQLN	521
20	527	_ARRVARAIGV	537
21	560	GKLKKIDGLKVGFLASVET	578
22	580	ASIEAASELSK	590
23	593	SEDGVDVVVVAE	604
24	618	DAQFDAVVAPGAEGLFSTLSF	640
25	648	SSGTLFPAQRPLQIVID	664
26	669	FGKPVGAVGSAATA	681
27	691	RDGVYYDK	698
28	700	V~SGHVD	706
29	713	RTFKFLDR	720

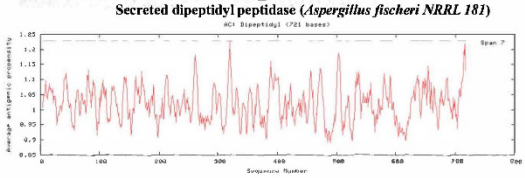
NCBI Accession no. XP_018385554.1
(GroES-like protein (*Alternaria alternata*)



n	Start Position	Sequence	End Position
1	18	GPVDYKKIPVQK	29
2	31	GPDEVLVNIKYSGVCHTDLHA	51
3	58	LATKLPLVGG	67
4	69	EGAGVVVARGDLVTD	83
5	94	WLNGSCLACDFCQQADEPLCPKPLLSGY	121
6	127	FQQYCIAKAAHVARIPKECDLAAIAPVLCAGITVYKG	163
7	169	VKPGQFAAIVGA	180
8	183	GLGSLACQY	191
9	217	GAEAFVDF	224
10	227	SKNLVADVQK	236
11	241	GLQPHVVILVAVNE	254
12	256	PFQQAAEYVRPRGTVICIGLPAGAYLKAPVFETVIKM	292
13	314	RRGLIKAPYKVVGLSELQMV	333
14	342	IAGRYVL	348

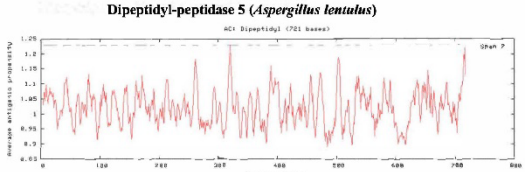
Figure B.3: Catalase B *(A. minisclerotigenes)* and GroES-like protein *(A. alternata)* determined antigenic peptides in predicted antigen proteins.

NCBI Accession no. XP_001260402.1
Secreted dipeptidyl peptidase (*Aspergillus fischeri NRRL 181*)



n	Start Position	Sequence	End Position
1	4	FRWLSIAAAASTALALTPEDLT	26
2	37	PSGKVAVFSTSQYSF	51
3	59	WWSLLDL	65
4	68	GQSKVLTN	75
5	78	SVSEIVWLSDDSILYV	93
6	100	IPGGVELWVTQ	110
7	119	KAASLPASFSG	129
8	139	DIRLVAYGQ	147
9	159	LAAAPLSSARIYDSIYVRHWD	179
10	184	TTFNAVFS	191
11	209	LKNLVSPVKN	218
12	237	DGKWVAFK	244
13	256	TTSYIYLVPH	265
14	290	SSSPVFS	296
15	298	KSDKLAY	304
16	314	SDRRVLYVYSL	324
17	350	GKTLVGS	357
18	364	RLFSLPA	370
19	385	GSVSAYYFLPDSSLLVTG	402
20	416	PEKGVIKTL	423
21	453	DIHAWVIY	460
22	468	K<YPLIFFIH	477
23	499	DQGYVVVAP	507
24	515	FGQALTD	521
25	531	PYDDLVKCWEYIHEHLDFVDTDHGVAAG	558
26	542	IHEHLDFVDTDHGVAAG	558
27	574	LGRKFKALVSHDGTPVADAK	593
28	624	APERILQFATPMLVIHS	640
29	643	DYRLPVAEGLSLFNVLQER	661
30	674	NHWVVNPENSLVWHQQAL	691
31	704	NPDAVSLEDTVVPV	717

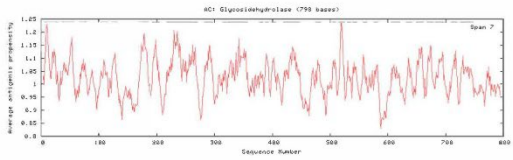
NCBI Accession no. GFF50131.1
Dipeptidyl-peptidase 5 (*Aspergillus lentulus*)



n	Start Position	Sequence	End Position
1	4	LRWLSIAAAASTALALTPEQLIT	26
2	37	PSGKVAVFSTSQYSF	51
3	59	WWSLLDL	65
4	69	GTKVLTN	75
5	78	SVSEIVWLSDDSILY	92
6	100	IPGGVELWVTQ	110
7	119	KAASLPASFSG	129
8	139	DIRPVAYGQ	147
9	159	LAAAPLSSARIYDSIYVRHWD	179
10	184	TTFNAVFS	191
11	209	LKNLVSPVKN	218
12	237	DGKWVAFK	244
13	256	TTSYIYLVPH	265
14	290	SSSPVFS	296
15	314	SDRRVLYVYSLSSKKTIPSV	333
16	350	GKTLIVGS	357
17	364	RLFSLPA	370
18	385	GSVSAYYFLPDSSLLVTG	402
19	416	PEKGVIKTL	424
20	453	DIHAWVIY	460
21	468	KKYPLIFFIH	477
22	499	DQGYVVVAP	507
23	515	FGQALTD	521
24	531	PYDDLVKCWEYIHEHLDFVDTDHGVAAG	558
25	574	LGRKFKALVSHDGTPVADAKVST	596
26	624	APERILQFATPMLVIHS	640
27	643	DYRLPVAEGLSLFNVLQER	661
28	674	NHWVVNPENSLVWHQQAL	691
29	704	NPDAVSLEDTVVPV	717

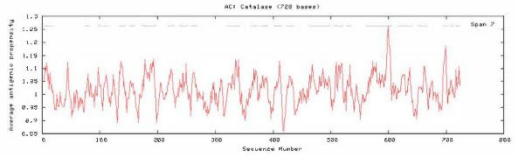
Figure B.4: Secreted dipeptidyl peptidase *(A. fischeri NRRL 181)* and Dipeptidyl-peptidase *5 (A. lentulus)* determined antigenic peptides in predicted antigen proteins.

NCBI Accession no. XP_018381330.1
glycoside hydrolase (*Alternaria alternata*)



n	Start Position	Sequence	End Position
1	6	RSLGHTIMATGAYAGPVPPCTG	29
2	36	TECASAEPLARAKSLVALYTL	57
3	67	GSMGVMRLGSMPY	79
4	103	SYSTSFPQFILMC	115
5	122	LTEVAKMS	131
6	170	DPYHLSCYVGALVHGL	185
7	192	KYKRVNMTCKHFAG	205
8	217	YGNDVCSQQCLVEYYLVPHGADVGAMVDAFMCEYNAVNGVPTCADPYLLGTL	270
9	283	VTSDCDAVGNVYLPH	297
10	315	TDLTCGT	321
11	323	MQEHLPAAFQQCLEESVLDGALVRQYSSLVRLGY	357
12	378	ASCALAR	384
13	388	ALGRVLL	394
14	399	ILHLSLDS	406
15	419	ATSQLGYYAGVSTYLHSPLYA_EQLNVT	448
16	474	STSDLMYYG	483
17	504	AGLDVT	510
18	519	TGKPVWWTG	526
19	530	DSETLVN	536
20	540	ISAEVNA	546
21	556	AIDNTG	563
22	575	YMENYAK	583
23	606	NGSAVYEFGHGLHYT	620
24	632	NSYASALTG	641
25	649	PLERDPAAVDVEV	662
26	668	VTSDYVALGYA	679
27	683	GHAPHHKSLNSYNR	697
28	705	ATLNLTLASLAR	726
29	726	NKVLYFGDYTLLDMHTLASIN	747

NCBI Accession no. XP_001273665.1
(Catalase (*Aspergillus clavatus NRRL 1*))



n	Start Position	Sequence	End Position
1	4	TYLPGLIGVANAMCPY	19
2	75	GPTLLED	81
3	97	PERAVHA	103
4	107	GAHGVFTS	114
5	133	ETPVFVRFSTV	143
6	152	LSRDVHGFAT	161
7	174	GNNIPVFFIQDAILFPDLIHAVK	196
8	217	FSQQPSALHTLFW	229
9	238	RSFRHVDGFGVHTFRF	253
10	259	ATKLVKFHWT	268
11	321	LRFGFDLLDPTKIVPEELVPIT	342
12	362	QVMFQPGHIVRG	373
13	378	EDPLLQGRIFSYLD	391
14	402	FEQLPINQPRVPV	414
15	424	QMFIPLNPHAYSP	436
16	463	VSGKLIRAVSPTFEDVWSQPRLFYNSLIPAEQQFIIDA	500
17	507	NVKSPIVKNNVVIQL	521
18	528	LARRVARA	535
19	562	KLKKLDGLKVGFFASVQHASSLDAASALRASLSKAGVDVVVAE	605
20	617	TSDAIQFDAIIA	629
21	631	GAETLFSLGS	640
22	652	TTSLYPTGRPLQILID	667
23	694	RDGVVVAQ	701
24	706	GFVEHIK	712
25	716	STFKFVDRF	724

Figure B.5: Glycoside hydrolase *(A. alternata)* and Catalase *(A. clavatus NRRL 1)* determined antigenic peptides in predicted antigen proteins.

Appendix C Buffer recipes

1. *ELISA buffers*

Carbonate bicarbonate buffer, pH 9.6:	0.795g $NaCO_3$
	1.465g $NaHCO_3$
	500ml distilled water

Blocking buffer:	100mls of 10X PBS
	900ml distilled water
	300ul Tween20
	50g Marvel
	Or (for CD23)
	100mls of 10X PBS
	900ml distilled water
	300ul Tween20
	20g BSA

Washing buffer:	500mls of 10X PBS
	4500ml distilled water
	1.5ml Tween 20

2. *Buffers for Western blot*

Transfer buffer (pH8.6):	25mM Tris
	192mM Glycine
	20% (v/v) Methanol

TBS (pH7.5):	10mM Tris-HCl (pH7.4) 150mM NaCl
TBSTT:	0.05% (v/v) Tween20 + 0.5 Triton-X 100 in TBS
Blocking buffer:	100mls of 10X PBS 900ml distilled water 300ul Tween20 20g BSA

Appendix D Publications

- **Pfavayi LT**, Burchmore R, Sibanda EN, Baker S., Woolhouse M., Mduluza T., Mutapi F. The Identification and Characterization of Immunoreactive Fungal Proteins Recognized by Sera from Zimbabweans Sensitized to Fungi. Int Arch Allergy Immunol. 2022;1-10. doi:10.1159/000524771
- **Pfavayi LT**., Sibanda EN., Baker S., Woolhouse M., Mduluza T., Mutapi F. Fungal allergic sensitisation in young rural Zimbabwean children: Gut mycobiome and seroreactivity characteristics. Curr Res Microb Sci. 2021;2:100082. doi:10.1016/j.crmicr.2021.100082
- Mutapi F., **Pfavayi L**., Osakunor D., Lim R., Kasambala M., Mutemeri A., Rusakaniko S., Chibanda D., Mduluza T. Assessing early child development and its association with stunting and schistosome infections in rural Zimbabwean children using the Griffiths Scales of Child Development. PLOS Neglected Tropical Diseases 2021;15(8): e0009660. https://doi.org/10.1371/journal.pntd.0009660
- Mushayi C., Nyabadza F., Chigidi E., Mataramvura H., **Pfavayi L**., Rusakaniko S., Sibanda EN. A mathematical model for the prediction of the prevalence of allergies in Zimbabwe. World Allergy Organ J. 2021;14(7):100555. doi:10.1016/j.waojou.2021.100555
- **Pfavayi LT**., Denning DW., Baker S., Sibanda EN., Mutapi F. Determining the burden of fungal infections in Zimbabwe. Sci Rep. 2021; 11, 13240. https://doi.org/10.1038/s41598-021-92605-1
- **Pfavayi LT**, Sibanda EN, Mutapi F. The Pathogenesis of Fungal-Related Diseases and Allergies in the African Population: The State of the Evidence and Knowledge Gaps. Int Arch Allergy Immunol. 2020;181(4):257-269. doi: 10.1159/000506009. Epub 2020 Feb 18. PMID: 32069461.

Clinical Allergy – Review Article

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Int Arch Allergy Immunol 2020;181:257–269
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The Pathogenesis of Fungal-Related Diseases and Allergies in the African Population: The State of the Evidence and Knowledge Gaps

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Keywords
Allergy · Fungi · Africa · Fungal diseases · Pathogenesis

Abstract
The prevalence of allergic diseases in the African continent has received limited attention with the allergic diseases due to fungal allergens being among the least studied. This lead to the opinion being that the prevalence of allergic disease is low in Africa. Recent reports from different African countries indicate that this is not the case as allergic conditions are common and some; particularly those due to fungal allergens are increasing in prevalence. Thus, there is need to understand both the aetiology and pathogenies of these diseases, particularly the neglected fungal allergic diseases. This review addresses currently available knowledge of fungal-induced allergy, disease pathogenesis comparing findings from human versus experimental mouse studies of fungal allergy. The review discusses the potential role of the gut mycobiome and the extent to which this is relevant to fungal allergy, diagnosis and human health.

Edited by: H.-U. Simon, Bern.

Introduction

Fungi are eukaryotic, filamentous and mostly spore-forming organisms that are ubiquitous in nature [1–3]. They are important disease-causing agents either directly as exemplified by cryptococcal meningitis [4], pneumocystis pneumonia [5], pulmonary aspergillosis [6–8] or indirectly as allergens that can induce or exacerbate respiratory diseases such as asthma [9].

Fungi are responsible for considerable morbidity as they cause a wide variety of diseases ranging from superficial skin mycoses [10–12] to potentially fatal systemic mycoses [13, 14]. Annual global mortality due to fungal diseases is estimated to be over 1.6 million [15, 16]. In Africa, tinea capitis dominates the overall burden with an estimated 8.6 million [17] affected in Ethiopia, Ghana [18] and South Africa [17, 19]. Despite this, the association between fungal pathogenesis and the adverse health sequelae remains poorly characterised partly because it frequently develops in patients with multiple morbidities including immunodeficiency [20, 21].

In the last decade, there has been an increase in the incidence of fungal diseases [22, 23]. The increase has partly been attributed to climate change with global warming

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believed to favour the propagation of fungal spores [22, 24]. Although fungi are a common and integral part of ecosystems, the impact of fungal diseases on the entire ecosystem can be devastating [7, 25–27]. Thus, fungi are considered a current and future public health problem that should not be underestimated [28].

Public Health Burden of Fungal-Related Diseases

The global prevalence of skin infections due to fungal infestation is estimated to be over a billion [7, 29] with an age-standardised disability-adjusted life year rate of 48.9 per 100,000 sixteen times less than that of malaria (794.7 per 100,000) [30]. More than 100 million people are said to be affected with mucosal fungal infections [16], whilst >10 million people succumb to severe allergies and a million die due to fungal infections [6]. As of 2017, global mortality owing to fungal infections was greater than that for malaria [31] and was equivalent to that for tuberculosis [16, 32]. The public health impact of this relatively silent cause of morbidity and mortality has not been adequately addressed.

In Africa, the precise prevalence of fungal diseases is currently unknown; however, the very large number of HIV [33] and pulmonary tuberculosis cases in most African countries leads to a large number of cases of opportunistic fungal infections [17]. These fungal infections have been observed in most African countries in studies carried out by the Global Action Fund for Fungal Infections [17, 34]. In Senegal, Nigeria, Malawi and South Africa, 12.5, 11.8, 7.54 and 7.1% [19, 35–37] of the populations respectively are estimated to suffer from serious fungal diseases each year. These infections include chronic pulmonary aspergillosis [18, 19, 35], pneumocystis pneumonia [18, 36, 38], cryptococcal meningitis [19, 39, 40], allergic bronchopulmonary aspergillosis (ABPA) [35, 40, 41] and recurrent vulvovaginal candidiasis [40–42]. However, the epidemiology of allergic diseases due to fungi exposure such as asthma and allergic rhinitis has not been fully elucidated [43]. This review focuses on immune-mediated fungal diseases.

Prevalence of the Immune-Mediated Fungal Diseases in African Populations

Allergy was thought to be rare in Africa in line with the hygiene hypothesis [44, 45] until the results of the International Study of Asthma and Allergies in Childhood, which showed an increase in the prevalence of allergic asthma, rhinitis and eczema in African countries [46–49]. Reports from different African countries indicate that allergic conditions are common [50–52]. However, there have been limited reports of allergy due to fungal allergens in the continent due to inadequate reporting, limited awareness and diagnostics [53, 54].

Recently, Kwizera et al. [55] carried out a systematic review and meta-analysis to estimate the burden of fungal asthma in Africa using data from cross-sectional studies and review articles. The data were obtained from 13 African countries, and this showed the average prevalence of fungal asthma as 28%. These results show that fungal asthma is a significant problem in Africa, but there is still a dearth of epidemiological data in most countries [55].

From previous studies in parts of sub-Saharan Africa, the prevalence of fungal sensitisation was high, being 14.9% [50], 53% [56] and 28% [57] amongst referral patients in Zimbabwe, South Africa and Botswana, respectively. The patients included in these studies were secondary referrals, so only those with severe symptoms that warranted specialist consultation and had the financial capacity to afford specialist care were included. Consequently, it is likely that the cost barriers meant only a small proportion of affected individuals were captured in the studies.

The optimum conditions for fungal spore growth are in the range of 12–30 °C [58], but some fungi species can tolerate lower or higher temperatures [22]. This climatic criteria encompass the majority of the African countries located in the subtropical zone, providing an optimum environment for fungal survival and growth [59, 60]. Hence, the data presented in these studies are likely to be an underestimation of the true extent of fungal sensitisation in sub-Saharan Africa.

Types of Immune-Mediated Fungal Diseases

The spectrum of immune-mediated fungal diseases is huge, and a number of these diseases have been widely studied [61, 62]. The main diseases that affect individuals are allergic rhinitis [63], allergic conjunctivitis, allergic fungal sinusitis [64], atopic dermatitis [65] and asthma [66]. Other less common immune-mediated diseases are allergic bronchopulmonary mycoses (ABPM) [67] and hypersensitivity pneumonitis (HP) [68]. These are briefly discussed.

Allergic Rhinitis

Allergic rhinitis is a common inflammatory disease of the nose [62, 69, 70]. It affects up to 40% of the population in Europe and the States [71]. Exposure to fungi/dampness has been associated with allergic rhinitis in epidemiological studies [63, 72]. In a longitudinal population-based study, Shaaban et al. [73] found that the presence of allergic rhinitis significantly increases the probability of adult-onset asthma [74].

Allergic Conjuctivitis

Allergic conjunctivitis is an inflammatory disease of the conjunctiva [75, 76]. It affects 15–40% of the population [75] and maybe associated with allergic rhinitis [72]. Symptoms of allergic conjunctivitis are usually aggravated by exposure to dry and windy climates [77].

Atopic Dermatitis

Atopic dermatitis is a chronic inflammatory skin disease characterised by pruritic skin lesion [78, 79]. Atopic dermatitis usually starts in early childhood and is frequently associated with allergic rhinoconjuctivitis and allergic asthma [65]. Most atopic dermatitis patients have been shown to be sensitised to the fungi *Malassezia* [80].

The small size of fungal spores (<10 μm) [81, 82] enable fungi to penetrate the bronchi, which may lead to allergic reactions of the lower respiratory tract resulting in allergic asthma, ABPM and allergic alveolitis [82].

Allergic Bronchopulmonary Mycoses

ABPM is a rare hypersensitivity disease of the lower airways characterised by sensitisation to fungi [83]. ABPM occurs in susceptible individuals with asthma and cystic fibrosis [84]. The most frequent ABPM is caused by *Aspergillus fumigatus* antigens and is commonly known as ABPA [85]. The pathogenesis of ABPA is characterised by colonisation of fungi in the lower airways and combines elements of Type I, III and IV hypersensitivity reactions [86].

Allergic Fungal Sinusitis

Allergic fungal sinusitis is a severe form of chronic rhinosinusitis in which individuals develop an intense inflammatory reaction to airborne fungi [87]. The pathogenesis is characterised by eosinophil-predominant Type I hypersensitivity reaction sustained by fungal antigens in the mucosa of the sinonasal tract in atopic individuals [64, 88].

Hypersensitivity Pneumonitis

HP also known as extrinsic allergic alveolitis [89] is an immunologically mediated lung disease, which predominantly occurs as an occupational disease [90]. The pathogenesis of HP is characterised by Type III and IV hypersensitivity reactions [68].

Allergic Asthma

Allergic asthma is an inflammatory disease of the airways characterised by bronchial hyperresponsiveness and airflow limitations [91, 92]. Fungal sensitisation maybe associated with severe asthma attacks requiring hospital admission [93]. Although the evidence that fungi can act as an asthma trigger is widely accepted, the mechanisms by which this occurs are still not clear [94, 95], nor has it been conclusively proven that fungi exposure is responsible for these clinical manifestations [96].

While effective therapies for controlling allergic reactions are available, none are curative. Consequently, allergic diseases such as asthma often persist from early childhood through to adulthood [97, 98]. Such allergies usually have a detrimental effect on the quality of life of the affected individual and have been known to affect their sleep, competencies at work or school as well as their social interaction [99].

Auto-Allergic and Autoimmune Conditions

Fungi contribute to auto-reactivity against self-antigens due to shared epitopes between fungal and human proteins [61] such as manganese superoxide dismutase [100], thioredoxin, cyclophins and acid ribosomal proteins. The underlying mechanism is thought to be molecular mimicry [61, 101] maintaining severe chronic allergic diseases such as atopic dermatitis [102].

Table 1. Species-specific allergens

Allergen source (species)	Allergen	Molecular weight range, kDa	Protein family	References
Alternaria alternata	Alt a 15*	50–58	Serine proteases	[1]
	Alt a 10*; Alt a 8*	28–53	Dehydrogenases	[2, 3]
	Alt a 4*	57	Disulfide isomerases	[2, 4]
	Alt a 7*	22	Flavodoxins	[2, 4]
	Alt a1*	11–45	Unknown	[2, 5]
Aspergillus fumigatus	Asp f 23*	44	Ribosomal proteins	[6]
	Asp f 17*	19.42	Galactomanno proteins	[6]
	Asp f 34*	19–20	Cellwall proteins	[7]
	Asp f 10*	34–35	Aspartic proteases	[6, 8]
	Asp f 15*	15–16	Cerato platanins	[6]
	Asp f 9*	33.7	Glycosyl hydrolases	[6, 9]
	Asp f 5*	42–43	Metallo proteases	[6]
	Asp f 2*	34–37	Fibrinogen-binding proteins	[6]
	Asp f 1*	16–18	Ribonucleases	[4]
	Asp f 4*; Asp f 7*	11–45	Unknown	[6]
Cladosporium herbarum	Cla h 9*	50–58	Serine proteases	[1]
	Cla h 8*; Cla h 10*	28–53	Dehydrogenases	[2, 3, 10]
	Cla h 7*	22	Flavodoxins	[2]
	Cla h HCh1	10.5	Hydrophobins	[11]
	Cla h2*	11–45	Unknown	[5]

* These allergens have been approved by the WHO/IUIS Allergen Nomenclature Committee [12]. All the other allergens can also be found in the Allergome database [13].

WHO/IUIS, World Health Organization and International Union of Immunological Societies.

Currently, the evidence for fungal exposure being linked to the induction of autoimmune diseases is controversial. Studies by Miyoshi et al. [103], and Myllykangas-Luosujarvi et al. [104] all suggest that fungal proteins have a role to play in autoimmune diseases. However, further studies are needed to establish the role of fungi in the immunopathology of autoimmune diseases.

Fungal Allergens

The most common fungi species implicated in allergic reactions are *Alternaria*, *Cladosporium*, *Aspergillus* and *Penicillium* [105, 106], which can be established by the use of a skin prick testing or allergen-specific IgE antibody detection [107, 108]. The allergenic proteins of these fungi [109] can induce sensitisation and result in immune-mediated diseases such as asthma [110, 111], allergic bronchopulmonary diseases [112–114] and/or HP [115, 116].

Although progress is being made in identifying and characterising the fungal allergens involved in eliciting allergic immune responses, fungal allergens are thought to be still neglected and underestimated, compared to other aeroallergens [117, 118] such as pollen or house dust mites.

Fungi polysensitisation (sensitisation to multiple fungi) or cross-reactivity is frequently observed in clinical cases. This makes the precise identification of a given fungal allergen challenging. This is further complicated by the fact that fungi share several potentially allergenic epitopes, making a precise diagnosis of a specific fungal allergy difficult [119]. The use of component-resolved diagnostic techniques [120] that involve mapping the allergen sensitisation of a patient at a molecular level using purified natural or recombinant allergenic molecules instead of allergenic extracts [121] has enabled progress in attributing fungal allergen sources to allergic manifestations.

Progress has also been made in the characterisation and identification of clinically relevant allergens. Nonetheless, to improve molecular diagnosis both the cross-reactive and the species-specific allergens need to be identified [118]. From the relevant literature, some of the fol-

Table 2. Cross-reactive allergens

Allergen source (species)	Allergen	Molecular weight range, kDa	Protein family	References
Alternaria alternata	Alt a 6*	45–48	Enolases	[2, 4]
	Alt a 12*; Alt a 5*	11–12	Ribosomal proteins	[2, 4]
	Alt a 3*	65–90	Heat shock proteins	[2, 5]
	Alt a TCTP	18–22	Translationally controlled tumour proteins	[14]
	Alt a NTF2	13–14	Nuclear transport factors	[15]
Aspergillus fumigatus	Asp f 22*	45–48	Enolases	[16]
	Asp f 11*; Asp f 27*	16–20	Cyclophins	[17, 18]
	Asp f 6*	22–25	Manganese superoxide dismutases	[6, 19, 20]
	Asp f 8*	11–12	Ribosomal proteins	[21]
	Asp f 12*	65–90	Heat shock proteins	[2]
	Asp f 3*	17–19	Peroxisomal proteins	[22]
	Asp f 13*; Asp f 18*	32–34	Serine proteases	[23, 24]
	Asp f 28*; Asp f 29	10–12	Thioredoxins	[25, 26]
	Asp f GST	26	Glutathione-S-transferases	[27]
Cladosporium herbarum	Cla h 6*	45–48	Enolases	[2, 28]
	Cla h 12*; Cla h 5*	11–12	Ribosomal proteins	[21, 26]
	Cla h TCTP	18–22	Translationally controlled tumour proteins	[29]
	Cla h NTF2	13–14	Nuclear transport factors	[15]

* These allergens have been approved by the WHO/IUIS Allergen Nomenclature Committee [12]. All the other allergens can also be found in the Allergome database [13].

WHO/IUIS, World Health Organization and International Union of Immunological Societies; GST, glutathione S-transferase; TCTP, translationally controlled tumour protein; NTF, nuclear transport factor.

lowing allergens have been identified from *Alternaria alternata*, *Aspergillus fumigatus* and *Cladosporium herbarum*. These are presented in Tables 1 and 2.

Exposure to Fungi and Fungal Species in Africa

The mid and hot tropical climates [122] in Africa provide favourable growth conditions for fungi species and as such it is possibly the most exposed of all continents [123]. In addition to the climatic conditions, factors such as poverty make it highly plausible for people in Africa to consume mycotoxin-contaminated food. These mycotoxins are produced by some fungal species as secondary metabolites [124]. Majority of the food crops [125] contaminated are part of the main ingredients in weaning porridge [126], and due to this, it has been suggested that exposure to the mycotoxins maybe a causative factor for child stunting and underweight [127, 128] observed in some African children.

Pathogenesis in Fungal Allergic Diseases

In this review, we are looking at the pathogenesis of fungal allergic diseases in a wider study to understand allergic reactivity in Africa. The allergic diseases can result from immune-mediated inflammatory responses to fungal allergen sources causing tissue damage [129]. The fungal allergens can elicit hypersensitivity reactions including of type I (IgE mediated), type III (IgG/IgM-mediated) and type IV (delayed type hypersensitivity), and these may act together to mediate the pathogenesis of different allergic diseases. A schematic illustration of these reactions is shown in Figure 1, but the specific allergens responsible for symptoms remain poorly characterised [95, 117, 130]. Additionally, it is not known why fungal allergens produce more severe airway diseases than other common aeroallergens [67]. One possible explanation could be that colonisation with fungi as well as their ability to actively germinate in the host predisposes the host to immune-related diseases and severe disease course [82, 131].

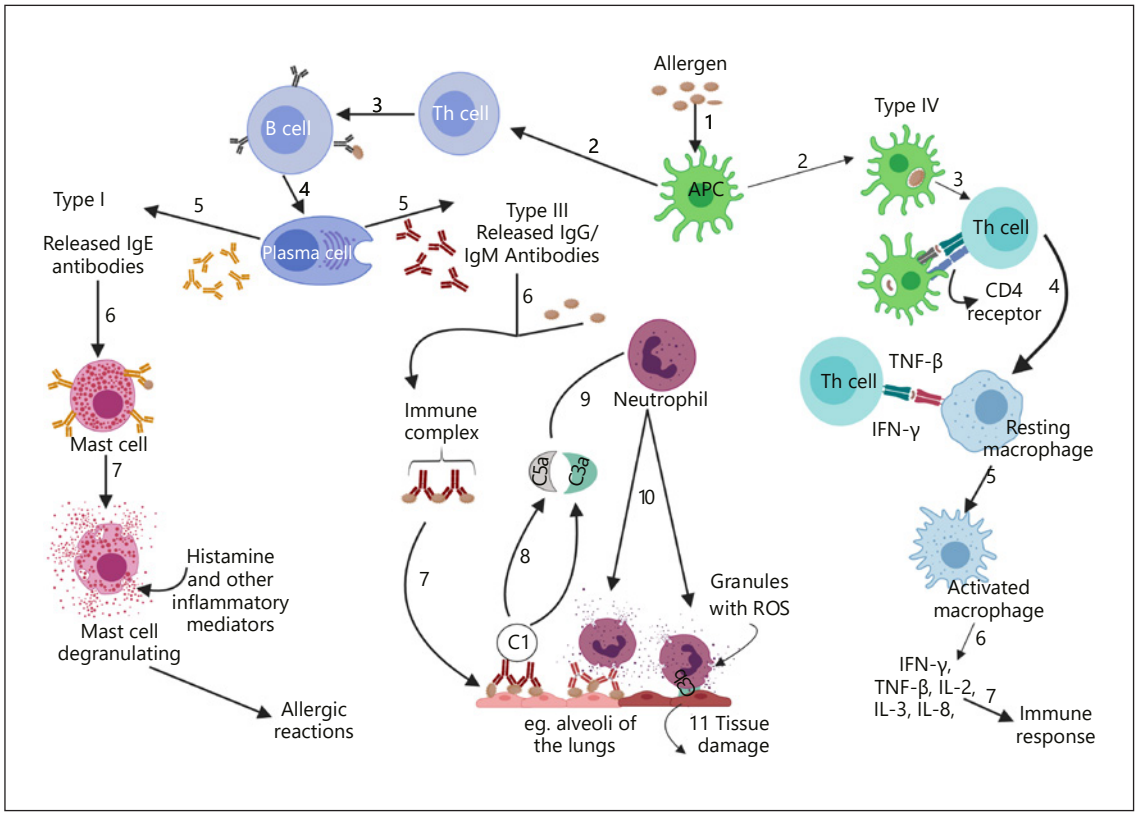

Fig. 1. Mechanisms of hypersensitivity reactions involved in fungal allergy. In the Type I hypersensitivity reaction, the mechanism of action involves preferential production of IgE (5), in response to allergens and the primary cellular component in this hypersensitivity is the mast cell (6). In Type III hypersensitivity reactions, primary components are soluble immune complexes and complement (C3a and 5a) and the injury is caused by neutrophils. In Type IV hypersensitivity reactions, injury is caused by activated macrophages. Diagram adapted from Rajan [185]. IFN-γ, interferon gamma.

Allergic sensitisation involves the development of allergen-specific Th2 responses and IgE production. IgE binds to the high-affinity IgE receptor (FcεRI) present on mast cells. Re-exposure to the specific allergen results in cross-linking of IgE on the mast cell surface, activation and rapid degranulation of the mast cells, with the secretion of active mediators such as histamine. The late phase response involves an influx of Th2 lymphocytes and eosinophils leading to a more prolonged response with tissue damage [132, 133].

Significant progress is currently being made into understanding the mechanistic pathways by which fungi cause or exacerbate allergic diseases such as asthma. It has been reported that fungal cell wall components such as β-glucans, chitin and proteases are the main source of pathogen-associated molecular patterns recognised by pattern recognition receptors as well as protease activated receptors on the host cells [134]. These cell wall components have been suggested to be widely conserved across the fungal kingdom and absent in humans, hence ideal targets for immune recognition [135]. When exposed to β-glucans, chitin and proteases, the epithelial cells mount an immune against these components by releasing chemokines, cytokines and antimicrobial peptides [136]. Repeated exposures to fungi allergens lead to the induction of Th1, Th2 and Th17 reactions and chronic airway inflammation [137–139] as shown in Figure 2.

Fungal proteases induce inflammatory responses by compromising mucociliary clearance, altering the permeability of epithelial barrier, and activating innate immune responses leading to asthma development [140, 141]. The β-glucans induce IL-6, IL-8 and CCL-20 from airway epithelial cells through Dectin-1 receptor [142, 143]. Chitin induces inflammatory responses characterized by IL-17, IL-23 and TNFα [144] as well induce the expression of IL-25, IL-33 and thymic stromal lympho-

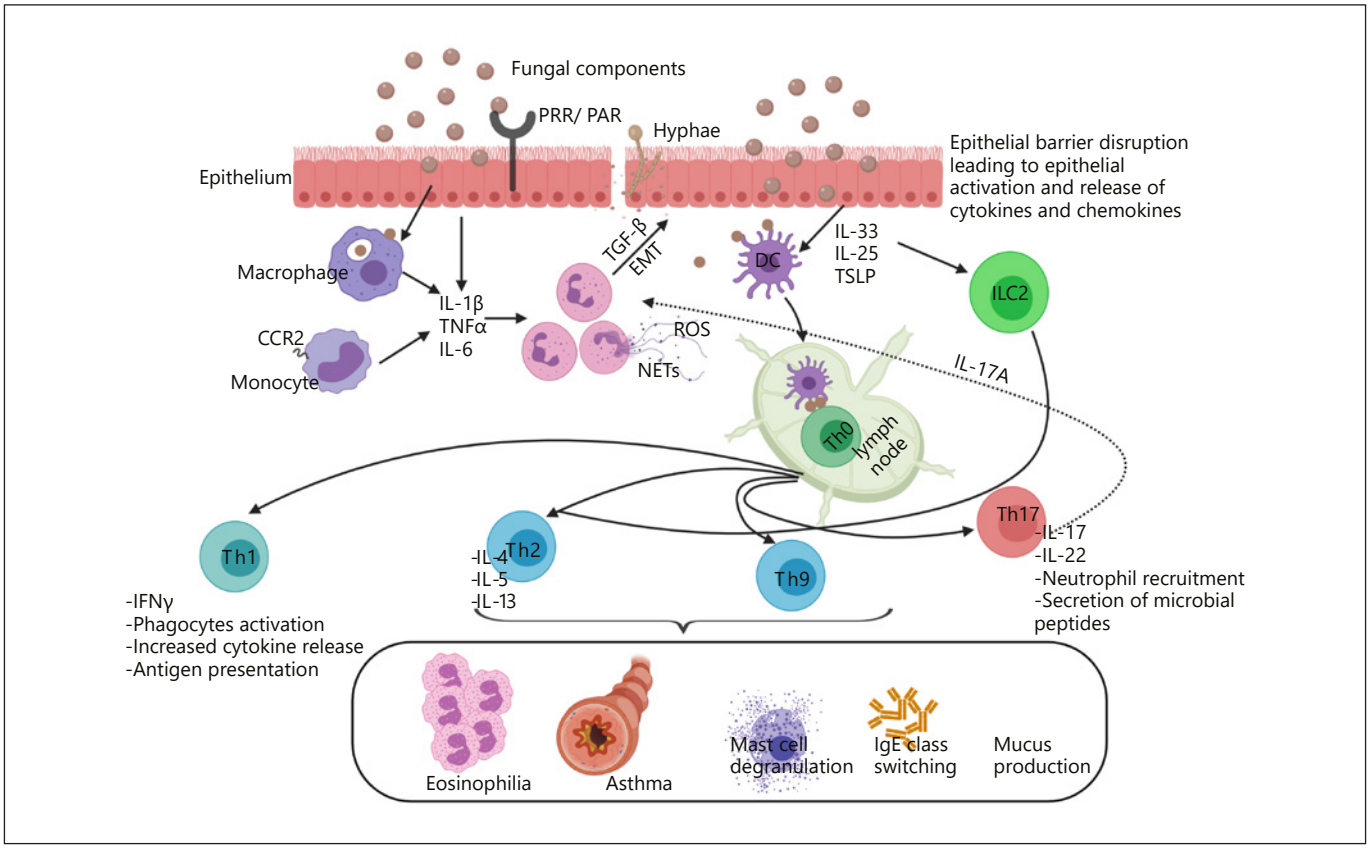

Fig. 2. Cells and cell mediators involved in fungal allergic inflammation. Possible effects of fungal components on the permeability of the airway epithelial and inflammatory responses. The epithelium is exposed to proteolytic enzymes from fungi, which digest proteins of the epithelial layer, making it more permeable. Exposure to fungal components induces the selective release and production of IL-33, IL-25 and thymic stromal lymphopoietin by the airway epithelial cells. Thymic stromal lymphopoietin, IL-33 and IL-25 activate ILC2s to produce type 2 cytokines such as IL-5 and IL-13, initiating allergic inflammation. Adapted from references [146, 148, 182, 186–191]. PRR, pattern recognition receptor; PAR, protease activated receptor; TGF-β, transforming growth factor beta; EMT, epithelial mesenchymal transition; IFN-γ, interferon gamma; ILC2, innate lymphoid cells; TSLP, thymic stromal lymphopoietin; CCR, CC chemokine receptor; NETs, neutrophil extracellular traps; ROS, reactive oxygen species.

poietin, which activate innate lymphoid cells (ILC2s) [145] to express IL-5 and IL-13, leading to eosinophilia [146] and accumulation of alternatively activated macrophages.

ILC2s have been shown to contribute to the initiation and persistence of fungus-mediated allergic immune responses in mice [147, 148], suggesting that they have a role to play in fungal allergy. However, the mechanism that explains how airway exposure to fungal allergens results in increased production and secretion of pro-type 2 cytokines, such as IL-33, leads to activation of ILC2s and other inflammatory cells in airway mucosa, are only partly understood [147]. Therefore, further studies are required to have a better understanding of the mechanistic pathways involved in the pathogenesis of fungal allergy.

Gut Microbiome and Fungal Allergy

There is increasing evidence that resident microbial communities in the gastrointestinal tract, airways and on the skin contribute to health and disease [149]. Several studies have highlighted that gut microbiome dysbiosis can influence susceptibility to non-infectious diseases [150] such as atopic dermatitis, allergy, cancer, obesity and diabetes [151, 152].

In context of the entire microbiota, fungi are considered a minor component [153] and hence rarely focused on when discussing microbiome which mainly refers to bacteria. The role of gut mycobiome in immune regulation and asthma development has been documented in murine experimental model studies. In particular, Wheel-

er et al. [154], investigated the importance of a "healthy mycobiota" in the gut in modulating immune function using mice. In this study they found that prolonged oral treatment of mice with anti-fungal drugs increased the abundance of *Aspergillus*, *Epicoccum* and *Wallemia* spp in the gut and exacerbated the development of allergic airway diseases [154]. The authors also reported that inducing alterations in the existing mycobiome could change the course of house dust mite-induced allergic diseases.

In addition, studies by Noverr et al. [155, 156] demonstrated that mice develop allergic airway responses if their endogenous microbiota is altered as compared to those with normal microbiota. All these studies suggest that there is a connection between the gut microbiome and allergy at least in animal models. The challenge remains how to interpret these sorts of results from experimental studies in terms of human patients.

It has been observed both in human and experimental models that allergic diseases correlate with widespread use of antibiotics [155–159] and alteration in faecal microbiome, which lead to overgrowth of yeast such as *Candida albicans*, which can secrete potent prostaglandin-like immune response modulators, involved in inflammation. Given the widespread use of antibiotics in African countries [160, 161] and the increasing prevalence of allergic diseases in this continent, there is a likelihood that gut mycobiome are involved in allergic diseases, though studies are needed to investigate this association.

The mycobiome has also been implicated in other diseases such as inflammatory bowel disease [162–164], Crohn's disease [165], Autism [166] as well as Rett syndrome [167]. Benito-Leon et al. [168] hypothesised that the gut mycobiome has a role to play in multiple sclerosis and this was observed in a case-control study [169]. However, further studies are necessary to comprehensively understand the role of the mycobiome in the pathophysiology of these diseases.

Limitations of Mouse Models

Studies of fungal exposure and allergy have benefited greatly from the use of murine models to evaluate fungal pathology [147, 170–173]. However, little is known about how these specific cell types translate to human patients who have asthma and other allergic diseases [147].

Murine research has contributed to defining the immunological mechanisms underlying allergic asthma and has provided some understanding of the disease [174]. Although mouse models are widely used, it is important to be cognizant of the fact that mouse airways differ significantly from human airways, in terms of the anatomy, development and physiology as well as in the nature of allergen exposure [174, 175]. These differences underly some of the challenges in translating findings from experimental models to human disease [176].

Mice do not have asthma and do not exhibit spontaneous "symptoms" consistent with asthma [177], and hence, are usually manipulated to develop allergic/Th2-type immune responses. This results in sensitisation of the animal by systemic administration of the allergen, whereas in humans there is no systemic administration of allergen. The allergic diseases in mice are acute and transient, so it is difficult to establish chronic allergic diseases in mice [178]. Furthermore, experimental mice are inbred strains whereas humans are not; hence, other environmental factors might also influence how humans respond to the allergens [175]. Overall, this leads to difficulties in transposing mice immunological responses into useful human data [179].

Knowledge Gaps Relevant to Improve Fungal Allergy and Human Health

Although fungal-related diseases are now recognised as a growing problem globally [6], there continues to be a paucity of epidemiological data in Africa as majority of the published data is from Europe and the States [16]. Additionally, in Africa, there are diagnostic challenges as most people have limited healthcare access due to cost barriers, poor healthcare infrastructures as well as lack of expertise [180].

In general, there is paucity of studies in relevant model systems for human fungal disease; hence, mechanism of pathogenesis remains unclear despite all the research progress made in experimental models. It has been suggested that human microbiome (ensemble of microbes that reside in and on and interact with the human body) [181] has a crucial role in the development and severity of allergic disorders and are involved in their resolution or chronicity. Currently, there is still a limited understanding of the interactions between the human microbiome, immune system and allergic disorders [182].

Allergic sensitisation and inflammation studies of human populations and experimental studies in animal models point to interactions between the external environment, the microbiome, and immune function in early life as causing an underlying predisposition to allergic sensitisation [98]. The majority of the studies report that an alteration in the microbiome [183] is associated with

development or exacerbation of allergic conditions such as asthma [154–156, 184]. Only a limited number of studies have been carried out in human populations, highlighting the need to further extend present knowledge regarding the relationship between the human microbiome and fungal allergy, which would give insight on the pathogenesis of fungal-induced allergies.

Thus, we will be investigating the role of human microbiome in fungal allergy. Of particular interest, is the association of gut mycobiome in the development of sensitivity or tolerance to fungal allergens.

Conclusion

Although progress has been made on identification and characterisation of fungal allergens, the pathogenesis of fungal allergic diseases still remains elusive because of the complexity of the immunological response to fungi exposure, especially in African populations. Understanding this will impact on the way allergic diseases are diagnosed and managed in these populations.

Furthermore, there is a need to further investigate the association between the gut mycobiome and fungal allergies as well as mechanistic pathways of interaction if any, between the two. This will inform the development of appropriate diagnostics and interventions for fungal allergic diseases, particularly those occurring as co- or multimorbidities. This is critical for African health systems where the growing burden of non-infectious diseases must be managed on a background of endemic and epidemic infectious diseases.

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Author Contributions

All authors contributed to the draft manuscript editing, reviewing and approval of the final version of the manuscript.

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Determining the burden of fungal infections in Zimbabwe

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Zimbabwe currently faces several healthcare challenges, most notably HIV and associated infections including tuberculosis (TB), malaria and recently outbreaks of cholera, typhoid fever and COVID-19. Fungal infections, which are also a major public health threat, receive considerably less attention. Consequently, there is dearth of data regarding the burden of fungal diseases in the country. We estimated the burden of fungal diseases in Zimbabwe based on published literature and 'at-risk' populations (HIV/AIDS patients, survivors of pulmonary TB, cancer, chronic obstructive pulmonary disease, asthma and patients receiving critical care) using previously described methods. Where there was no data for Zimbabwe, regional, or international data was used. Our study revealed that approximately 14.9% of Zimbabweans suffer from fungal infections annually, with 80% having tinea capitis. The annual incidence of cryptococcal meningitis and *Pneumocystis jirovecii* pneumonia in HIV/AIDS were estimated at 41/100,000 and 63/100,000, respectively. The estimated prevalence of recurrent vulvovaginal candidiasis (RVVC) was 2,739/100,000. The estimated burden of fungal diseases in Zimbabwe is high in comparison to other African countries, highlighting the urgent need for increased awareness and surveillance to improve diagnosis and management.

Africa has an estimated population of 1.3 billion people and accounts for about 75% of all the 38 million human immunodeficiency virus (HIV)-infected people in the world. Notably, approximately 50% of all fungal-related deaths due to HIV infections are thought to occur in Africa; however accurate data are lacking[1,2]. Data generated by the Global Action Funds for Fungal Infections (GAFFI), suggests an estimated 47.6 million Africans suffer from fungal diseases, of which 1.7 million suffer annually from a serious fungal infection[3]. However, these estimates are based on data from only a few African countries, and most likely underestimates the true prevalence.

Fungal diseases are life-threatening and are responsible for a largely silent epidemic, often hidden killers causing substantial morbidity and mortality in susceptible individuals[4]. Patients with fungal infections occur across a huge spectrum of medical conditions often as co-infections or opportunistic infections[5] and are thus treated as separate entities, hindering progress in diagnosis and management of these patients[6]. Only skin, hair, nails and mucosal infections can be clinically diagnosed (with much imprecision) without specific laboratory testing or medical assessment (radiology, mycology, histopathology) with expensive technologies requiring trained personnel. On the other hand, most life-threatening infections require the referred methods to be diagnosed, which is often out of the reach of patients in poor resource settings.

Nonetheless, substantial progress is being made to prevent and manage some of these fungal diseases. Mycetoma and chromoblastomycosis have been included in the World Health Organisation (WHO) list of neglected tropical diseases[7,8] and new guidelines for the prevention and management of cryptococcal meningitis were recently issued[3].

Fungal infections such as histoplasmosis, mycetoma, chromoblastomycosis, sporotrichosis, cryptococcal meningitis and tinea capitis[9–13] have been reported in Zimbabwe, albeit in few and dated reports. Therefore, there

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Number of Infections per underlying disorder per year								
Infection	None	HIV/AIDS	Respiratory disease	Cancer	Critical care + surgery	Totals	Burden	Rate/100,000
Cryptococcal meningitis		6086				6086	I	41
Pneumocystis pneumonia		9429				9429	I	63
Invasive aspergillosis		800	19	46	1582	2448	I	16
CPA			6182			6182	P	42
ABPA			14,892			14,892	P	100
SAFS			19,657			19,657	P	132
Candidaemia				520	223	743	I	5.0
Candida peritonitis					111	111	I	0.8
Oral candidiasis		77,143				77,143	I	519
Oesophageal candidiasis		63,571				63,571	I	427
RVVC (≥ 4x/year)	203,585					203,585	P	2739[a]
Mucormycosis				30		30	I	0.2
Histoplasmosis		57				57	I	0.4
Fungal keratitis	2081					2081	I	14
Tinea capitis	1,806,700					1,806,700	P	12,156
Total burden estimated	2,012,366	157,086	40,750	596	1916	2,212,715		16,255.4

Table 1. Estimated burden of fungal diseases in Zimbabwe. *I* Incidence; *P* Prevalence; *ABPA* Allergic bronchopulmonary aspergillosis; *SAFS* Severe asthma with fungal sensitisation; *CPA* Chronic pulmonary aspergillosis; *RVVC* Recurrent Vulvovaginal Candidiasis [a]Rate among all females.

is need to update the information on the burden of these infections in Zimbabwe if they are to be prioritised for health intervention.

In the last three decades Zimbabwe's health system has faced considerable issues[14], most notably the demand for providing healthcare services for the control of HIV and associated tuberculosis (TB) as well as for other endemic infections such as malaria and schistosomiasis. The recent cholera[15] and typhoid fever[16] outbreaks further exacerbated Zimbabwe's health challenges. The impact of the current severe acute respiratory syndrome coronavirus (SARS-CoV-2) pandemic[17] on the health system has yet to be fully realised. Thus, given the current health prioritisations in the context of limited resources, controlling fungal infections is currently not a national priority[6]. This may only change with the quantification of the burden of these diseases and their impact on human health in Zimbabwean population.

Thus, in this study, we sought to provide estimates of the burden of fungal infections by using local published data; for those diseases with no existing local data, we used data from neighbouring countries, or international sources.

Results

Using previously described methods, we were able to estimate the occurrence of 2,212,715 cases of fungal infections each year in Zimbabwe (Table 1). The rate of each fungal disease per 100,000 people in Zimbabwe is also represented in Fig. 1.

HIV-related fungal infections. *Cryptococcus neoformans* complex, *Pneumocystis jirovecii* (previously *Pneumocystis carinii*) and oropharyngeal candidiasis are the fungal diseases most commonly associated with AIDS. According to the UNAIDS 2019 report, 1.4 million Zimbabweans were living with HIV, 85% were on ART and 42,857 new AIDS cases at risk of opportunistic fungal infections[18]. *C. neoformans* complex is the most common cause of meningitis globally and is a leading cause of mortality among HIV-infected adults[11,19,20] in these patients. We estimated 6,086 cases (40/100,000) of cryptococcal meningitis. PCP is a major cause of infection in those with HIV/AIDS, and unfortunately, most of these patients are undiagnosed or diagnosed late, particularly in resource-limited settings[21,22]. In Zimbabwe, the largest reported series was 8 (22%) cases of PCP in 1989 of HIV-infected individuals with respiratory symptoms[23]. Assuming 11% of newly diagnosed HIV/AIDS adults[24], develop PCP over 2 years, we estimated 9429 cases (63/100,000) of PCP. PCP may be proportionately more common in children with HIV and was likely a significant contributor to the 3000 children who died of AIDS in 2019[18], but we did not estimate this separately due to the absence of data. We estimated oral candidiasis to affect 77,143 individuals and oesophageal candidiasis 63,571 people living with HIV (PLHIV).

Invasive aspergillosis (IA) and mucormycosis. We estimated a total of 2448 cases of IA annually (16/100,000) including 45 cases in haematological malignancy, 19 cases among those with lung cancer, 800

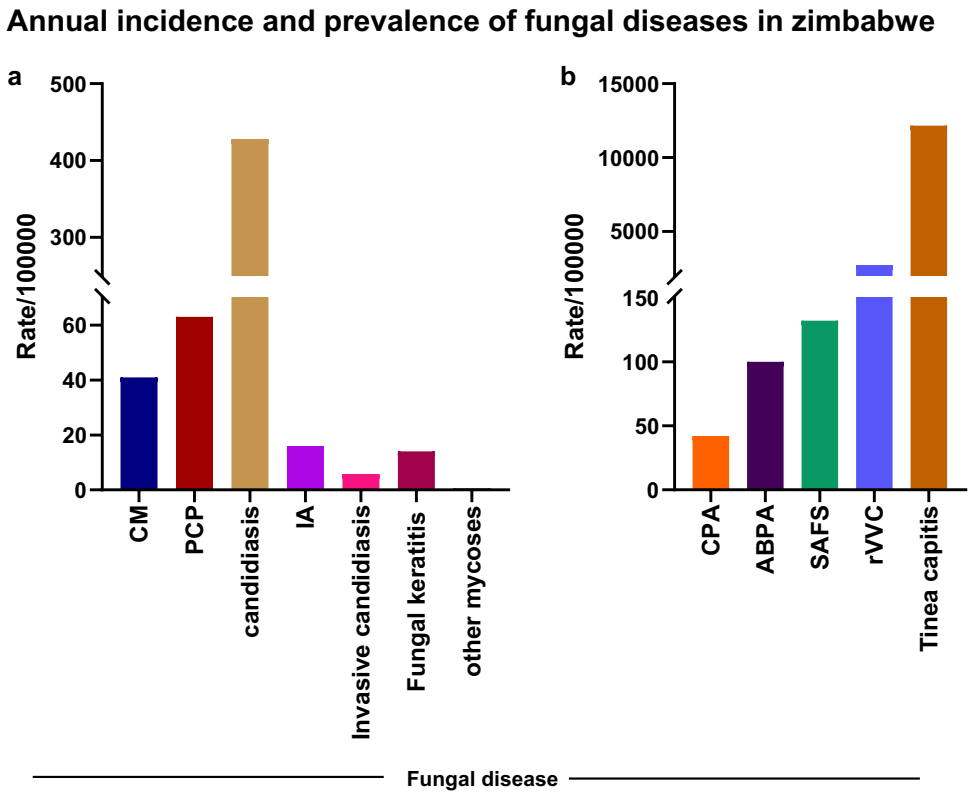

Figure 1. Annual incidence and prevalence of fungal infections in Zimbabwe. Bar charts representing the burden of fungal diseases per 100,000 people (**a**) incidence and (**b**) prevalence for each fungal disease with data available. CM, Cryptococcal meningitis; PCP, *Pneumocystis* pneumonia; candidiasis (oesophageal candidiasis) ; Invasive candidiasis (candidaemia and *Candida* peritonitis); IA, Invasive aspergillosis; other mycoses (histoplasmosis and mucormycosis); CPA, Chronic pulmonary aspergillosis; ABPA, Allergic bronchopulmonary aspergillosis; SAFS, Severe asthma with fungal sensitisation; rVVC, recurrent vulvovaginal candidiasis.

cases among people who died HIV/AIDS and 1582 cases among persons admitted to hospital with COPD. The recognized association with transplantation procedures could not be estimated since these procedures were not undertaken in Zimbabwe during the relevant period. For mucormycosis we conservatively estimated only 30 cases[25].

Histoplasmosis. Histoplasmosis is poorly described in Zimbabwe with limited epidemiological data. However, a study by Oladele et al.[26] reports that *Histoplasmosis capsulatum* var *capsulatum* (HCC) and *Histoplasmosis capsulatum* var *duboisii* (HCD) coexist in Zimbabwe. We estimate 57 cases of histoplasmosis per year. This estimate excluded non-disseminated forms of histoplasmosis.

Non-HIV-related fungal disease burden. Chronic pulmonary aspergillosis (CPA) is a complication of pulmonary TB that is often diagnosed late and may mimic pulmonary TB. It also affects patients with other pulmonary disorders, notably COPD, after pneumothorax and occasionally those with ABPA and asthma[27]. We estimated 6840 of CPA cases per year. Fungal allergy exacerbates asthma, especially in adults. The prevalence of asthma in adults in Zimbabwe was estimated to be 6.9% using data from Democratic Republic of the Congo[28]. We estimated 14,892 and 19,657 cases per year of ABPA and SAFS respectively. There may be some duplication between these entities as many ABPA patients have severe asthma. Therefore, the true 'fungal asthma' prevalence may be 75% of their total. Cystic fibrosis has not reported from Zimbabwe.

Candidaemia and *Candida* peritonitis were estimated to affect 743 and 111 patients, respectively. We did not estimate *Candida* peritonitis complicating chronic ambulatory peritoneal dialysis.

RVVC is defined as four or more episodes of vulvovaginal candidiasis per year[29]. We estimated 203,585 RVVC to occur among adult women in the general healthy female population in their fertile years, which may be conservative. Hormone replacement therapy can precipitate RVVC[30], but we did not estimate this.

Fungal keratitis often occurs following ocular trauma from vegetable material[31,32] and male agricultural workers are at a greater risk[33]. It often leads to blindness and a recent global estimate found a culture and microscopy positive annual incidence of 14/100,000, which translates into approximately 2080 cases in Zimbabwe. However, assuming that culture and microscopy negative cases are usually cases of fungal keratitis[34] in high incidence areas,

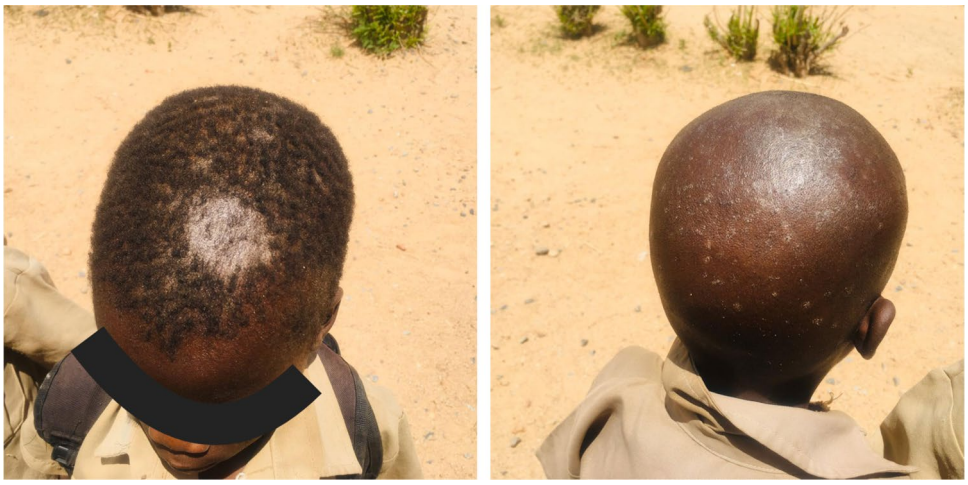

Figure 2. Tinea capitis infection in two young boys from rural Zimbabwe.

this number rises to approximately 2930 cases. Most of the infected eyes will go blind and some will perforate and require removal[34].

Cutaneous fungal infections are very common in southern Africa, but here we focus only on tinea capitis, given its transmissibility, scarring potential and occasional complications of kerion[35]. We estimated 1,806,700 schoolchildren suffering from tinea capitis. The two most frequent dermatophyte species isolated from tinea cases in Zimbabwean children were *Trichophyton violaceum* and *Microsporum audouinii*[9]. These species have been shown to be the most frequent dermatophyte species involved in tinea capitis among children in southern Africa[36–39]. This observation is in accordance with a recent systematic review estimating the burden of tinea capitis among children in Africa[40]. Figure 2 shows tinea capitis infection in two young school boys from rural Zimbabwe.

There were 14 chromoblastomycosis, 3 sporotrichosis and 5 mycetoma cases as reported by Ross and Gelfand in a 10-year survey of histological material[12,13]. Only four cases of blastomycosis were reported in 1991[41], however some more recent isolates such as *Blastomyces gilchristii* have been described[42,43]. Given that these older estimates are the only available data in Zimbabwe, we cannot use them for a reliable estimate of the current burden. The reports however show that these conditions occur in this population and may be underdiagnosed and under reported.

Discussion

A diverse range of fungal infections commonly occurs in Zimbabwe. Frequent HIV and TB co-infections contribute to a higher prevalence of some fungal diseases. Each of these conditions on its own or in combination can predispose individuals to fungal diseases[44]. To date, the burden of fungal infections in Zimbabwe has not been documented. Therefore, we conducted this study to estimate the burden of fungal infections/diseases in the country. Our study indicated that over 2 million people [2,212,715 (14.9%)] suffer from fungal disease annually, a higher number than most African countries. This figure is comparable to that of Senegal[45] and Nigeria[46] with tinea capitis being the most predominant fungal infection.

In Zimbabwe, we found that, following tinea capitis, the most frequent serious fungal diseases were recurrent vulvovaginal candidiasis, oral and oesophageal candidiasis. Vulvovaginal candidiasis (VVC) is a common gynaecological problem occurring among women globally, most commonly caused by *Candida albicans*[47,48]. In previous studies carried out in Zimbabwe among women presenting with symptomatic vaginal discharge, VVC prevalence rates ranged between 25 and 40%[49,50] and we have estimated that 203,585 Zimbabwean women suffer from recurrent episodes. While recurrent vulvovaginal candidiasis (RVVC) is not life threatening, it is a significantly more severe clinical form than VVC. This is because of the recurrence of symptoms defined as four or more episodes per year[29,51] and it is a major health problem for sexually active women. RVVC affects quality of life and is associated with anxiety, depression and a loss of productivity[52–54]. Our estimates, make the burden of RVVC in Zimbabwe the fifth highest among the Southern African Development Community (SADC) countries with available estimated burdens[44,55–57] and some data indicate that the prevalence of RVCC may be higher in Africa than other continents[54,58,59]. Genetic factors have been suggested to be related to the susceptibility of Black/ African women to RVCC. However, comprehensive assessment of the role of genetics in RVCC is still lacking. Likewise, host-related and behavioural factors could also have a role to play[51,60,61].

Oral and oesophageal candidiasis occurs commonly in AIDS patients or those with other immunosuppressive conditions. Oral candidiasis is one of the most common fungal opportunistic infections in immunocompromised individuals[62,63] and was found to be the most common opportunistic infection in Nigeria[64] and Uganda before the initiation of highly active antiretroviral therapy (HAART)[65]. Fluconazole is the drug of choice in the treatment of oral candidiasis[62] because of its bioavailability and efficacy compared with other antifungal drugs[66,67]. However, an increase in resistance of *Candida* species to fluconazole has been reported in some parts of Africa[68–71] and this is important to note because of the implications for morbidity and mortality rates[71,72].

Cryptococcal meningitis is the leading cause of meningitis in sub-Saharan Africa[44,73]. Recent studies have shown that an increasing proportion of patients with cryptococcosis are ART-experienced[74,75]. In Uganda 3% of ART-experienced patients with virological failure were cryptococcal antigen (CrAg) positive[76] with a background rate of 5–10% cryptococcal meningitis. Consequently, to estimate the incidence of cryptococcal meningitis in Zimbabwe we doubled our at-risk population (ART naïve), being cognisant of the fact that virological failure does not always translate to immunodeficiency. We estimated the occurrence of 6086 cases of cryptococcal meningitis per year. We estimated 9429 cases of PCP in HIV/AIDS patients only. Although, PCP is common in children[77–79] and also occurs in non-HIV infected patients we did not include these in our estimates due to paucity of data.

The prevalence of CPA was estimated at 42/100,000, which was relatively high compared to other African countries[55–57]. South Africa had the highest prevalence, at 176/100,000[44]. The high number in Zimbabwe could reflect the relatively high burden of tuberculosis[80] and further studies are required to validate this. The diagnosis requires a combination of imaging and *Aspergillus* IgG antibody testing recently recognised as an Essential Diagnostic by WHO[81], however these are not routinely done in Zimbabwe. Notably a fifth (19%) of CPA patients who were TB smear negative and GeneXpert negative in Nigeria were incorrectly diagnosed as having pulmonary TB[82] with consequent inappropriate treatment.

Asthma is a significant public health problem in Zimbabwe and is often poorly controlled[83]. Here we estimated that 595,677 adults have asthma. In Zimbabwe fungal sensitisation studies have not been conducted, nonetheless we were able to estimate the burden for ABPA and SAFS, which are collectively known as 'fungal asthma'. Fungal asthma differs from allergic asthma. Although the bronchoconstriction can be alleviated by the bronchodilators and inhaled glucocorticosteroids used in the management of asthma, fungal diseases requires the administration of antifungal agents such as oral itraconazole[84–87] and voriconazole, which can only be prescribed if an accurate diagnosis has been made. These antifungal agents act by reducing the fungal load, thus minimizing the stimulus for the ongoing inflammatory activity[88]. If inadequately managed fungal asthma can lead to significant complications such as long term steroid toxicity, bronchiectasis and CPA[89].

A review on the role of antifungals in the management of patients with severe asthma has recently been published[87]. The paper highlights significant studies that confirm lower toxicity of treatment with azoles, particularly itraconazole for ABPA and provide recommendations for the use of antifungal agents in patients with severe asthma, airways fungal infection and fungal colonisation[87].

Dermatophyte infections, especially tinea capitis, are common among children all over Africa, particularly in areas with poor socioeconomic and sanitary conditions[90–92]. They are a public health problem due to their contagious nature[55]. Prevalence rates range from 10% to more than 70% in different regions of Africa[9,40,93,94]. The estimate of tinea capitis in this study was based on Zimbabwean data from 1990[9]. However, there may have been changes over time and these figures may not be a true presentation of the current situation in the country. Nonetheless, it remains a common clinical problem.

Mycetoma is a neglected tropical disease caused by fungi or bacteria and mainly affects the skin as well as the underlying tissues[95]. The morbidity due to mycetoma is high[96] and there are currently no control programmes except in Sudan where it is highly endemic[7,8,97]. Cases of mycetoma have been reported in many African countries[45,98–103] including Zimbabwe[12]. Cases of chromoblastomycosis, sporotrichosis and blastomycosis have also been reported in Zimbabwe. However, to date there are no reports of disseminated *Emergomyces* infections in Zimbabwe albeit there are some reports from South Africa[104]. Contributory factors include a low index of clinical suspicion, limited diagnostic capacity and a dearth of the requisite clinical and diagnostic expertise.

Most fungal infection studies in Zimbabwe are dated having been carried out about two decades ago[9–11,23]. While these indicate susceptibility in this population, they do not accurately represent the current situation in the country, especially with changes in the epidemiology of HIV whose prevalence has gone down from more than 15%[14] to about 8.7% with 85% of these receiving ART[18]. The health and economic challenges faced by the country with respect to public health priorities, clinical and laboratory expertise, the inadequacy of financial resources militate against the early diagnosis and treatment of fungal infections. Consequently, these health system limitations contribute to high rates of morbidity and mortality[105].

As most serious fungal infections are opportunistic infections, a majority of the affected individuals are immunocompromised[4]. For example, cryptococcal meningitis, oesophageal candidiasis, PCP as well as aspergillosis are among the most common systemic fungal infections observed in HIV and AIDS patients, thus a combination of the underlying immunocompromised and superimposed fungal infection contributes to a higher risk of mortality[106]. The comorbidities necessitate the co-administration of drugs, resulting in drug-drug interactions (DDIs). In some cases, optimum therapy for fungal infections is contraindicated in conjunction with medicines used to treat co-morbid conditions in an attempt to prevent potential adverse effects and treatment failure[107,108].

The preferred treatment for these fungal infections includes the administration of amphotericin B or azole antifungals such as fluconazole, itraconazole and voriconazole. All of these except voriconazole are on Zimbabwe's essential list of medicines[109] and studies from other countries have suggested that renal function should be closely monitored with concomitant use of amphotericin B and tenofovir as both drugs can cause nephrotoxicity. Similarly, combination therapy of zidovudine and amphotericin B may result in anaemia and neutropenia[108]. Hence, as DDIs are often unavoidable in HIV-infected patients, the potential effects of these DDIs cannot be ignored[106] especially in Africa where very few drugs used have been evaluated for DDIs and pharmacogenetics[110]. This will potentially help in the management of patients suffering from co-morbidities as well as broaden our understanding of the effect of these DDIs in different populations. In addition to DDIs there is also a possibility of antifungal resistance[111]. Previous studies have reported fluconazole resistance among *C. neoformans* complex isolates from Africa[112] and among *Candida* spp. isolated from women with VVC[113]. This has a large impact on health and well-being of affected individuals.

Study limitations. Despite an exhaustive search in this study, we could not obtain enough local data to use for a precise estimate of the current burden, as most of the available studies were outdated. So, majority of the data used was obtained from other countries, which may introduce some inaccuracies when estimating the burden in Zimbabwe due to socioeconomic and geographical differences[114]. Another significant limitation is the incomplete nature of the estimates: for example, we could not estimate the burden of PCP in children or non-HIV patients, the burden of mycetoma, chromoblastomycosis as well sporotrichosis could not be estimated due to paucity of data despite reports of cases in the country. Nonetheless, our results show that fungal diseases are probably much more common than are documented in clinical practice. Our estimates provide a starting point from which to better understand the extent of the problem in the country and create awareness and propose appropriate studies and interventions to address fungal diseases that are of significant public health importance.

Recommendations. Educating the community about fungal diseases is an important step in raising awareness about the morbidity and mortality associated with these diseases. For most villages and communities across Zimbabwe, the entry point to health and health information dissemination is vested in the Community/Village Health Workers. These people are therefore integral in promoting awareness and assisting in early detection of symptoms associated with fungal diseases. Their knowledge of a community's languages and customs means that they are able to deliver health messages to groups in a culturally appropriate manner, which is also easily understood. This is an effective means of disseminating information to community members resulting in community-based surveillance, which improves the likelihood of early case detection[115], as well as reducing the stigma associated with some of the fungal diseases. The medical mycology community in Zimbabwe should therefore work closely with local organisations and community health workers to raise awareness of fungal diseases.

Schools are also a good place to promote campaigns aimed at raising awareness about fungal infections. For example, the neglected tropical disease, schistosomiasis, is a public health burden in Zimbabwe[116]. Educational campaigns in schools including essay competitions, drama and 'edutainment' are ways in which awareness about the disease is raised in communities. A similar approach could be used to create awareness about fungi diseases among school children.

To increase the access to better diagnostics locally, a standardised diagnostic algorithm based on clinical signs and symptoms that can be easily identified by primary healthcare professionals[117] can be developed. The algorithm will help in early diagnosis and treatment of fungal diseases as well as ensure wealth of information on fungal diseases affecting the African population. This will allow interventions to be implemented in the primary healthcare setting and has the potential to significantly reduce morbidity and improve quality of life.

Conclusion

This study is the first to estimate the burden of fungal diseases in Zimbabwe and to provide an estimation of its impact on public health. The paucity of data on fungal infections in the country warrants for further epidemiology studies and better diagnostics to aid patient management.

Methods

The prevalence and incidence of fungal disease in Zimbabwe were calculated following methods previously described[118,119] and the applied formulae will be detailed below. The burden was estimated for the general healthy population and for the 'at- risk' populations including HIV/AIDS patients, survivors of pulmonary TB, cancer, chronic obstructive pulmonary disease (COPD), asthma and patients receiving critical care. National or local data were preferred, but where these were unavailable, data were extrapolated from other sources. The annual burden was estimated for each fungal disease and presented as: (i) absolute number of cases per year in the country and (ii) annual rates. The absolute cases were presented as either incidence or prevalence depending on the nature of infection. The annual rates (incidence or prevalence) were calculated using the absolute annual number of cases as the numerator and the entire Zimbabwean population as the denominator. For simplicity, the 2019 Zimbabwean population (n = 14,863,000[120]) was used regardless of the year from which the numerator data originated. The United Nations population estimates 2019, WHO reports, and The Joint Nations Programme on HIV/AIDS (UNAIDS) were used for the population demographics.

Calculating fungal disease burden. Prevalence or incidence was calculated using data from published studies. Prevalence was calculated for allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitisation (SAFS), chronic pulmonary aspergillosis (CPA), recurrent vulvovaginal candidiasis (RVCC), and tinea capitis and the remaining estimates were calculated as annual incidence. Due to paucity of data, we were not able to calculate both prevalence and incidence for each disease.

Prevalence. To calculate the prevalence of ABPA, SAFS, CPA, RVCC, and tinea capitis we applied the same formulae used by GAFFI members to estimate the prevalence of fungal diseases in other countries[55,60,121,122].

Annual incidence. To calculate the incidence of invasive aspergillosis, oesophageal candidiasis, candidemia and *Candida* peritonitis we applied the same formulae used to estimate the incidence in other countries[55,60,121,122]. For cryptococcal meningitis, *Pneumocystis* pneumonia and oral candidiasis, instead of calculating a figure based on the denominator, we doubled our at-risk population (ART naïve), being cognisant of the fact that virological failure does not always translate to immunodeficiency.

Diagnostic	Score
PCR + laboratory + clinical + imaging	2
Culture, smear, histology	1
Clinical suspicion only	0
Patient sample size	Score
≥ 10	1
< 10	0
Year of study	Score
< 5 years	2
5–10 years	1
> 10 years	0
Country (data used)	Score
Zimbabwe	2
Any other African country	1
Rest of the world	0
Methodology (well designed)	Score
Yes	1
No	0
Type of publication	Score
Research paper	2
Case study/short reports	1
Review papers	0
Possible total score	**10**

Table 2. Scoring system for modified GRADE criteria.

Cryptococcal meningitis

$$= \left(\text{Annual new AIDS cases} \times \text{Proportion of AIDS patients presenting with cryptococcal meningitis}\right) \times 2$$

Pneumocystis **pneumonia**

$$= \left(\text{Annual new AIDS cases} \times \text{Proportion of AIDS patients presenting with } \textit{Pneumocystis} \text{ pneumonia}\right) \times 2$$

$$\textbf{Oral candidiasis} = \left(\text{Annual new AIDS cases} \times 0.9\right) \times 2$$

The values above and the assumptions made to obtain the accurate denominators were obtained from a systematic literature search detailed below. The assessment of the quality of the source data, country profile and assumptions made for the analyses are detailed below.

Data sources and search terms. Published papers were identified from four databases: PubMed, Web of Science, EMBASE and Google Scholar. The following search terms were used: fungal infection, fungal burden, fungal epidemiology, Zimbabwe, Southern Africa, and Africa. A second search included the same searches using the following diseases: *Cryptococcus*/cryptococcal, *Candida*/thrush, *Aspergillus*/aspergillosis, histoplasmosis, asthma, leukaemia, chronic obstructive pulmonary disease (COPD), *Pneumocystis* pneumonia/*Pneumocystis jirovecii* pneumonia (PJP)/*Pneumocystis carinii* pneumonia, chronic pulmonary aspergillosis (CPA), aspergilloma, allergic bronchopulmonary aspergillosis (ABPA), severe asthma with fungal sensitisation (SAFS), tinea/ringworm. We used HIV data to estimate the burdens of cryptococcal meningitis (CM), candidiasis and *Pneumocystis jirovecii* pneumonia (PCP). Asthma, chronic obstructive pulmonary disease and tuberculosis data were used to estimate the presumed burden of allergic bronchopulmonary aspergillosis (ABPA) and chronic pulmonary aspergillosis (CPA). Burdens of candidaemia and *Candida* peritonitis were derived from critical care and/or cancer patients' data.

Papers presenting incidence or prevalence of any fungal disease were evaluated using an adapted Grading of Recommendations, Assessment, Development and Evaluations (GRADE) score[123] based on the following features: diagnostic accuracy, study size (using a cut-off of > 10 cases), year of study, with more recent studies scoring higher, type of publication, with original research article scoring more, methodology and country, with studies from Zimbabwe scoring higher (Table 2). Those with an adapted GRADE mean score of > 2 were deemed acceptable and enabled a minimum estimation of the country burden of fungal diseases (Table 3). Papers with a mean score < 2 were excluded in the estimation of the country's burden of fungal diseases but were discussed in the review.

Disease	Diagnostic accuracy	Patient sample size n > 10	Up to date	Type of publication	Methodology	Country	Overall score	References
PCP	2	1	1	2	1	1	8	24
Histoplasmosis	1	1	1	1	0	2	6	10
Invasive aspergillosis	2	1	1	2	1	0	6	124
	1	1	0	1	1	0	4	125
	1	1	1	1	1	0	5	126
	0	1	1	1	1	0	4	127
Candidaemia	–	–	1	0	1	–	2	128
Candida peritonitis	1	1	1	1	1	0	5	129
ABPA	–	–	1	0	1	–	2	130
SAFS	–	–	1	0	1	–	2	89
CM	–	–	2	0	1	–	3	131
RVVC	–	–	2	0	1	0	3	132
Tinea capitis	1	1	0	2	1	2	7	9
Mucormycosis	0	1	1	1	1	0	4	133
Fungal keratitis	–	–	1	0	1	–	2	34

Table 3. Modified GRADE score for the papers used for estimating burden of fungal diseases in Zimbabwe. *CM* Cryptococcal meningitis, *PCP* Pneumocystis pneumonia, *CPA* chronic pulmonary aspergillosis, *ABPA* allergic bronchopulmonary aspergillosis, *SAFS* severe asthma with fungal sensitisation, *IA* invasive candidiasis, *RVVC* recurrent vulvovaginal candidiasis.

		Patient numbers and rates	Source
Demographics	Total population	14,863,000	120
	Children (< 15 years),	6,230,000	
	Total number of adults,	8,633,000	
	Adult women	4,489,160	
HIV/AIDS	Current total HIV/AIDS	1,400,000	18
	Children with HIV	84,000	
	Proportion of diagnosed cases on ARVs	85%	
	Number of diagnosed cases receiving ARVs	1,100,000	
	Proportion of those on ARVs who fail or have ARV resistance Number of diagnosed cases not receiving ARVs	11% 300,000	135
	Annual new AIDS cases (at risk of OIs)	42,857	
	AIDS-related deaths	20,000	
Respiratory diseases	Pulmonary tuberculosis annual incidence (survivors)	20,430	80
	Prevalence of asthma in adults	6.9%	28
	COPD prevalence (all GOLD stages) COPD hospital admissions	7.8% 121,728	136 137,138
Lung cancer		744	134
Diabetes		4.6%	139
Leukaemia	AML	230	140

Table 4. Country's profile. Populations and rates required to calculate burden fungal-related diseases in Zimbabwe. *COPD* chronic obstructive pulmonary disease, *GOLD* Global initiative for Obstructive Lung Disease, *ARV* antiretroviral, *OI* opportunistic infection, *AML* acute myeloid leukaemia.

Country profile. Zimbabwe is a landlocked country situated in Southern Africa, between the Zambezi and Limpopo Rivers, bordered by Botswana, Mozambique, South Africa and Zambia. In 2019, the Zimbabwean population was projected to be 14.9 million, with 58% adults[120]. The number of people living with HIV/AIDS (PLWH) as of 2019 was estimated to be 1.3 million. The population estimates and HIV-related deaths were obtained from World Population Prospects and UNAIDS respectively and are shown in Table 4 [18,120]. National TB data were obtained from the World Health Organization (WHO)[80]. National prevalence data for lung cancer, chronic obstructive pulmonary disease (COPD), diabetes and incidence data for acute myeloid leukemia (AML) were obtained from the 2016 Global Burden of Disease study[134]. To estimate the burden for HIV-related fungal diseases, we have assumed a 7-year linear decline in CD4 count to $< 200 \times 10^6$/l, of those not on ART, doubled to reflect those on ART who fail with ARV resistance or default (at risk of opportunistic infections).

Fungal disease	Underlying condition	Assumptions made	References
Cryptococcal meningitis	HIV/AIDS	Assumes 7.1% of AIDS patients	131
Pneumocystis pneumonia	HIV/AIDS	Assumes 11% PCP as newly diagnosed HIV/AIDS adults over 2 years	24
Invasive aspergillosis	HIV/AIDS; COPD; Leukaemia; lung cancer	Assumes 10% of AML patients develop IA. Rate in non-AML same as in AML. 1.3% of admitted COPD patients, 2.6% of lung cancer patients and 4% of HIV/AIDS deaths	124–127
Chronic pulmonary aspergillosis	Tuberculosis, COPD	Assumed that 22% of those with and 2% of those without cavities after TB develop CPA; that pulmonary tuberculosis is the underlying diagnosis in 67% of all CPA cases	141
Allergic bronchopulmonary aspergillosis	Asthma	Assumed to occur in 2.5% of adult asthmatics	28,130,143
Severe asthma with fungal sensitisation	Severe asthma	Assumes 33% of worst 10% of adult asthmatics	89
Candidemia		5/100,000 (mean of 2-11/100,000) with 30% in ICU (critical care and post-surgical patients) and 70% in cancer and other immunocompromised patients	128
Candida peritonitis	Pancreatitis, major abdominal surgery	Assumes 1 patient with hospital-acquired (almost all post-operative) Candida peritonitis for every 2 patients with candidaemia, in ICU	129
Oral candidiasis	HIV/AIDS	Assumes it occurs in 90% of untreated HIV patients, over 2 years	144
Oesophageal candidiasis	HIV/AIDS	20% of patients not on ARVs, and 0.5% of those on ARVs	145,146
Recurrent Vulvovaginal Candidiasis (≥ 4x/year)		6% of adult women	132
Mucormycosis		Assumes that it affects 2 per million of the population based on data from Europe	133
Histoplasmosis	HIV/AIDS	Based on literature	10
Tinea capitis		Assumes 29%, based on a study by Robertson and Wright (1990)	9

Table 5. Assumptions on which estimates of fungal diseases were made. *COPD* chronic obstructive pulmonary disease, *CPA* chronic pulmonary aspergillosis.

Assumptions from other published reports were used to identify the most accurate denominators to use for our estimates and these are summarised in Table 5**.** In brief, PCP frequency was estimated by assuming 11% of newly diagnosed HIV/AIDS adults with the risk spread over 2 years[24]. The prevalence of AIDS patients presenting with cryptococcal meningitis was assumed to be 7.1% based on a study by Rajasingham et al.[131] among antiretroviral therapy (ART)-naive HIV patients. Chronic pulmonary aspergillosis (CPA) prevalence was estimated using the previously described approach taken by Denning et al.[141], where the number of annual PTB cases with cavities (22%) was multiplied by the incidence of CPA in cavities (22%) and the number of PTB cases without cavities (78%) was multiplied by CPA incidence (2%). An estimation of a 5-year prevalence of CPA was made, assuming a 15% annual mortality or surgical cure rate[130]. To calculate all cases of CPA, PTB was assumed to be the underlying disorder in 67% of cases[142]. Invasive aspergillosis was estimated in haematological and lung malignancies, HIV/AIDS and COPD. It was assumed that 10% of acute myeloid leukemia (AML) patients develop IA and that an equal number of cases are found in non-AML haematological patients while 1.3% of admitted COPD patients[124], 2.6% of lung cancer patients[127] and 4% HIV/AIDS patients who died develop IA[125]. ABPA estimation was made assuming that 2.5% of adult asthmatics have ABPA[130,143] and although ABPA also occurs in cystic fibrosis, no estimate of the prevalence of this disease in Zimbabwe was attainable. The estimate of SAFS was as estimated at 33% of the most severe asthmatics (10%)[89].

Oral candidiasis was assumed to affect 90% of untreated HIV patients over 12 months, based on a study in Tanzania[144]. Oesophageal candidiasis was assumed to affect 20% of advanced HIV disease patients and 0.5% of HIV patients on ARV treatment[145,146]. Mucormycosis was estimated to occur at a rate of 0.2/100,000 (literature estimate)[25]. Candidaemia cases were estimated assuming it occurs at a rate of 5 per 100,000 with 30% in ICU (critical care and post-surgical patients) and 70% in cancer and other immunocompromised and hospitalised patients[128]. For *Candida* peritonitis (intrabdominal candidiasis), we assumed that the rate was half of the ICU candidemia rate[129]. The estimated prevalence of RVVC was established assuming a frequency rate of 6% in adult women[132]. Tinea capitis was estimated at 29% prevalence amongst schoolchildren in Zimbabwe[9].

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Author contributions

F.M. and D.W.D. conceptualized and designed the study. L.P. and D.W.D. analysed the data. L.P. and F.M. prepared the draft manuscript. S.B., E.N.S. and D.W.D. reviewed and edited the draft manuscript. All authors read and approved the final version of the manuscript.

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Competing interests

LTP, FM., SB. and ENS have no competing interests to declare. DWD and family hold Founder shares in F2G Ltd, a University of Manchester spin-out antifungal discovery company. He acts or has recently acted as a consultant to Pulmatrix, Pulmocide, Zambon, iCo Therapeutics, Mayne Pharma, Biosergen, Bright Angel Therapeutics, Cipla and Metis. He sits on the DSMB for a SARS CoV2 vaccine trial. In the last 3 years, he has been paid for talks on behalf of Dynamiker, Hikma, Gilead, Merck, Mylan and Pfizer. He is a longstanding member of the Infectious Disease Society of America Aspergillosis Guidelines group, the European Society for Clinical Microbiology and Infectious Diseases Aspergillosis Guidelines group.

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Fungal allergic sensitisation in young rural Zimbabwean children: Gut mycobiome and seroreactivity characteristics

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SUMMARY

Background: The prevalence of allergic diseases has increased over the last few decades, with sensitisation to fungal allergens and gut microbiome dysbiosis implicated in this trend. The fungal community in the gut (mycobiome) has yet to be characterised and related to fungal allergic sensitisation. Thus, we characterised the gut mycobiome and related it to fungal sensitisation and seroreactivity among Zimbabwean children. We further determined the effect of host age, sex, *Schistosoma haematobium* infection and mycobiome composition on fungal sensitisation and seroreactivity.

Methods: Using shotgun metagenomic sequencing, we characterised the gut microbiome of stool samples of 116 preschool aged children (PSAC) (≤5 years old, 57(49.1%) male and 59 (50.9%) female). Sensitisation to common fungi in Zimbabwe was assessed using skin prick tests (SPTs). Allergen-specific IgM, IgA, IgG, IgE and IgG4 antibodies were quantified by ELISA. We analysed the relationship between fungal genera and SPT reactivity by ANOVA; fungal genera and IgE antibody reactivity by linear regression; variation in mycobiome abundance with host and environmental factors by PERMANOVA; SPT reactivity and host and environmental factors by logistic regression; seroreactivity and host and environmental factors by ANOVA.

Results: The mycobiome formed <1% of the sequenced gut microbiome and 228 fungal genera were identified. The most abundant genera detected were *Protomyces, Taphrina,* and *Aspergillus. S.haematobium* infection had a significant effect on fungal genera. Prevalence of SPT sensitisation to ≥1 fungal species was 96%, and individuals were frequently sensitised to *Saccharomyces cerevisiae*. Antibodies were detected in 100% of the population. There was no relationship between mycobiome abundance and IgE titres or IgE/IgG4 ratios for each fungal species; no significant differences between SPT reactivity and abundance of fungal species except for *S. cerevisiae*; and fungal seroreactivity did not significantly differ with age. There were some sex (*m>f* for, *Epicoccum nigrum* and *Penicillium chrysogenum*) and SPT reactivity –related differences in seroreactivity.

Conclusion: This is the first comprehensive characterisation of gut mycobiome and fungal allergic sensitisation of rural children in Zimbabwe. Although reported allergic disease is low there is a high percentage of sensitisation. Further studies with larger populations are required to understand the role of the mycobiome in allergic diseases.

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1. Introduction

The prevalence of allergic diseases in childhood has increased over the last few decades (Wong et al., 2012). Sensitisation to allergens occurring in the first few years of life is suggested to be a major risk factor for developing allergic diseases (Holt et al., 2010). These allergic conditions have complex etiologies that involve both genetic and environmental factors (Ober and Yao, 2011). The major factors contributing to increases in allergic diseases are proposed to be related to changes in lifestyle and environmental exposure. Among these, it has been proposed that increases in allergic diseases may be related to gut microbiome dysbiosis (Huang et al., 2017), as well as the immunological effects of a decline in exposure to infectious diseases in more developed and urban environments (Mpairwe et al., 2008).

To date, allergic sensitisation and inflammation studies report that an alteration in the microbiome (Fujimura et al., 2016; Arrieta et al., 2018) is associated with the development or exacerbation of allergic conditions such as asthma (Noverr et al., 2004, 2005; Wheeler et al., 2016; Arrieta et al., 2018). However, these studies have yielded variable findings, due to the heterogeneity of study populations, differences in profiling and study designs, variable definitions of allergy and varying sample sizes (Zhao et al., 2019). Furthermore, few allergy studies have specifically examined the interaction between gut mycobiome and allergen sensitisation (Fujimura et al., 2016), highlighting a need for more research in this area. Investigating the gut mycobiome in individuals with fungal sensitisation may identify associations between the gut mycobial composition and sensitivity or tolerance to fungal allergens. Additionally, a number of unculturable fungal species whose influence on fungal sensitised people is yet unknown may be identified.

Data from previous studies suggests that microbial dysbiosis occurs early in life, preceding the onset of sensitisation. The dysbiosis results in differences in gut microbial composition which have been associated with allergy skin prick test (SPT) response (Balenga et al., 2015), specific IgE levels, (Adlerberth et al., 2007) and allergy status (Thompson-Chagoyan et al., 2010). However, much of this previous work focused on the bacterial microbiome and relied on culture methods, excluding the large majority of organisms that cannot be cultured (Bunyavanich et al., 2016).

Thus, in this descriptive study we characterise the gut mycobiome using shotgun metagenomics and relate this to fungal sensitisation and fungal seroreactivity among Zimbabwean preschool-aged children (PSAC). We further assess whether seroreactivity varies with age, sex, *Schistosoma haematobium* infection status, gut mycobial composition or fungal sensitisation in children who were born in and were permanent residents of the study area. Thus, had comparable dietary and environmental exposure patterns as gathered from questionnaires administered at the time of recruitment of the main study.

2. Methodology

2.1. Ethical approval and consent

This study was part of a larger paediatric schistosomiasis study in children aged 5 years and below. Ethical and institutional approval for this study was granted by the Medical Research Council of Zimbabwe (MRCZ/A/1964) and University of Edinburgh. Permission to conduct the study in the province was obtained from the Mashonaland Central Provincial Medical Director. Before enrolling in the study, all participants and their parents/guardians were informed of the study aims, and procedures in their local language, Shona. Enrolment and participation was voluntary with written informed consent being obtained from the participants' parents/guardians. Participants were free to withdraw from the study at any time with no further obligation.

2.2. Study design, population and site

This cross-sectional study was conducted in Shamva district, one of the seven districts in the Mashonaland Central province of Zimbabwe. It was part of a larger research project, the Paediatric schistosomiasis study, where the overall health impact of paediatric schistosomiasis in children aged 5 years and below was investigated. Within this broader framework, the structure and diversity of the gut microbiome and resistome was characterised in two complementary studies; the first study investigated the association between schistosome infection and the gut microbiome dysbiosis and resistome in PSAC (Osakunor et al., 2020). Whereas, the second study, which is the current study investigates the relationship between gut mycobiome and fungal sensitisation and seroreactivity.

At baseline, the study enrolled children aged 6 months to 5 years who met the following inclusion criteria. The children had to, i) be lifelong residents of the study area, ii) no history of recent major illness/ surgery and iii) guardian/career had given consent for them to participate in the study. The samples used by Osakunor et al. (2020), and subsequently the present study, had to meet further criteria of; iv) consent for stool samples to be used for microbiome characterisation. Following these inclusion criteria, 116 stool samples from 1 to 5 year olds (57 males (49.1%), 59 (50.9%) females) children were included in the study.

To be included in the current study, children who fulfilled the inclusion criteria described above had to meet the additional criteria of; v) consent for serum samples to be used for serological assays, vi) availability of socio-demographic data and; vii) consent to perform skin prick testing (SPT) using allergen extracts. Following these inclusion criteria, the same 116 children were included in the current cross-sectional study.

2.3. Sample collection, processing and DNA extraction

Urine and stool samples were collected from all the participants to screen for schistosomiasis and soil-transmitted helminths as previously described by our group (Osakunor et al., 2020). For the characterisation of the gut microbiome, a small sample of each stool was transferred into a 2 mL cryovial tube and DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's instructions. To evaluate DNA purity, each sample was quantified at the University of Edinburgh using the Qubit fluorometer (Thermo Fisher Scientific) prior to shipment for DNA sequencing. For serological assays, up to 5 mL of venous blood was collected from each participant into serum separator blood collection tubes (BD Vacutainer®) and serum was separated for analysis.

2.4. Next-generation sequencing

Stool samples for next-generation sequencing (NGS) were prepared as previously described (Osakunor et al., 2020). Sequencing quality control and trimming of the reads was conducted using FASTQC and BBduk2 [BBMap—Bushnell B.—https://sourceforge.net/projects/bbmap/] respectively. The trimmed reads were used as input to align direct to reference sequence databases downloaded via NCBI GenBank clade specific assembly_summary.txt files (ftp://ftp.ncbi.nlm.nih.gov/ genomes/genbank) using k-mer alignment (KMA) (Osakunor et al., 2020). The primary alignment obtained for mapped sequences was used to assign a putative taxonomy, based on the taxonID obtained. TaxonIDs and associated taxonomy classifications were obtained from downloaded reference microbial genomes from NCBI (ftp://ftp.ncbi.nih.gov/pub/taxonomy/taxdump.tar.gz), these were assigned to all taxonomic levels.

To obtain information about the abundances of features in the datasets relative to each other, datasets were treated as compositional (Gloor et al., 2017) and prior to transformations, a pseudo-count of half the smallest non-zero abundance per feature was added to each feature

for all the normalised abundance matrices. Microbiota abundance data tables with counts and number of populations (taxa members), were centred log ratio (clr) transformed (Calle, 2019). The clr matrices were used for all downstream analyses.

2.5. Skin prick tests (SPTs)

SPTs were conducted using six different fungal allergen extracts (Stallergenes Greer, France): *Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans* and *Saccharomyces cerevisiae* were chosen based on the Zimbabwean sensitisation profile (Westritschnig et al., 2003). Briefly, drops of each allergen extract were placed on the forearm 2 cm apart and pricked using a calibrated lancet that introduces approximately 1 μg/mL of allergen into the dermis. Histamine dihydrochloride (10 mg/mL) was used as a positive control and a saline solution as a negative control. Reactions were considered valid if the histamine wheal diameter was greater than the negative control. Results were read at 15 min. The largest diameter of the wheal for each allergen extract was measured and a wheal of $\geq$ 3 mm scored positive.

2.6. Serology assays

2.6.1. Fungus-specific responses

Humoral responses (IgA, IgG, IgM, IgE and IgG4) to fungal antigens were assessed using commercial extracts of *Aspergillus fumigatus, A. alternata, C.herbarum, E.nigrum, P.chrysogenum, R.nigricans* and *S.cerevisiae* (Stallergenes Greer, USA) in a standard indirect enzyme-linked immunosorbent assay (ELISA). In brief, ELISA plates were coated overnight at 4 °C with 50 μl/well of fungal antigen diluted in PBS at 5 μg/mL. Serum samples and secondary antibodies (anti-human horse-radish peroxidase conjugated) were diluted according to the nominal antigen. The substrate 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase was used for the colorimetric reaction and the absorbence was read at 450 nm.

Optimum dilutions of antigens, sera and secondary antibodies were determined by several serial dilutions, based on protocols previously developed by others. To minimize variations across plates, positive and negative controls were repeatedly run on each plate and plotted to detect any outliers (in which case assays were repeated). The positive controls were pools of sera from six individuals presenting with the highest skin- prick reactivity while negative controls were pools of sera from six individuals with the lowest skin-prick reactivity. To account for background variation, a blank well (containing no sera) was included on each plate and this absorbence reading was subtracted from all other plate-readings. All the samples and the blank were run in duplicate on each plate and reported values are means of duplicates.

2.7. Data analysis

Statistical analyses were performed using SPSS v22 (IBM Corp.) and various Bioconductor packages within the R environment v3.6.1. Data visualization was performed within the R environment and GraphPad Prism v7.02 (GraphPad Software, Inc.). To test whether sample-related metadata predicted within-group dispersion of the microbiome, the Euclidean distances were calculated using R. The effect of such metadata on sample dissimilarities were determined using permutational multivariate analysis of variance (PERMANOVA; adonis2 function in the vegan package) using $p <$ 0.05 as the significance threshold. A false discovery rate (FDR (Benjamini–Hochberg FDR)) correction was applied to counteract multiple testing (Benjamini and Hochberg, 1995). Bar plots from normalised, zero-corrected abundance matrices were used to give an overview of the microbiota gene abundances across all samples.

To determine whether age, sex, *S.haematobium* infection status had an effect on SPT reactivity binary logistic regression was performed. To test whether serological reactivity to fungi varies with age, sex, *S. haematobium* infection status as well as gut microbiome structure, analysis of variance (ANOVA) was used. Differences were considered to be significant at $p < 0.05$. The experimental data are presented as the mean ± standard error of the group means. Due to the skewed nature of the fungus-specific antibody responses values were square root-transformed. To characterise patterns of the different fungus-specific antibodies, all titres (IgM, IgA, IgG, IgG4 and IgE), were reduced into axes to facilitate interpretation of patterns and differences among groups using non-metric multidimensional scaling (NMDS). NMDS was run in R using a Bray Curtis distance method and 1–6 dimensions were trialled. The stress scores were recorded to determine the number of axes needed. The stress score produced is a goodness of fit statistic based on the differences between the actual distances and their predicted values. A stress score >0.1 is 'poor, <0.1 is 'fair' and <0.05 is 'good' (Kruskal, 1964). Pearson's correlations were used to determine the relationship between the original variables and the axis and only antibodies with an $r^2 < -0.5$ or > 0.5 were considered adequately reflected by the axis.

NMDS scores were compared by sex, age group, SPT reactivity and *S. haematobium* infection.

Table 1
Demographic characteristics of study population.

Demographic categories		Frequency	Percentage
Gender	Female	59	50.9
	Male	57	49.1
Age group (years)	≤3	52	44.8
	4	33	28.4
	5	31	26.7
S.haematobium infection status	Negative	98	84.5
	Positive	18	15.5
Nutritional and growth factors			
Breastfed (months)	<6	1	1.1
	≥ 6	89	98.9
Solid food introduction (months)	<6	79	77.5
	≥ 6	23	22.5
Malnourished (WHA)	Yes	4	3.7
	No	103	96.3
Stunted (HAZ)	Yes	16	14.7
	No	93	85.3
Total		116	100

Role of funding

The funders played no part in the design of the study, collection, analysis and interpretation of data, and in writing the manuscript.

3. Results

3.1. Population characteristics

Table 1 details the demographic characteristics of the study population. The mean age of the 116 participants was 3.7 ± 1.1 years. Out of the 116 participants majority were female (50.9%, $n = 59$). In regards to the children's dietary habits and nutritional status, the majority were breastfed for six or more months. Children were introduced to solid foods between 1 and 24 months after birth. The main component of the diet was traditional maize flour porridge. Anthropometric measurements, adjusted for age, were used to assess nutritional status (MOH Malawi, 2016; Osakunor et al., 2018). Table 1 shows the children's demographics characteristics.

3.2. Taxonomic composition of the microbiome

Using some of the data analysed and recently published by our research group (Osakunor et al., 2020), the relative abundance was calculated for each microbial community in all samples. An average of 45.1% of read pairs were mapped to specific reference sequences in the

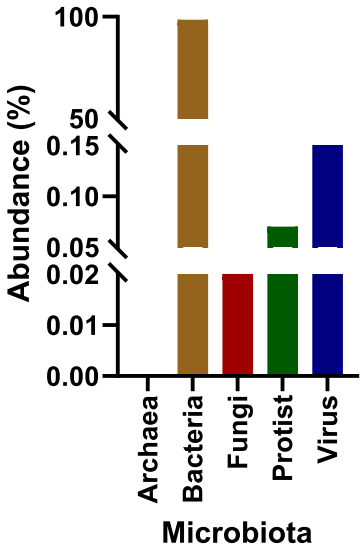

Fig. 1. Composition of the gut microbial communities.

genomic database (Osakunor et al., 2020), similar to values reported in other studies (Afshinnekoo et al., 2015). The mycobiome made up less than 1% of the sequenced gut microbiome as shown in Fig. 1. However, it showed high diversity (Fig. 2). In the 116 stool samples analysed, 228 fungal genera (from six unique phyla) were detected.

3.2.1. Relative abundance fungal phyla and genera in the gut microbiome

Abundance was calculated for each microbial taxon across all samples. The most prevalent phyla were Ascomycota (genera: *Protomyces* (present in 100% of the samples), *Taphrina* (98%), *Aspergillus* (91%), *Saccharomyces* (91%)), Microsporidia (*Enterocytozoon* (100%)), and Zoopagomycota (*Entomophthora* (100%)) Fig. 2a-b. These phyla dominated the microbiome. Charts were generated using normalised, zero-corrected abundance matrices. "Other" represents abundance data for all other taxa in the abundance data set.

Fig. 1: Overview of fungal microbiota abundance and composition Stacked bar charts show the most abundant fungal (a) phyla and (b) genera respectively per sample, proportional to the total microbiota within each sample.

3.3. Variation in the mycobiome and association with participant metadata

To initially examine variability and patterns in the data set, principal

Table 2
Summary of sample metadata and association with gut mycobiome.

Variable	n	p-value	Explained sum of squares	Total sum of squares	FDR
Gender	116	0.370	85.8	9673.3	0.726
Age (years)	116	0.098	123.0	9636.1	0.343
Malnourished (WHA) yes/no	107	0.830	59.5	9148.0	0.830
Stunted (HAZ) yes/no	109	0.624	71.5	9229.7	0.728
Breast-fed (months)	90	0.415	75.2	6986.5	0.726
Solid food introduction (months)	102	0.602	73.6	8794.3	0.728
S.haematobium status	116	0.001	339.1	9281.2	0.007

Classification of nutritional status was based on a cut off <−2 Z scores (MOH Malawi, 2016). WHA, weight-for height Z scores; HAZ, height-for-age Z scores; p-value-unadjusted p-value; FDR- adjusted p-value (FDR-corrected).

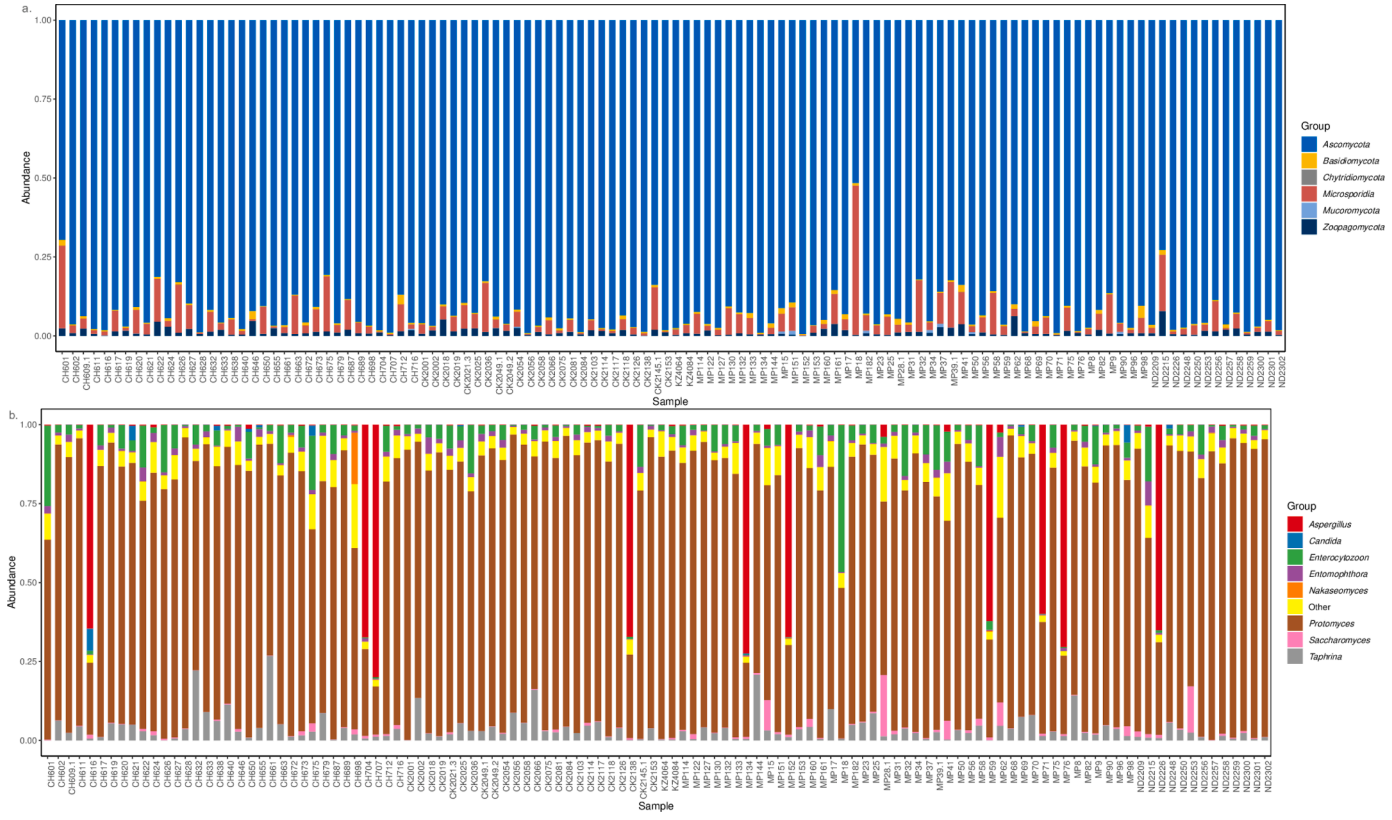

Fig. 2. Overview of fungal microbiota abundance and composition Stacked bar charts show the most abundant fungal (a) phyla and (b) genera respectively per sample, proportional to the total microbiota within each sample.

Table 3
Prevalence of fungal sensitisation based on skin prick testing (SPT) of the study population.

Total	48(96.0)	ONE-FS* 7(14.6)	TWO-FS* 9(18.6)	MFS* 32(66.7)
A.alternata	16 (33.3)	0	0	16(50.0)
C.herbarum	28(58.3)	3 (42.9)	1(11.1)	24(75.0)
E.nigrum	27(56.25)	1(14.3)	5(55.6)	21(65.6)
P.chrysogenum	28(58.3)	0	4(44.4)	24(75.0)
R.nigricans	24(50.0)	0	4(44.4)	20(62.5)
S.cerevisiae	29(60.4)	2(28.6)	4(44.4)	23(71.9)

* ONE-FS: one positive SPT to a single fungal species; TWO-FS: two positive SPT to fungi; MFS: more than two positive SPT to fungi (multiple fungi sensitization).

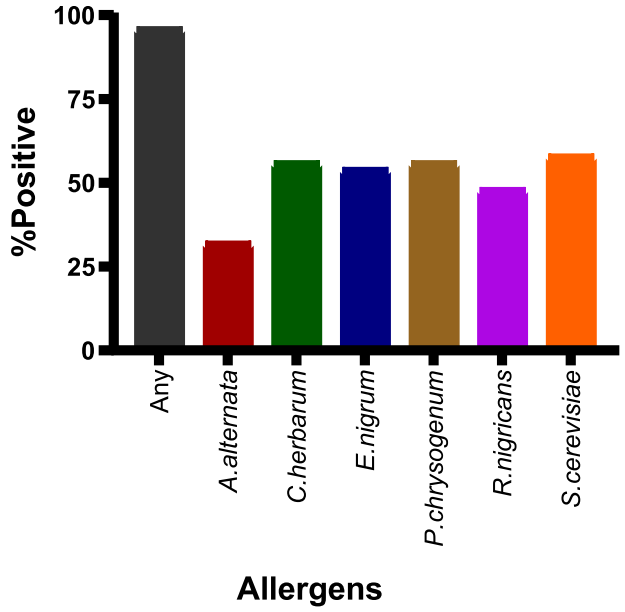

Fig. 3. Prevalence of fungal sensitisation based on skin prick testing (SPT) of the study population; Prevalence of positive SPT to different fungal species tested.

component analysis was used and the model showed homogeneity with no distinct clustering according to metadata as previously reported by our research group (Osakunor et al., 2020). The cluster dendrograms are shown in Supplementary Figure S1.

PERMANOVA analysis showed a significant effect of *S.haematobium* infection status (FDR= 0.007) on fungi genera, across samples, however, no such effects were observed for age, sex, nutritional and growth variables and feeding. Summary output for the analysis is shown in Table 2.

Of the 116 children with a characterised microbiome, 71 were included in the subsequent analysis. The decrease in sample size was due to loss of SPT follow-up or inadequate serum sample volumes. The age range for the final samples was 2–5 years, with a mean age of 3.98 years.

3.4. Skin prick reactivity profiles

Of the 71 children included in the serological study, only 50 had SPTs performed, as other participants withdrew due to aversion to the procedure. Forty-eight study participants (96%) were SPT-positive to at least one fungal source. Ages ranged from 3 to 5 years, with a female/male ratio of 1.5. Table 3 summarises the prevalence of different patterns of fungal sensitization. Majority of the participants were sensitised to two (TWO-FS=18.6%) or multiple fungi (MFS = 66.7%). *S.cerevisiae, C.herbarum*, and *E.nigrum* were positive in 85.7% of the participants. All participants sensitized to *A.alternata* were poly-sensitised. Fig. 3 summarises the sensitisation pattern observed in the study population.

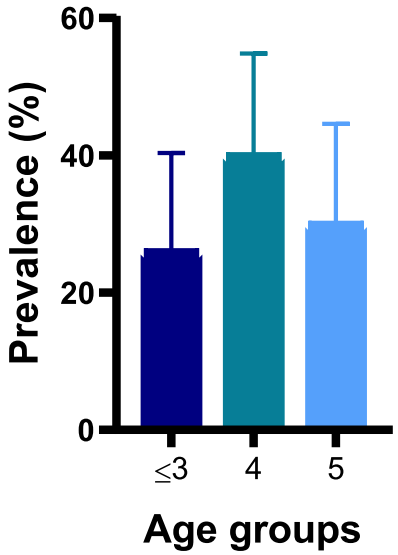

Fig. 4. Prevalence (%) of positive SPTs for the different age groups with 95% CI.

The prevalence of positive SPTs decreased with age (26% for the <3 years group, 40% for the 4 years group, and 30% at 5 years; Fig. 4.

3.4.1. Effects of age, sex and S.haematobium infection on SPT reactivity

A binary logistic regression was performed to ascertain the effects of age, sex, and *S.haematobium* infection on SPT reactivity. In all cases, the predictor variables (age, sex, and *S.haematobium* infection) had no significant effect on SPT reactivity ($p > 0.05$) (see Supplementary Table S1).

3.5. Antibody profiles for fungal-specific immune responses

Overall, fungal seroprevalence was 100% (95% Confidence Interval (CI) 94.94–100). IgE antibodies were detected in 85.9% (95% CI 75.6–93.0) of the study population whereas IgG4 antibodies were detected in 32.4% (95% CI 21.8- 44.6). Ages ranged from 2 to 5 years, with a mean age of 3.98 years with a female/male ratio of 1.2. Figs. 5 and 6 summarise the frequency distribution of children producing each antibody (with 95% CI) and mean antibody levels (absorbence) against each fungal species respectively.

The response to allergens induces different levels of Immunoglobulin classes, depending on the specific allergen. IgM and IgG, which are produced predominantly in the first exposure to an allergen, (Scott--Taylor et al., 2018) had higher mean antibody responses for all fungal species except *A. fumigatus,* where IgM titre was low. In all cases where IgM and IgG responses were high, IgE titres were lower or in some cases, not detected altogether (*R.nigricans, P.chrysogenum,* and *E.nirgum*) as shown in Fig. 6.

3.5.1. Characterisation of fungus-specific antibodies

To characterise patterns of the different fungus-specific antibodies, all antibodies produced by the participants were reduced into NMDS axes using the vegan package in R v3.6.1 (metaMDS) which follows the ordination with a rotation via principal components analysis. This is a useful feature because it ensures that NMDS axis 1 (NMDS1) reflects the principal source of variation (McCune and Grace, 2002). Prior to analysis all antibodies were square-root transformed for each participant to reduce the influence of outlier values.

NMDS1, which accounted for the most variation within the data, was positively correlated with *A.fumigatus (A.f)* –IgG4; *E.nigrum (E.n)*-IgG; *P. chrysogenum (P.c)* - IgG and IgG4, whilst negatively correlated with *A.*

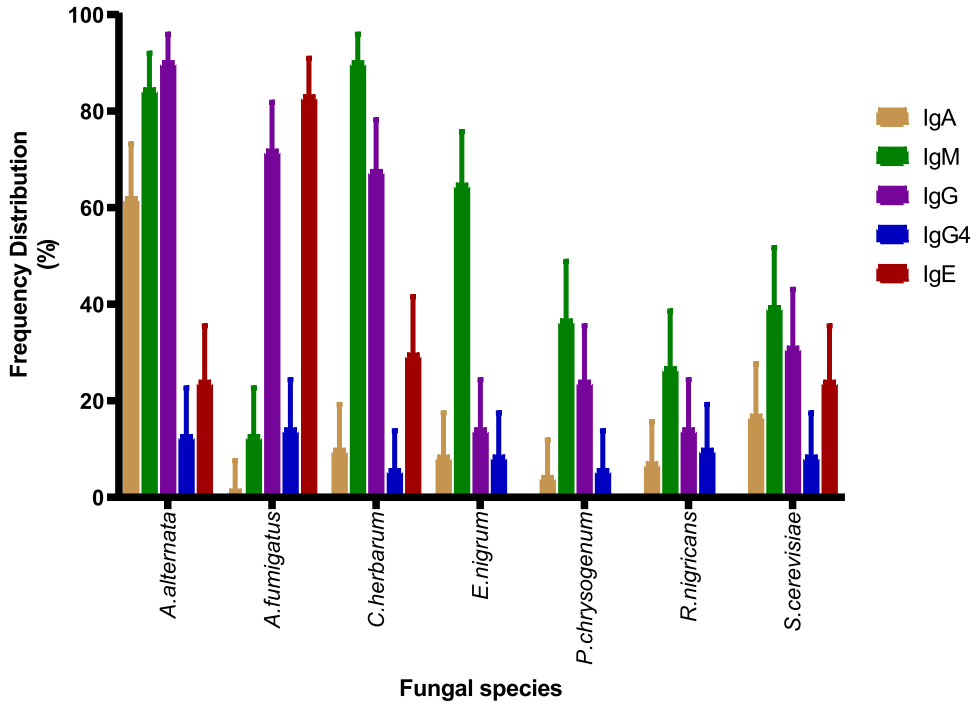

Fig. 5. Frequency distribution of children producing each antibody against each fungal species with 95% CI.

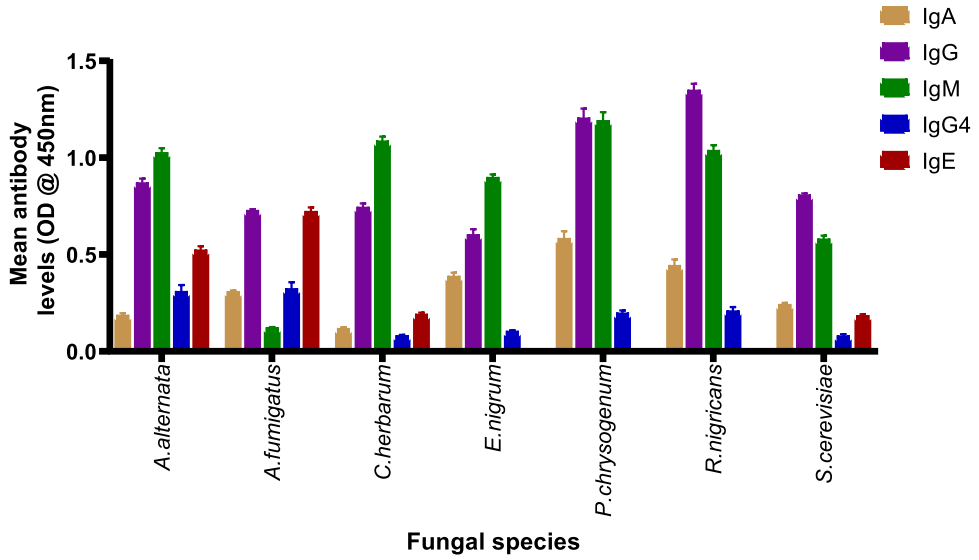

Fig. 6. Mean antibody production with SE bars for each antibody for each fungal species.

alternata (A.a)-IgG4 and IgE; *C.herbarum (C.h)* - IgM and IgG; *S.cerevisiae (S.c)*- IgM, IgA and IgG; *P.chrysogenum* (P.c)-IgM; *R.nigricans (R.n)*-IgA, IgG and IgG4. NMDS2 was positively correlated with *A.fumigatus* –IgA and IgE; *P.chrysogenum*- IgA; *S.cerevisiae*- IgE and IgG4, whilst negatively correlated with *C.herbarum*- IgG4; *E.nigrum*- IgM and IgA; *R.nigricans* –IgM. NMDS3 was positively correlated with, *E.nigrum*-IgG4 and IgM; *P. chrysogenum*- IgA, *A.alternata*-IgG whilst negatively correlated with *A. fumigatus* –IgM and *S.cerevisiae*- IgA Fig. 7. Summary of the NMDS scores are shown in Supplementary Table S2.

As IgM and IgG are often associated with first exposure to allergen, *C. herbarum, P.chrysogenum* (IgM), *R.nigricans* and *S.cerevisiae* were negatively associated with allergen exposure. Whereas *A.alternata, P.chrysogenum* (IgG) and *E.nigrum* were positively associated with allergen exposure. As IgE is associated with allergic sensitisation, *A.fumigatus* and *S.cerevisiae* were associated with allergic sensitisation.

3.5.2. *Serological reactivity to fungi varies with sex, S.haematobium infection and SPT reactivity*

To determine whether SPT reactivity influenced the antibody profiles within our study population and to identify whether age, sex as well *S.haematobium* infection status affected the antibody profiles identified, three factorial ANOVA models were used: assessing the influence of age and SPT status on NMDS scores, sex and SPT status on NMDS scores as well as *S.haematobium* infection status and SPT status on NMDS score.

When examining the effects of age followed by SPT status, our results showed that age had no significant effect on NMDS scores for all the fungal species included in the analysis (Figs. 8–11(a, d and g) and Figs. S2–S3).

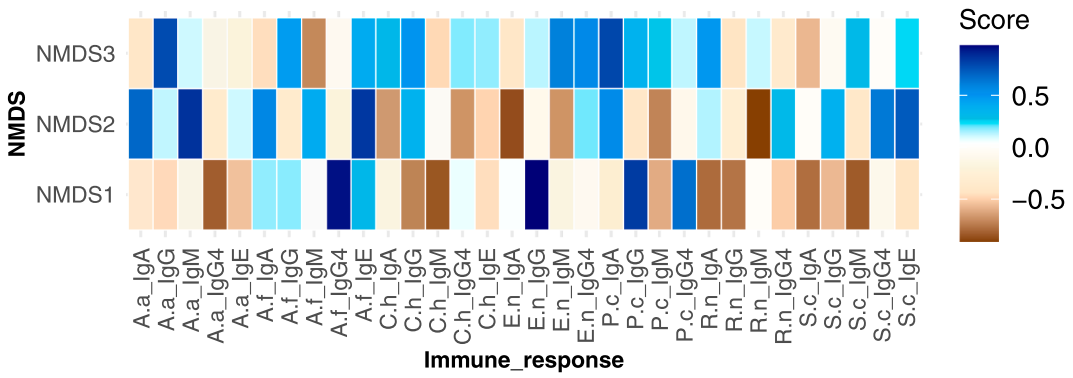

Fig. 7. The NMDS scores of the original variables for each axis calculated after NMDS of fungal-specific antibodies. Those variables which were significantly positively correlated score >0.5) onto an axis are shown in dark blue and those variables which were significantly negatively correlated (score <−0.5) are shown in dark brown. There is a colour gradient, whereby the darker the colour, the higher the score (if >0) or lower the loading score (if <0).

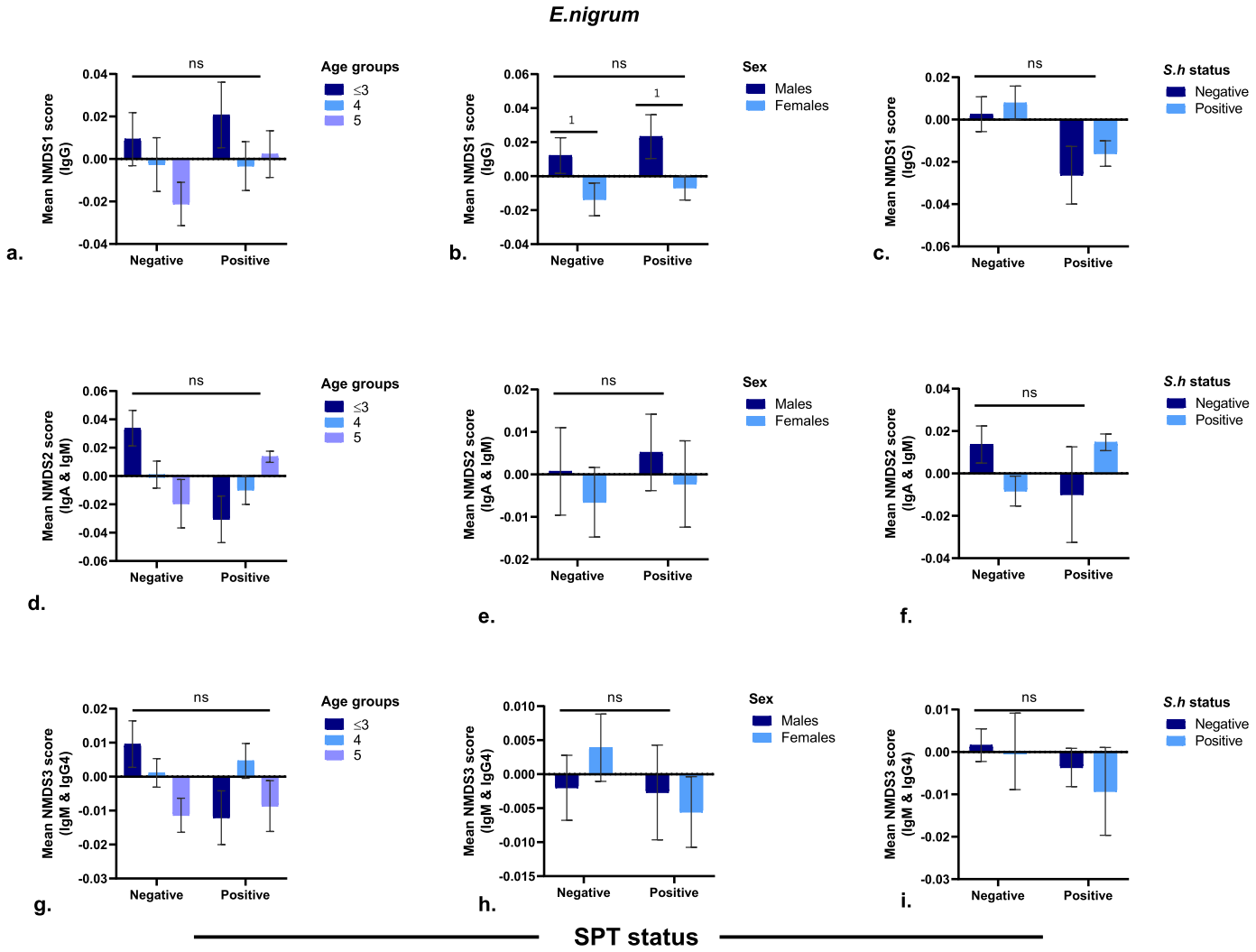

Fig. 8. The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S.haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses. Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S.haematobium* infection status and SPT reactivity, are indicated on the graph using *, $p < 0.05$ =*, $p > 0.05$=ns. Standard error of mean is indicated on the graphs for each group.

When examining the effects of sex and SPT status, our results showed that sex had a significant effect on NMDS score for NMDS1 (comprising of IgG) for *E.nigrum* and (IgG, IgG4 and IgM) for *P. chrysogenum* with males having a significantly higher NMDS score compared to females (ANOVA, F (1, 46) =8.035, $p = 0.007$; Fig. 8b and F (1, 46) =4.398, $p =$ 0.042; Fig. 9b respectively).

For *C.herbarum*, SPT status had a significant effect on NMDS score for NMDS1 (IgG and IgM) with SPT-positive individuals having significantly higher NMDS score compared to SPT-negative (ANOVA, F (1, 46) =4.727, $p = 0.035$; Fig. 10b). There was a statistically significant

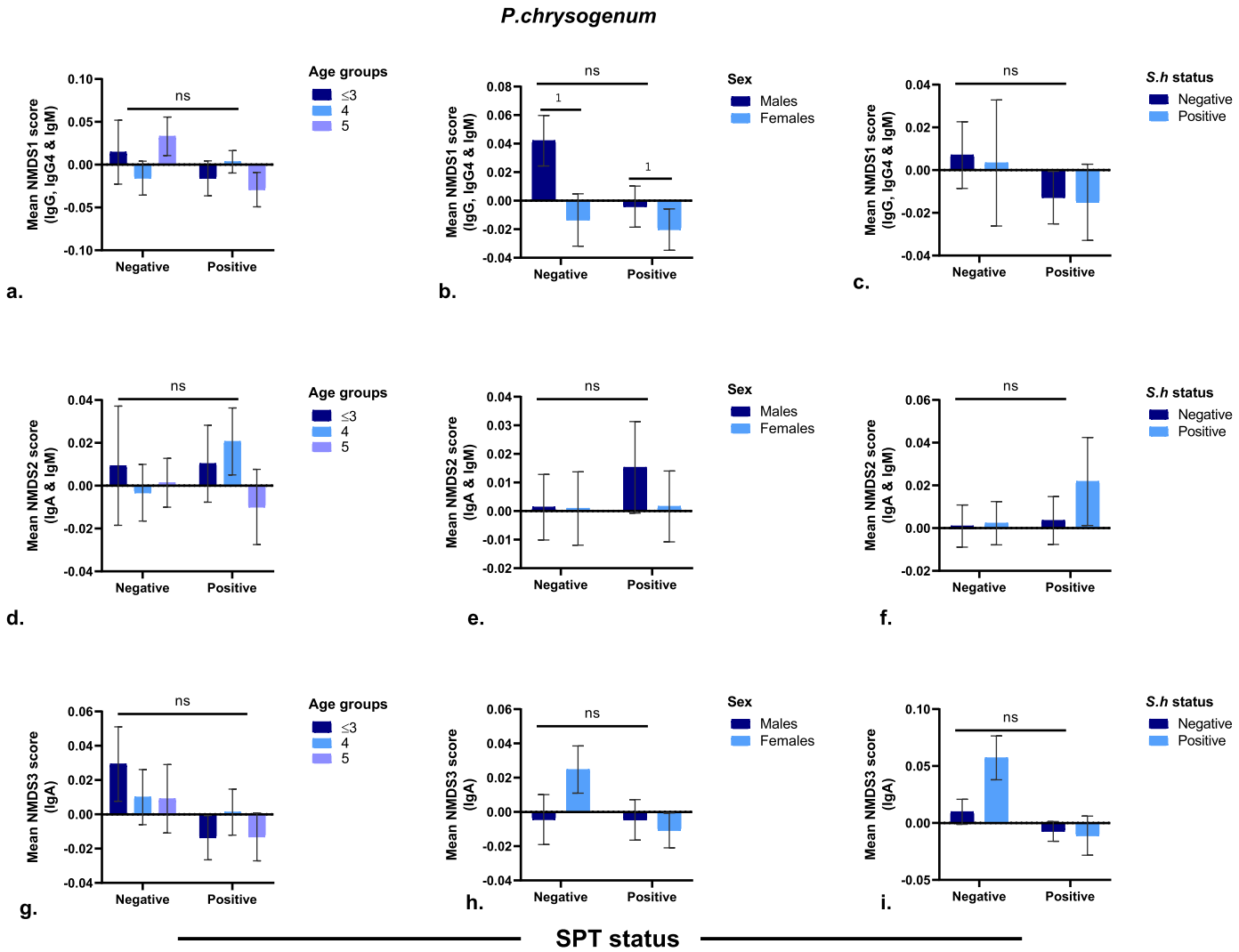

Fig. 9. The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S.haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses. Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S.haematobium* infection status and SPT reactivity, are indicated on the graph using *, $p < 0.05$ =*, $p > 0.05$=ns. Standard error of mean is indicated on the graphs for each group.

interaction between sex and SPT status on NMDS2 for *C.herbarum* (F (1, 46) = 5245, $p = 0.027$). A pairwise comparisons was conducted and revealed that there was a statistically significant difference in mean NMDS2 (IgG4) scores between males and females who were SPT positive (F (1, 46) = 4.305, $p = 0.044$; Fig. 10e).

When examining the effects of *S.haematobium* infection status and SPT status our results showed that SPT status had a significant effect on NMDS score for NMDS3 (IgA) for *S.cerevisiae* with SPT-negative individuals having significantly higher NMDS score compared to SPT-positive (F (1, 46) =4.330, $p = 0.043$; Fig. 11i).

3.6. Association between gut mycobial abundance with SPT reactivity and fungal-specific antibody responses

There was no association between mycobiome abundance and SPT reactivity or fungal-specific antibody responses as determined by PERMANOVA analysis, Tables 4 and 5 respectively.

3.6.1. Association between of fungal abundance and SPT reactivity with skin prick reactivity, and IGE response and IGE/ IGG4 ratio to specific fungal species

We further assessed the association of abundance of specific fungal genera with fungal SPT reactivity and antigen-specific IgE responses by ANOVA and linear regression analysis, respectively.

No significant differences were found between species abundance in skin test-positive and skin test-negative children for *A.alternata* and *C. herbarum* (Fig. 12 [1a and b]). In contrast, species abundance of S.cerevisiae was significantly higher in children who were skin test-negative (Fig. 12[1c]). No significant correlation was found between the species abundance and IgE reactivity or IgE/Ig4 ratios for *A.alternata, C.herbarum or S.cerevisiae* (Fig. 12 [2a-f])

4. Discussion

This study is to our knowledge the first comprehensive descriptive study of the relationship between gut mycobiome, fungal sensitisation and fungal seroreactivity in rural pre-school aged African children. The key findings were that fungal sensitisation is common and that gut mycobial abundance and diversity is not associated with SPT reactivity or seroreactivity.

In the current study, the mycobiome of the children was heterogeneous and comprised less than 1% of the sequenced gut microbiota, which is comparable to previous studies (Qin et al., 2010; Cardinelli et al., 2015). Our dendrograms showed no distinct clustering (Figure S1) reflecting high inter-individual variability and this is similar to what was reported in the Human Microbiome Project (HMP) cohort. In the HMP it

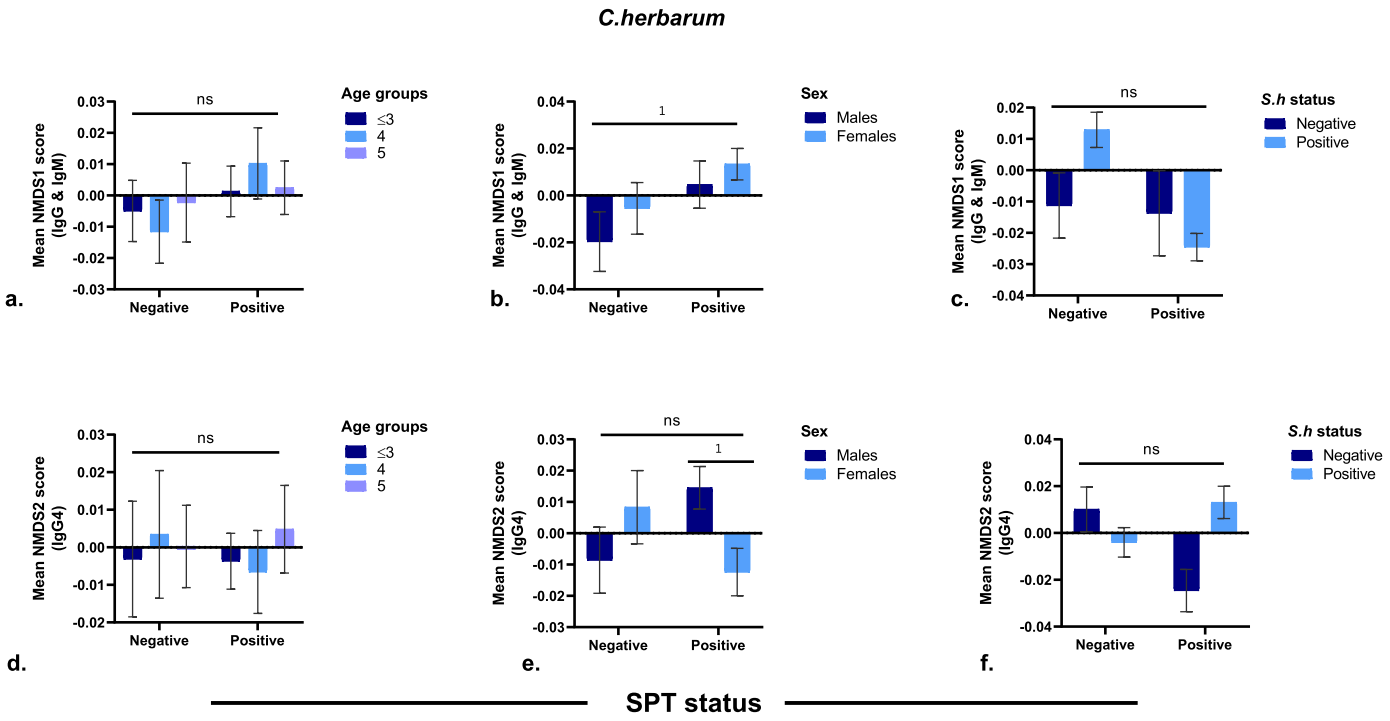

Fig. 10. The mean NMDS scores for each axes, by age and SPT reactivity (a & d), by sex and SPT reactivity (b & e), and *S. haematobium* infection status and SPT reactivity (c & f) for the NMDS of antibody responses. Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S.haematobium* infection status and SPT reactivity, are indicated on the graph using *, $p < 0.05$ =*, $p > 0.05$=ns. Standard error of mean is indicated on the graphs for each group.

was reported that, in contrast to bacteria, the mycobiome has both high inter- and intra-individual variability (Nash et al., 2017).

Previous studies have shown that diet (Hoffmann et al., 2013) age, (Rodríguez et al., 2015) environmental patterns, (De Filippo et al., 2010) geography (Yatsunenko et al., 2012; Kabwe et al., 2020) and sex (Strati et al., 2016) are determinants of the gut microbial community structure (Conlon and Bird, 2014; Vangay et al., 2018). In the current study, there was a significant association between *S.haematobium* infection status and gut mycobial abundance. However, there were no significant differences observed with age, sex and nutritional and growth variables possibly due to the comparable dietary and environmental exposure as the children were born in and were permanent residents of the study area. Moreover, they were all $\leq$ 5 years, thus comparable age-range and sex-specific microbiome profiles have been suggested to emerge later after puberty (Markle et al., 2013; Kim et al., 2020).

The most prevalent genera were *Protomyces, Aspergillus, Saccharomyces,* and *Taphrina*. In the Hoffmann study in USA on diet as a determinant of the gut mycobial community structure, the most abundant fungal genera noted were *Saccharomyces, Candida* and *Cladosporium* (Hoffmann et al., 2013) whereas *Pichia, Candida, Aspergillus* and *Cladosporium*, dominated the South African gut microbiome when geographical location was studied (Kabwe et al., 2020). Our findings are comparable with these previous studies which show that *Aspergillus, Candida, Malassezia, Penicillium, Pichia,* and *Saccharomyces* genera are among the most prevalent fungal genera (Hallen-Adams et al., 2015). The differences observed may be due to factors highlighted such as diet, lifestyle or age (Yatsunenko et al., 2012; Hoffmann et al., 2013; Rodríguez et al., 2015; Kabwe et al., 2020).

We found that *Aspergillus* was among the most abundant fungal genera, and an increase in fungus populations such as *Aspergillus* has been associated with increased eosinophil levels and an exaggerated Th2 response (Wheeler et al., 2016), both of which are characteristic of allergic responses. However, it remains to be established if our observations were due to primary changes in the fungal population or were secondary to changes to other microbial communities such as bacteria (Osakunor et al., 2020). Further exploration of the microbiome using clinically defined allergy cases would be informative.

We investigated whether abundance of specific fungal genera was associated with fungal skin prick reactivity and specific IgE antibody reactivity as well as IgE/ IgG4 ratio. No significant correlation was found between the species abundance and IgE reactivity or IgE/Ig4 ratios. Although we observed no significant differences between children who were SPT negative or positive for abundance of *A.alternata* and *C.herbarum,* we found significant differences for *S.cerevisiae* (Fig. 12). For SPT-negative children with higher species abundance, and failing to elicit an immune response, there is a possibility of tolerance arising from an active control mechanism, or a state of non-responsiveness whereby IgG4 antibodies inhibit IgE receptor-facilitated allergen binding to B cells thereby diminishing SPT reactivity. This has been demonstrated in immunotherapy studies (Wachholz and Durham, 2004; Shamji and Durham, 2011) which have also shown how this could diminish mast cell and basophil activation (Wisniewski et al., 2013).

Atopic diseases are common chronic childhood disorders and sensitisation to allergens is recognised as the most important risk factor (Chiu et al., 2014). From our data, a 96% prevalence of SPT reactivity to at least one of the six selected fungal species was found. Grouping the fungi-sensitised individuals according to the pattern of SPT reactivity showed that the largest subgroup of fungi-sensitised individuals was reactive to multiple fungi species which could have been either due to genuine sensitisation to a variety of fungi, or due to cross-reactivity between fungal allergens. Our high rate of SPT reactivity is in contrast to rates reported by other countries, ranging from 3% to 58% (D'Amato et al., 1997; Arbes et al., 2005; Bousquet et al., 2007; Wiszniewska et al., 2013; Fernández-Soto et al., 2018; Kwizera et al., 2019). These results highlight differences between different populations and thus emphasize the need for studies to characterise sensitisation patterns in different regions of the world (Eriksson and Holmen, 1996; Mpairwe et al., 2008). Many studies have suggested that the occurrence and severity of atopic disease symptoms in later childhood are directly related to allergen

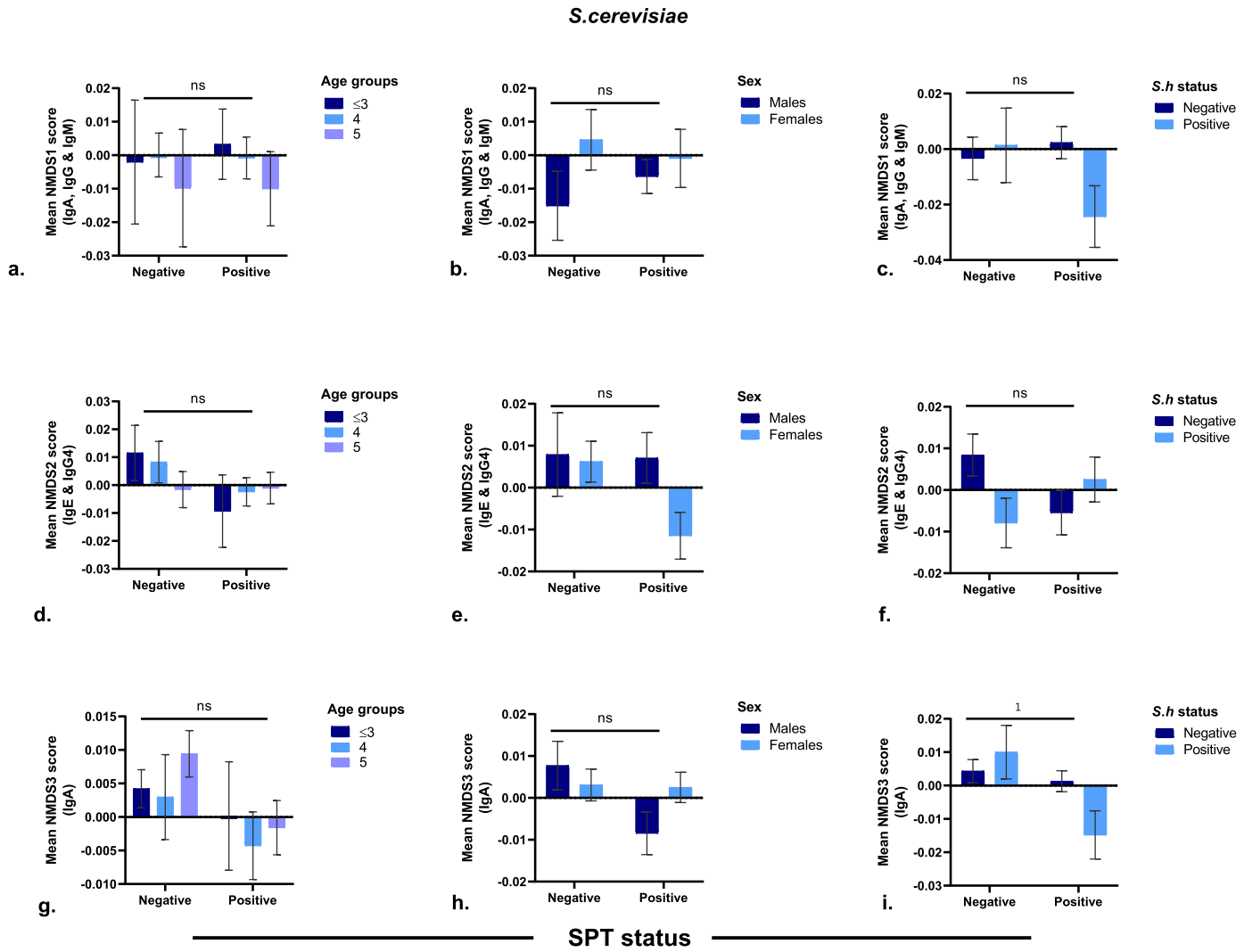

Fig. 11. The mean NMDS scores for each axes, by age and SPT reactivity (a, d & g), by sex and SPT reactivity (b, e & h), and *S.haematobium* infection status and SPT reactivity (c, f & i) for the NMDS of antibody responses. Significance between the main effects, age and SPT reactivity, sex and SPT reactivity as well as *S.haematobium* infection status and SPT reactivity, are indicated on the graph using *, $p < 0.05$ =*, $p > 0.05$=ns. Standard error of mean is indicated on the graphs for each group.

Table 4
Summary of SPT reactivity and association with gut mycobiome.

Variable	n	p-value	Explained sum of squares	Total sum of squares	FDR
A.alternata	50	0.155	117.2	4323.4	0.458
C.herbarum	50	0.944	52.9	4387.8	0.944
S.cerevisiae	50	0.252	109.6	4331.0	0.458
P.chrysogenum	50	0.270	101.8	4338.9	0.458
R.nigricans	50	0.371	92.1	4348.5	0.458
E.nigrum	50	0.382	91.4	4349.2	0.458

p-value-unadjusted p-value; FDR- adjusted p-value (FDR-corrected).

sensitisation in infancy (Kulig et al., 1998; Sun et al., 2014). Thus, knowledge of the sensitisation patterns may provide a means by which to establish allergic diseases management strategy for allergists (Lou et al., 2017).

Empirical studies have indicated sex and age differences in the prevalence of allergy sensitisation and diseases (Govaere et al., 2007; Chen et al., 2008) with males being more atopic in childhood than females (Govaere et al., 2007). However, this trend changes after puberty with allergies becoming more apparent in females (Shah, 2012). In our study, there were no significant effects of sex and age on SPT reactivity; however, prevalence of SPT reactivity decreased with age. There were no significant effects of *S.haematobium* infection status on SPT reactivity and this might have been due to the small sample size of *S.haematobium* infected people.

We were able to detect IgA, IgG, IgM and IgG4 antibodies to all the fungal allergens but not IgE in participants who were SPT positive for *E. nigrum, P.chrysogenum, R.nigricans*. This is not surprising because a positive SPT without detectable IgE in the ELISA is indicative of non IgE-mediated response (Denning et al., 2006) and the presence of high titres of allergen-specific IgG antibodies can interfere with IgE reactivity through competition with IgE for binding to the solid phase-bound allergens (Van der Zee et al., 1988). Seroprevalence was high in the

Table 5
Summary of fungal antibody responses and their association with gut mycobiome.

Variable		n	p-value	Explained sum of squares	Total sum of squares	FDR
A.alternata	IgA	71	0.507	72.6	5556.5	0.839
	IgG	71	0.956	47.3	5581.7	0.956
	IgM	71	0.881	54.2	5574.8	0.924
	IgG4	71	0.381	80.2	5548.9	0.839
	IgE	71	0.708	62.5	5566.6	0.888
A.fumigatus	IgA	71	0.471	76.5	5552.5	0.839
	IgG	71	0.740	61.6	5567.5	0.888
	IgM	71	0.830	56.6	5572.4	0.924
	IgG4	71	0.495	74.2	5554.9	0.839
	IgE	71	0.582	69.3	5559.7	0.839
C.herbarum	IgA	71	0.403	79.9	5549.1	0.839
	IgG	71	0.562	70.1	5558.9	0.839
	IgM	71	0.306	85.9	5543.2	0.839
	IgG4	71	0.242	94.3	5534.8	0.839
	IgE	71	0.898	50.7	5578.4	0.924
S.cerevisiae	IgA	71	0.545	71.7	5557.4	0.839
	IgG	71	0.739	61.3	5567.7	0.888
	IgM	71	0.192	97.3	5531.8	0.839
	IgG4	71	0.283	88.5	5540.6	0.839
	IgE	71	0.458	75.9	5553.2	0.839
E.nigrum	IgA	71	0.529	72.2	5556.9	0.839
	IgG	71	0.150	108.0	5521.1	0.839
	IgM	71	0.689	64.0	5565.0	0.888
	IgG4	71	0.168	101.2	5527.9	0.839
P.chrysogenum	IgA	71	0.868	54.6	5574.5	0.924
	IgG	71	0.678	64.5	5564.5	0.888
	IgM	71	0.273	90.3	5538.7	0.839
	IgG4	71	0.236	94.1	5535.0	0.839
R.nigricans	IgA	71	0.151	106.8	5522.3	0.839
	IgG	71	0.472	75.9	5553.2	0.839
	IgM	71	0.838	55.6	5573.5	0.924
	IgG4	71	0.537	70.9	5558.1	0.839

p-value-unadjusted p-value; FDR- adjusted p-value (FDR-corrected).

population and this could have been due cross-reactivity as some of these fungal allergens are known to cross-react with each other resulting in false positives (Crameri et al., 2009; Fukutomi and Taniguchi, 2015).

To characterise patterns of the different fungus-specific antibodies in the participants, NMDS was used to reduce antibody responses into variables. We went on to determine whether skin prick test (SPT) reactivity influenced the antibody profiles within our study population and identify whether age, sex as well *S.haematobium* infection status affected the antibody profiles identified in PSAC. From the analysis, we observed that age had no significant effect on NMDS scores for all fungal species investigated. The absence of an association with age is not surprising since the age range studied is narrow.

When examining the effects of sex and SPT status, our results showed that sex had a significant effect on NMDS score for NMDS1 (comprising of IgG) for *E.nigrum* and (IgG, IgM and IgG4) for *P. chrysogenum* with males having significantly higher responses compared to females. It has been suggested that the risk of being allergic is greater for males in childhood (Jensen-Jarolim and Untersmayr, 2008), and this may explain our observation of significantly higher antibody responses in males. This gender difference seems to be less pronounced after puberty as girls become more likely to be atopic throughout the reproductive years (Govaere et al., 2007) and this has been suggested to be due to differences in sex hormones during onset of puberty (PausJenssen and Cockcroft, 2003; Chen et al., 2008). However, this cannot explain the differences observed in our study population and therefore the exact pathophysiologic mechanism of gender differences in atopy remains unclear (Kim et al., 2014).

SPT status had a significant effect on NMDS score for NMDS1 (IgG and IgM) for *C.herbarum*, with SPT-positive individuals having significantly higher responses compared to SPT-negative. Our findings are consistent with the suggestions that IgM and then IgG are produced primarily in the first exposure to an allergen and associated with allergic diseases (Scott-Taylor et al., 2018). Therefore, a higher response in SPT-positive individuals would be expected as observed with *C. herbarum*.

Several epidemiology studies have shown inverse associations of chronic parasitic worm infections with allergy and atopy in regions of high prevalence of such infections (van den Biggelaar et al., 2004; Leonardi-Bee et al., 2006; Mpairwe et al., 2008; Rujeni et al., 2012; Webb et al., 2016). Other studies in schoolchildren have produced conflicting results with regard to the effects of deworming on SPT responses (van den Biggelaar et al., 2004; Cooper et al., 2006) A study in Indonesia found that SPT reactivity increased after 1 year of albendazole treatment, in Gabon SPT reactivity was significantly reduced in children infected with *S.haematobium* and in Ecuador, albendazole treatment had no effect on the prevalence of SPT reactivity (Lynch et al., 1993; van den Biggelaar et al., 2000; Cooper et al., 2006; Staal et al., 2018). In this study, when we examined the effects of *S.haematobium* infection status and SPT status on NMDS scores our results showed that SPT status had a significant effect on NMDS scores for NMDS3 (IgA) for *S.cerevisiae* with SPT-negative individuals having significantly higher IgA response compared to SPT-positive individuals. This observation might be due to the protective role of IgA, which has been suggested to contribute to the maintenance of mucosal tolerance by dampening immune responses and hence is thought to prevent the development of hyperinflammatory responses towards environmental allergens that would otherwise cause allergic inflammation (Gloudemans et al., 2013).

Finally, a substantial number of studies have suggested that SPT-positive individuals sensitised to aeroallergens are more prone to developing allergic disease (Hagy and Settipane, 1976; Bodtger et al., 2003). Important questions our study raises are; what is the long-term consequence of being SPT positive in childhood. Does this predispose to future atopic disease? What is the role of the mycobiome in inhalant allergy sensitisation?

There are some limitations to the present study. Not all study participants with the characterised mycobiome could be included for the

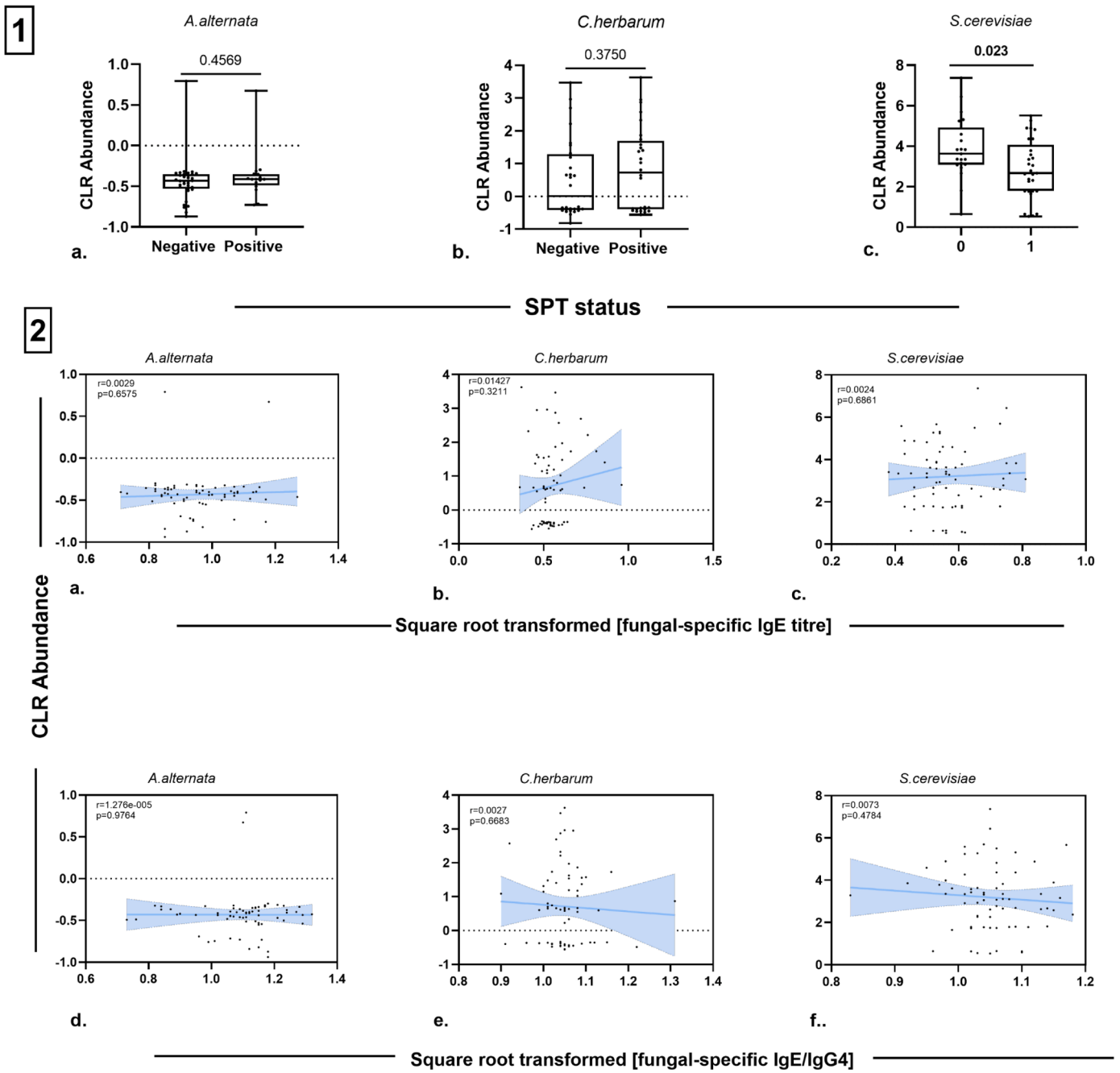

Fig. 12. Relationship between fungal species abundance and [1] skin prick test reactivity, [2] antigen-specific IgE response and IgE/IgG4 ratios Box plots showing the mean abundance of specific fungal genera, grouped by skin prick reactivity [1a-c]. The horizontal box lines represent the first quartile, the median and the third quartile. Whiskers denote the range of data within the first quartile −1.5 × the interquartile range and the third quartile +1.5 × the interquartile range. [2a-c] Scatter plots showing linear regression analysis of fungus-specific IgE antibody titres and fungal genera abundance. [2d-e] Scatter plots showing linear regression analysis of fungus-specific IgE/IgG4 ratios and fungal genera abundance Shaded areas indicate the 95% CI. Significant *p*-value is indicated in bold.

subsequent analysis due to loss of follow up, inadequate sample volumes or participants who withdrew due to aversion to the SPT procedure. The descriptive study design allowed the characterisation of the gut mycobiome and its relationship with SPT and seroreactivity to fungi as well as the determination of effects of host factors on seroreactivity and SPT reactivity of preschool-aged children at a single time point. A longitudinal study would be more useful for evaluating the relationship between the host factors and the development of fungal sensitivity over time as well as assessing its clinical relevance. Furthermore, the lack of participants with clinical symptoms meant the clinical relevance of the SPT could not be ascertained.

A further study evaluating the mycobiome in healthy and confirmed fungal-allergic individuals would be interesting to see whether variations in the mycobiome will be observed depending on allergy status. This study did not evaluate for possible interactions between other biomes such as bacteria, viruses and fungi. Investigating this would help evaluate what interactions occur between these biomes and the effect they may have on the host (Chin et al., 2020). Furthermore, future studies should assess the profile of fungal sensitisation amongst atopic children in other parts of Zimbabwe. These studies will aid in improved awareness of fungal sensitisation, early diagnosis of atopic diseases and implementation of preventative measures in the country.

5. Conclusions

This study provided the first comprehensive characterisation of the gut mycobiome and fungal allergic sensitisation of rural children in Zimbabwe. This indicated a high percentage of sensitization but reported allergic disease is low. Further studies with a larger number of well-characterised patients and controls are needed to understand the role of mycobiome in allergic diseases.

Author contributions

FM, TM & EN conceived the study. FM, TM, EN & LP conducted the fieldwork, LP, conducted the laboratory work and LP & FM conducted the data analysis. FM, SB, MW & EN; Supervision. LP & FM drafted the manuscript and all authors contributed to the final version.

Data sharing

All the individual participant data that underlie the results reported in this manuscript (after de-identification) will be fully available with publication, without restriction through the University of Edinburgh datashare.

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Declaration of Competing Interest

The authors have declared that no competing interests exist.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2021.100082.

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The Identification and Characterization of Immunoreactive Fungal Proteins Recognized by Sera from Zimbabweans Sensitized to Fungi

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Abstract

Background: Exposure to fungal allergens poses a serious threat to human health, especially to mould-allergic individuals. The prevalence of fungal allergic disease is increasing globally but is poorly studied in Africa. Here, we aimed to identify and characterize fungal proteins that were immunoreactive against serum samples from fungal-sensitized Zimbabweans from Shamva district to inform the development of diagnostics and therapeutics. ***Methods:*** Crude protein extracts of the Ascomycota *Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum,* and *Saccharomyces cerevisiae* as well as mucoromycota *Rhizopus nigricans* were individually separated by one-dimensional gel electrophoresis for protein staining and immunoblotting. A pool of eight sera from fungi-sensitive Zimbabwean children aged 3–5 years was used to screen the crude extracts to determine their immunoreactivity. Protein bands recognized by the sera were subjected to mass spectrometry to identify the individual proteins reactive with the sera. ***Results:*** The pooled serum sample reacted with 20 bands, which resolved to 34 distinct proteins, most of which were novel immunogens. The pool was most reactive to *A. alternata*. The proteins identified included peptidases (8/34), hydrolases (6/34), oxidoreductases (5/34), and glucosidases (4/34), while 11/34 were unknown. Eight of the proteins were predicted to be allergens using the Structural Database of Allergenic Proteins (SDAP). ***Conclusions:*** We identified novel immunogens from fungi expanding the number of known fungal allergens. These form a potential basis for diagnostics specific for the Zimbabwean popula-

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tion. Validation assays will now need to be carried out to further evaluate the cross-reactivity of the identified allergen candidates as well as investigate their potential recognition in a larger cohort of patients. Furthermore, there is now a need to conduct studies relating sensitization to these immunogens and clinical diseases in the population.

Introduction

Allergic diseases associated with IgE-mediated sensitization to fungal allergens are increasing worldwide [1]. With global warming and climate change thought to favour the propagation of fungal spores, allergenicity to fungi has become a serious health concern, triggering or exacerbating respiratory allergic disorders [2, 3].

The prevalence of sensitization to allergens varies across regions worldwide. However, due to a lack of immunological and allergological research facilities in most African countries, there is paucity of allergen prevalence data in this continent [4]. Though documentation is poor, a wide range of aeroallergens has been observed across the continent [5–12] and it has been suggested that there are differences regarding sensitization to allergen molecules in Africa versus Europe [13]. To date, several studies have identified fungal allergens, yet the fungal allergen repertoire has not yet been characterized in an African population.

Fungi are associated with allergic respiratory diseases in humans, such as asthma, rhinitis, allergic bronchopulmonary mycoses, and hypersensitivity pneumonitis [14, 15]. These diseases can result from exposure to different developmental stages of fungi, including spores or vegetative cells [16]. The different developmental stages of fungi produce diverse proteins whose ability to bind to immunoglobulin E (IgE) is heterogeneous [17, 18]. Consequently, commercially available fungal extracts from different suppliers generate discordant results in skin tests and serologic examinations, resulting in pressing diagnostic and therapeutic challenges.

Currently, the management of allergies includes corticosteroids, antihistamines, and in acute cases, non-steroidal anti-inflammatories which may have side effects. To date, the only disease-modifying approach is allergen-specific immunotherapy (AIT) in which allergy-inducing molecules are used for vaccination [19]. AIT is widely used for bee and wasp, house dust mites, pollen, and pet allergies [20]. To date, there is limited evidence supporting the use of AIT in the management of fungi-induced asthma and allergic rhinitis. An exception is *Alternaria alternata* as this is the only standardized allergen extract available [21].

The standard diagnostic test for allergies has been a skin prick test (SPT) using crude extract and/or detection of allergen-specific serum IgE antibodies. However, species-specific IgE reactivity is difficult to confirm due to cross-reactivity between crude allergen extracts from different fungi, representing a significant problem for evaluating IgE tests in clinical practice [22, 23]. Therefore, there is a need to identify specific allergenic fungal proteins that can be used to produce recombinant proteins for therapy and diagnosis [24].

Furthermore, the characterization of the allergenic molecules that an individual is sensitized to can help discriminate between the likelihood of local versus systemic reactions and the persistence of clinical symptoms [25]. For example, some allergens, such as storage proteins in edible nuts (e.g., Ara h 2 and Cor a 9), have been shown to be associated with severe reactions, while other allergens cause sensitization mostly without a clinical reaction.

Most fungi possess multiple and diverse allergens that can be divided into several classes according to their function, e.g., protein hydrolysis (proteases, peptidases), carbohydrate hydrolases (chitinases, glucosidases), and antioxidants (superoxide dismutase, catalases) [26]. These have been described and officially named according to the IUIS nomenclature (http://www.allergen.org), including both major and minor allergens from *Aspergillus fumigatus, Penicillium chrysogenum, Cladosporium herbarum, Rhizopus, Alternaria alternata, and Epicoccum nigrum* [27–36]. However, it has been suggested that many patients display IgE reactivity towards unknown proteins, which may vary between populations [37]. As no studies have been conducted characterizing fungal allergic sensitization in a Zimbabwean population, the present study was conducted to identify allergenic proteins from seven fungal species (*Aspergillus fumigatus, Alternaria alternata, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans,* and *Saccharomyces cerevisiae*) in Zimbabwe.

Methodology

Study Population

This study was conducted in Shamva district, one of the seven districts in the Mashonaland Central province of Zimbabwe. It was part of a larger study investigating fungal sensitization in pre-

school age children. The area was selected for this current study because the prevalence of fungal sensitization was high, as determined by our previous study [38]. The inhabitants from the study area are of similar ethnicity (Shona) and socioeconomic background (primarily subsistence farmers).

Ethical Approval and Consent

Ethical and institutional approval was obtained from the Medical Research Council of Zimbabwe (MRCZ/A/1964) and the University of Edinburgh. Permission to conduct the study was obtained from the Mashonaland Central Provincial Medical Director. The study aims and procedures were explained to all participants and their parents/guardians in their local language (Shona) before obtaining consent. Written informed consent was obtained from the participants' parents/guardians and recruitment was voluntary with participants free to withdraw from the study at any stage.

Inclusion Criteria

To be included in the study cohort, participants had to meet all of the following criteria: (1) provide a serum sample; (2) ELISA results showing IgE-specific binding to different fungal species; (3) be sensitive to fungi (i.e., IgE positive as determined by SPT). Eight individuals met these criteria (3–5 years old).

Antigen

Freeze-dried crude extracts of *Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus nigricans,* and *Saccharomyces cerevisiae* were obtained from Stallergenes Greer (USA). These extracts were individually reconstituted in Phosphate Buffered Saline and separately run on sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Gel Electrophoresis

One-dimensional gel separation was performed in two parallel samples: one for protein identification and the other for Western blotting. Gel electrophoresis was performed on a 12% polyacrylamide 13-cm gel in a Hoefer SE600 system using sodium dodecyl sulphate (SDS) buffer. The proteins on the gel used for identification were stained with Coomassie blue to visualize them whereas proteins on the gel used for Western blot were transferred onto nitrocellulose membrane as described below.

Immunoblotting

Proteins were transferred from the gel onto a nitrocellulose membrane using semi-dry system (Hoefer) in transfer buffer (Invitrogen) containing 10% methanol at 30 V for 1 h. The membrane was stained with Ponceau S solution to check transfer efficiency and was then blocked at room temperature for 1 h in Tris-buffered saline (TBS) blocking buffer and 0.05% Tween 20. After blocking, the membrane was subjected to 2 separate 10-min washes with TBS, 0.05% Tween, and 0.5% Triton-X 100 (TBS/TT). A pool of serum samples (diluted 1:100 in blocking buffer) was added to the membrane and the membrane was incubated overnight at 4°C and then washed 3 times for 10 min each time in TBS/TT. Horseradish peroxidase-conjugated rabbit anti-human IgE and IgG were diluted 1:1,000 and 1:4,000, respectively, in TBS blocking buffer. The membrane was incubated at room temperature for 1 h and then washed 4 times for 10 min in TBS/TT and 1 time for 10 min in TBS alone. The proteins were visualized using the chemiluminescence product ECL Plus (Amersham), in accordance with manufacturer's instructions. The blots were analysed using Gel Doc from Bio-Rad Image Lab Software.

Image Analysis

The bands on the Coomassie blue-stained gel that matched those on the Western blots were excised and then were analysed by mass spectrometry (MS).

Mass Spectrometry

The excised bands were separately subjected to in-gel trypsin digestion and the resulting peptides solubilized in 20-μL 5% acetonitrile with 0.5% formic acid using the auto-sampler of a nano-flow uHPLC system (Thermo Scientific RSLCnano). Online detection of peptide ions was by electrospray ionization-mass spectrometry MS/MS with an Orbitrap Elite MS (Thermo Scientific). Data were submitted for an MS/MS ion search via the Mascot search engine (http://www.matrixscience.com).

Database Search and Allergen Prediction

For protein identification, the MS/MS data were uploaded on the Mascot search engine (v2.6.2, Matrix Science) and compared against protein sequences in the NCBIprot database using taxonomies *Alternaria alternata, Aspergillus fumigatus, Cladosporiaceae, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus stolonifera (nigricans), and Saccharomyces cerevisiae*. Searches were performed using the following parameters: trypsin as the proteolytic enzyme, allowing for one missed cleavage; for fixed and variable modifications, carbamidomethylation of cysteine and methionine oxidation were used, respectively. The precursor mass tolerance was set at 10 ppm and 0.3 Da for MS/MS matching. Proteins identified with a Mascot score greater than 200 (significant at 95% confidence interval) are reported.

To identify the likely allergen proteins present in the fungal species, the recognized proteins were searched through the structural database of allergenic proteins (SDAP) (http://fermi.utmb.edu/SDAP/index.html), a Web server that provides rapid, cross-referenced access to the sequences, structures, and IgE epitopes of allergenic proteins. The proteins were predicted under the following conditions: (1) sequence similarity >35% between presently obtained proteins and reported allergen proteins and (2) a minimum of 80 amino acid overlap length [39, 40]. Furthermore, predictions for antigenicity were obtained using an online software (http://imed.med.ucm.es/Tools/antigenic.pl) based on the algorithm of Kolaskar and Tongaonkar [41] where the predictions are based on a table that reflects the occurrence of amino acid residues in experimentally known segmental epitopes. Segments are only reported if they are at least eight residues. By these criteria, some of the fungal proteins identified by the sera were classified as likely corresponding to an allergenic protein.

Results

One-Dimensional Gel Electrophoresis Analysis

One-dimensional gel electrophoresis of crude extracts from fungal cells resulted in separation of several proteins

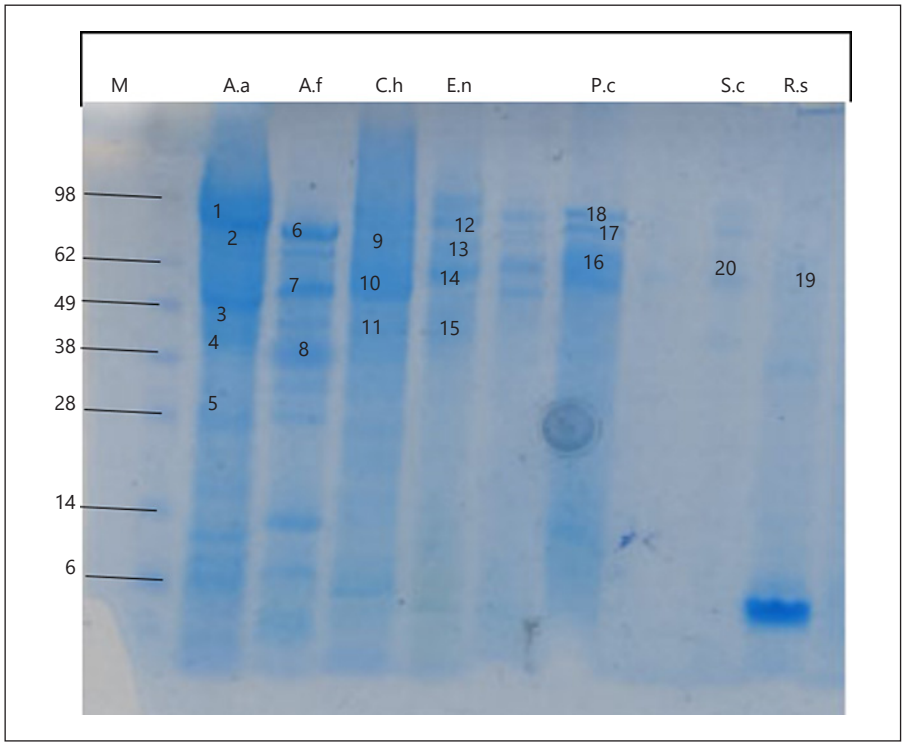

Fig. 1. Coomassie blue-stained one-dimensional gel showing bands matched to the Western blots. Bands on the gel were excised and identified. Molecular weight markers (in kilodaltons) are given on the left. M, marker; *Aa, Alternaria alternata; Af, Aspergillus fumigatus; Ch, Cladosporium herbarum; En, Epicoccum nigrum; Pc, Penicillium chrysogenum; Rs, Rhizopus stolonifer (nigricans)*; *Sc, Saccharomyces cerevisiae*.

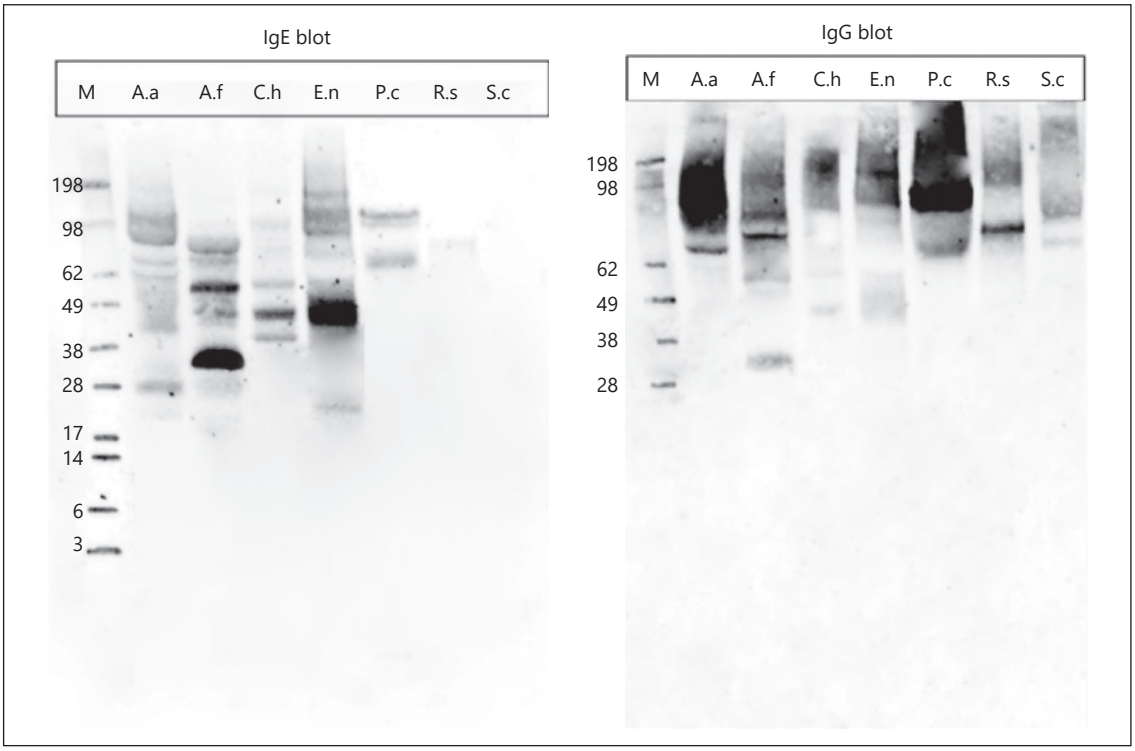

Fig. 2. Western blot analyses of serological reactivity of serum samples. M, marker; *Aa, Alternaria alternata; Af, Aspergillus fumigatus; Ch, Cladosporium herbarum; En, Epicoccum nigrum; Pc, Penicillium chrysogenum; Rs, Rhizopus stolonifer (nigricans)*; *Sc, Saccharomyces cerevisiae*.

Table 1. Fungal proteins identified by MS

Band[a]	Protein name	Species	Accession	Hit score[c]	MW[d]	Molecular function	Reactivity	
							IgE	IgG
1	Glycoside hydrolase	*Alternaria alternata*	XP_018381330.1	2,128	87,237	Hydrolase activity	Yes	No
1	Trehalose	*Alternaria alternata*	XP_018389336.1	2,326	77,106	Alpha, alpha trehalase activity	Yes	No
1	Peptidase s41 family protein	*Alternaria alternata*	OWY41590.1	4,458	83,904	Unknown	Yes	No
2	M6 metalloprotease	*Alternaria alternata*	OWY56860.1	2,911	74,095	Metallopeptidase activity	Yes	No
2	FAS1 domain-containing protein	*Alternaria alternata*	XP_018380929.1	2,217	50,925	Unknown	Yes	No
2	Cyclohexanone 1,2-monooxygenase	*Alternaria alternata*	OWY42352.1	1,663	128,346	Unknown	Yes	No
2	Meiotically up-regulated 157 protein	*Alternaria gaisen*	KAB2110334.1	1,290	57,040	Unknown	Yes	No
3	Vanadium chloroperoxidase	*Alternaria tenuissima*	RYN52497.1	7,156	67,460	Peroxidase activity	Yes	Yes
3	Meiotically up-regulated 157 protein	*Alternaria gaisen*	KAB2110334.1	5,868	57,040	Unknown	Yes	Yes
3	FAS1 domain-containing protein	*Alternaria alternata*	XP_018380929.1	897	50,925	Unknown	Yes	Yes
4	Subtilisin-like serine protease-like protein PR1A	*Alternaria alternata*	XP_018384475.1	2,893	40,384	Serine-type endopeptidase activity	Yes	No
4	Concanavalin A-like lectin/glucanase	*Alternaria alternata*	XP_018390955.1	1,408	46,193	Hydrolase activity	Yes	No
5	GroES-like protein	*Alternaria alternata*	XP_018385554.1	1,675	38,120	Oxidoreductase activity	Yes	No
5	Glycoside hydrolase	*Alternaria alternata*	XP_018382523.1	1,426	44,564	Hydrolase activity	Yes	No
6	Dipeptidyl-peptidase 5	*Aspergillus lentulus*	GFF50131.1	5,526	79,688	Serine-type peptidase activity	Yes	Yes
6	Secreted dipeptidyl peptidase	*Aspergillus fischeri NRRL 181*	XP_001260402.1	5,477	79,675	Serine-type peptidase activity	Yes	Yes
7	Secreted dipeptidyl peptidase	*Aspergillus fischeri NRRL 181*	XP_001260402.1	2,304	79,675	Serine-type peptidase activity	Yes	Yes
7	Catalase B	*Aspergillus minisclerotigenes*	KAB8269428.1	1,472	79,856	Catalase activity	Yes	Yes
7	Dipeptidyl-peptidase 5	*Aspergillus lentulus*	GFF50131.1	1,366	79,688	Serine-type peptidase activity	Yes	Yes
8	Chitinase	*Aspergillus fumigatus*	AAP23218.1	6,271	47,708	Chitinase activity	Yes	Yes
9	GPI-anchored cell wall beta 1,3 endoglucanase EglC	*Aspergillus fumigatus var. RP-2014*	KEY82708.1	2,198	44,923	Unknown	Yes	No
10	Hypothetical protein CNMCM8714_006,228	*Aspergillus fumigatus*	KAF4253478.1	1,319	105,137	Unknown	Yes	No
11	Hypothetical protein CDV57_00,056	*Aspergillus fumigatus*	OXN30505.1	1,063	16,162	Unknown	Yes	No
12	Catalase	*Aspergillus clavatus NRRL 1*	XP_001273665.1	521	80,097	Catalase activity	Yes	No
13	Alpha-glucosidase	*Rachicladosporium antarcticum*	OQO11764.1	625	67,385	Hydrolase activity	Yes	Yes
14	Hypothetical protein B5807_10,540	*Epicoccum nigrum*	OSS44738.1	7,238	112,128	Beta-glucosidase activity	Yes	No
15	S-adenosyl-L-homocysteine hydrolase	*Aspergillus homomorphus CBS 101889*	XP_025550664.1	3,028	49,516	Adenosylhomocysteinase activity	Yes	No
16	Hypothetical protein B5807_10,540	*Epicoccum nigrum*	OSS44738.1	1,485	112,128	Beta-glucosidase activity	Yes	Yes
17	Glycoside hydrolase family 31	*Aspergillus oryzae*	OOO09042.1	2,609	106,688	Beta-glucosidase activity, maltose alpha-glucosidase activity	Yes	Yes
17	Putative dipeptidyl peptidase	*Penicillium chrysogenum*	KZN92610.1	4,136	85,215	Serine-type peptidase activity	Yes	Yes
18	Putative dipeptidyl peptidase	*Penicillium chrysogenum*	KZN92610.1	15,819	85,215	Serine-type peptidase activity	Yes	No
18	Glucose oxidase	*Penicillium chrysogenum*	AFA42947.1	669	66,471	Glucose oxidase activity	Yes	No
19	RecName: full = alpha-(1–6)-linked fucose-specific lectin; AltName: full = RSL	*Rhizopus stolonifer*	P83973.1	7,343	3,199	Unknown	No	Yes
20	Glr1p	*Saccharomyces cerevisiae YJM320*	AJV98761.1	1,540	53,790	Unknown	No	Yes

[a] Band numbers indicated in Figure 1. [b] Accession numbers according to NCBInr database. [c] Mascot score reported after database search, score >200 indicates identity or extensive homology at $p < 0.05$. [d] Theoretical mass retrieved from NCBInr database.

Table 2. Predicted allergen-related proteins in the fungal species investigated

Predicted allergens				Corresponding known allergens				
band[a]	accession No[b]	description	AA[c]	allergen[d]	accession no[b]	AA[c]	bit score[d]	*E* score[e]
1	XP_018381330.1	Glycoside hydrolase (*Alternaria alternata*)	798	Asp n 14	CAB06417	804	500.0	2.8e–142
				Asp n 14	AAD13106	804	498.7	6.5e–142
4	XP_018384475.1	Subtilisin-like serine protease-like protein PR1A (*Alternaria alternata*)		Asp f 13	P28296	403	212.5	2.2e–56
				Asp v 13.0101	ADE74975	403	203.2	1.4e–53
				Pen c 13.0101	AAD25926	397	203.0	1.6e–53
				Pen ch 13	AAF23726	397	200.2	1.1e–52
				Tri r 2.0101	AAD52013	412	197.1	1.0e–51
				Asp fl protease	AAD47202	403	196.8	1.1e–51
				Asp o 13	CAA35594	403	196.8	1.1e–51
				Pen ch 18	AAF71379	494	186.1	2.4e–48
				Cur l 4.0101	ACF19589	506	113.2	2.2e–26
				Asp f 18.0101	Y13338	495	108.9	4.2e–25
				Pen o 18	AAG44478	503	107.2	1.4e–24
5	XP_018385554.1	GroES-like protein (*Alternaria alternata*)	352	Cand a 1	AAA53300	350	307.0	6.3e–85
				Cand a 1	P43067	350	304.3	4.0e–84
6	GFF50131.1	Dipeptidyl-peptidase 5 (*Aspergillus lentulus*)	721	Tri r 4.0101	AAD52012	726	666.6	1.5e–192
7	XP_001260402.1	Secreted dipeptidyl peptidase (*Aspergillus fischeri NRRL 181*)		Tri r 4.0101	AAD52012	726	677.2	9.5e–196
7	KAB8269428.1	Catalase B (*Aspergillus minisclerotigenes*)		Pen c 30.0101	ABB89950	733	930.0	0.0e+00
12	XP_001273665.1	Catalase (*Aspergillus clavatus NRRL 1*)	728	Asp f 15	O60022	152	224.0	1.4e–60
18	KZN92610.1	Putative dipeptidyl peptidase (*Penicillium chrysogenum*)	772	Tri r 4.0101	AAD52012	726	530.8	1.2e–151

[a] Band numbers indicated in Figure 1. [b] Accession numbers according to NCBInr database. [c] Amino acid sequence. [d] Bit score-sequence similarity. [e] *E* score-homology.

that were visible after staining (shown in Fig. 1). Of these, 20 bands reacted with human serum samples from fungal-sensitized individuals as determined by anti-human IgE/IgG immunoblotting (shown in Fig. 2; Table 1).

Immunogenic Protein Identities

The 20 bands identified as serologically reactive by Western blot were excised from the Coomassie blue-stained gel and were subjected to in-gel trypsin digestion. Subsequently, the peptides were analysed by MS/MS and the peptide data obtained were used to search NCBIprot databases. Most of the bands were successfully matched to specific fungal proteins and the identifications of these bands are shown in Table 1. The identity given for each band corresponding to the top hit score (the Mascot output statistic) that had a score >200 (significant at 95% confidence interval), the predicted MW, as well as the associated species are also provided in Table 1. The MS/MS analysis revealed cases in which different bands were derived from the same protein, for example, bands 2 and 3 (FAS1 domain-containing protein and eiotically up-regulated 157 protein) as well as 6 and 7 (secreted dipeptidyl peptidase). The MS/MS analysis also revealed bands that resolved to the same protein but with different accession numbers (e.g., bands 1 and 5, both glycoside hydrolase). The 20 bands recognized by serum samples gave rise to 34 protein identifications. Of the 34 proteins identified, 3 were hypothetical proteins, 11 had no known function,

and the remaining 20 proteins could be grouped by molecular function (Table 1. The identified proteins included enzymes and most of these proteins have not been previously shown to be immunogenic.

Prediction of Allergens Fungi (Alternaria alternata, Aspergillus, Cladosporium herbarum, Epicoccum nigrum, Penicillium chrysogenum, Rhizopus stolonifera, and Saccharomyces cerevisiae)

Fungal species are known to contain several proteins that act as allergens [42]. Using structural and sequence predictive tools, eight fungal proteins were predicted to be allergens (Table 2. Four of these corresponded with serine proteases from various fungal species, sharing 39.9–58% sequence homology to known fungal allergenic proteases.

The predicted antigenic peptides are shown in online supplementary Figures S1–S4 (for all online suppl. material, see www.karger.com/doi/10.1159/000524771). The remaining proteins were not identified as allergens using these tools.

Discussion

Many allergens have been reported from fungi, including >40 from the fungal species investigated in this study. These include allergens identified functionally as enzymes and regulatory proteins, proteases, enolases, and heat shock proteins, while others currently have unknown biochemical functions and activities [43]. Among the recognized enzymes, the predominant allergens are proteases, ribonuclease, chymotrypsin, catalase, and superoxide dismutase [44, 45].

In this study, the pooled sera reacted mostly with *A. alternata;* we were able to identify 34 immunogenic proteins across the functional spectrum [46]. Among the identified proteins, several were known allergens from other fungal, plant, and insect species, e.g., catalase, chitinase, subtilisin-like serine protease, beta glucosidase, and dipeptidyl-peptidase 5 [47–49]. However, we also identified novel IgE-binding proteins, i.e., allergens. For example, M6 metalloprotease and cyclohexanone 1,2-monooxygenase from *A. alternata* as well as exo-beta-1,3-glucanase and GPI-anchored cell wall beta 1,3 endoglucanase from *A. fumigatus.*

For the majority of detected proteins, there was a disparity between the observed and theoretical molecular weights. These discrepancies may have been due to post-transcriptional modification and/or the structural subunits required for appropriate functioning [50]. Of the 34 proteins recognized by Zimbabwean sera, eight were identified as putative allergens through the allergen-predicting software.

The sera in our study also reacted with Catalase B, which is consistent with other studies that have identified other fungal catalases as allergens including *A. fumigatus* [51], *Aspergillus versicolor* [52], and *Penicillium citrinum* [53]. In the present study, Catalase B exhibited high sequence identity (74.5%) with catalase from *P. citrinum* (Pen c 30.0101) [26]. The high sequence homology observed between these enzymes may represent a conserved allergenicity of the catalase protein. Catalases are ubiquitous iron-containing enzymes that protect cells from oxidative damage through hydrogen peroxide hydrolysis [54, 55]. However, in fungi, catalases have been suggested to play additional roles in conidial germination [56], sporulation [57], and pathogenesis [58], implying that fungal catalases contain some unique epitopes that may be immunogenic [59].

Of all the putative allergens identified here, only one, which was a glycoside hydrolase, corresponded to an occupational (workplace-related) allergen (xylanase [Asp n 14]) from *Aspergillus niger* [60], which is typically associated with baking, farming, and cereal handling [61]. This observation may be associated with para-occupational exposure. Previous studies have shown that occupational allergens can be transported home, presumably on contaminated clothing and skin, with subsequent sensitization of other household residents, including children, leading to severe allergic diseases in atopic patients if not diagnosed and treated [62].

Cross-reactive proteins in fungal allergens complicate the diagnosis and management of fungal allergy and this limitation results in patients having allergic sensitization to many biologically related fungi. In our study, several immunoreactive protein bands of the crude extracts belonged to two main fungi, *Alternaria* and *Aspergillus,* which are likely associated with cross-reactivity amongst the fungal species, as this has been shown between phylogenetically close and even distant species [22]. While molecular diagnostics have improved the ability to identify clinically relevant cross-reactivity, there is still a need to understand the fungal-specific (degree of homology, abundance) and patient-specific factors (immune response, augmentation factors), as well as the epidemiology of cross-reactivity that determines clinical relevance [63].

To further proceed with this work, we will analyse the samples individually for each patient in the study, which

could provide some additional information about the status and frequency of the specific recognition of these candidate allergens. In addition, we will conduct ELISA inhibition studies to further evaluate cross-reactivity of these candidate allergens. Furthermore, research into the potential recognition of these allergens in larger cohort populations will be key in validating these allergens.

In summary, the fact that several immunogenic proteins were novel allergens indicates a need to expand the reference database for the allergen prediction software and highlights potential population differences in genetic variations in the major histocompatibility complex specificity. Therefore, there is need to consider these differences when developing diagnostics and therapeutics for fungal allergy in African populations. The presence of cross-reactivity of allergens amongst related fungal species gives the potential for developing cross species therapeutics and diagnostics.

Conclusion

We identified 34 fungal proteins reactive with serum from a population of Zimbabweans sensitized to fungi. Based on the structural and sequence predictive tools, eight of these were identified as putative allergens. Validation assays will now need to be carried out to further evaluate the cross-reactivity of the identified allergen candidates as well as investigate their potential recognition in a larger cohort of patients. Furthermore, there is need to investigate the role of these immunogens in the aetiology of allergic disease and mechanistic pathways to inform the development of diagnostics and therapeutics appropriate for African populations.

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Statement of Ethics

Ethical and institutional approval was obtained from the Medical Research Council of Zimbabwe (MRCZ/A/1964) and the University of Edinburgh. Permission to conduct the study was obtained from the Mashonaland Central Provincial Medical Director. Written informed consent was obtained from the participants' parents/guardians and recruitment was voluntary with participants free to withdraw from the study at any stage.

Conflict of Interest Statement

The authors have declared that no competing interests exist.

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Author Contributions

Francisca Mutapi, Takafira Mduluza, and Elopy Sibanda conceived the study. Francisca Mutapi, Takafira Mduluza, Elopy Sibanda, and Lorraine Pfavayi conducted the fieldwork; Lorraine Pfavayi curated the field data. Lorraine Pfavayi and Richard Burchmore conducted the laboratory work; Lorraine Pfavayi, Richard Burchmore, and Francisca Mutapi conducted the data analysis. Lorraine Pfavayi and Francisca Mutapi drafted the manuscript; Stephen Baker, Mark Woolhouse, Richard Burchmore, Francisca Mutapi, Takafira Mduluza, Lorraine Pfavayi, and Elopy Sibanda discussed, reviewed, amended, and approved the final version of the manuscript.

Data Availability Statement

All the data that support the findings of this study will be fully available with publication, through the University of Edinburgh Datashare.

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A mathematical model for the prediction of the prevalence of allergies in Zimbabwe

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ABSTRACT

Background: The prevalence of allergies has been observed to be increasing in the past years in Zimbabwe. It is thus important to consider the long term prevalence of allergies. Our interest is in investigating the trends of allergies in the next 2 decades.

Method: We formulate a deterministic model with 6 compartments to predict the prevalence of allergies in Zimbabwe. The human population is divided into 4 distinct epidemiological, classes based on their exposure to 2 allergen groups (food and inhalants), represented by 2 compartments. The model is used to predict the prevalence of allergen sensitization. The number of human allergen groups in each compartment are tracked through a system of differential equations. Model parameters were obtained by fitting observed data to the model. Graphical solutions of the model were developed using Matlab and Excel.

Results: The rate of sensitisation to food allergen sources is found to be lower than the rate of sensitisation to inhalant allergens. The rate at which individuals develop tolerance to food allergen sources is found to be almost twice the rate of developing tolerance to inhalant allergies. The equilibrium solutions (the long-term states of the populations) of the model are found to be non-zero implying that there will never be an allergy-free population. Our results also show that the prevalence of food allergy is likely to increase in the next 2 decades while inhalant allergy prevalence is expected to decrease.

Conclusion: Our long-term solutions show endemicity in allergies in Zimbabwe. So, allergy will be endemic in the Zimbabwean population; hence there is a need for allergy care and management facilities to be increased. These results are critical in policy development and planning around allergies in the near future.

Keywords: Food allergy, Inhalant allergy, Mathematical model, Zimbabwe

INTRODUCTION

Type 1 allergic conditions are characterized by the expression of allergen specific IgE antibodies to triggering allergen sources that may be through inhalation or ingestion. The most frequent inhaled allergen sources locally are house dust mites,

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grass or tree pollen, and molds. The range of food allergen sources includes egg white, milk proteins, wheat, nuts, fruits, and fish. The food allergen sources to which an individual is exposed is influenced by dietary options that change as the population migrates from rural settings where traditional food items dominate to urban settings where the choices are greater and the variety ever increasing.[1] The flora and fauna of urban settings is dominated by ornamental trees, weeds, and lawn grass and is different from that obtaining in often parched rural settings.

Studies of allergic conditions have shown that these have erratic progression, are difficult to diagnose, and have potentially distressing consequences on the quality of life of affected individuals as well as an increasing health care burden of most countries worldwide.[2] Some descriptive studies that were conducted in Sub-Saharan countries including South Africa,[3] Nigeria,[4] Zimbabwe,[1] and Botswana,[5] all suggested that there is an increase in the prevalence of allergies.

In Zimbabwe, health priorities are, however, biased towards tropical infectious diseases. The relevance of allergic diseases in general and their impact on the quality of life is not prioritized in a country seized with major infectious diseases including HIV and AIDS, tuberculosis, and malaria. As a result, essential tiers of allergy care including human resource planning, training, and deployment for optimum mitigation, diagnosis, and disease management are overshadowed and lowly prioritized to the detriment of millions of affected individuals of all ages. Allergy data that are routinely captured by Ministries of Health are guided by World Health Organization (WHO) templates and tend to be limited to asthma. The prevalence, prevalence rates, periodicity, and seasonal fluctuations in numbers of patients with inhalant allergic diseases and the respective case fatality rates from these non-communicable allergic diseases are not systemically collected at the national level, and such tallying is not recommended by WHO. Resultantly relevant, informative data are not captured to the detriment of allergy patient care. We have observed substantial increases in the prevalence of allergy morbidity primarily in the private sector setting. Such data are not captured by the Ministry of Health; therefore, the extent to which non-communicable allergic diseases contribute to morbidity and mortality is not appreciated and opportunities to intervene are lost. The inclusion of the diagnosis and management of allergic diseases in a list of Ministry of Health priorities can only be informed by an understanding of the magnitude of the problem and its trajectory. We used data collected from the only allergy clinic in the country to attempt to project the trajectory of food and inhalant allergies over the next 20 years.

We have observed that numbers of sensitized individuals in the country are increasing. When a susceptible, usually atopic individual is exposed to allergen sources, sensitization can occur, and upon re-exposure, allergic symptoms can manifest. There is a large variety of allergen sources and a wide population of susceptible individuals, and the interplay between them is dynamic and variable. Additionally, as the environment continuously changes, so are the allergens available to interact with individuals which poses an increasing burden of allergy in a population. It is important to understand the rates at which susceptible individuals get sensitized to allergen sources as this is important information for public health planning and decision making. The few descriptive studies carried out in Zimbabwe lack the element of mathematical dynamics between allergens and the human population. Mathematical modelling studies that have been pivotal to the understanding of infectious diseases may also be useful in understanding the current and projected burden of allergies, whose results should inform relevant public health policy formulation.

This study was carried out to characterize the trends of food and inhalant allergy prevalence, to project the prevalence and to determine estimates of allergy epidemiology. The causal effect of allergy susceptibility and exposure were incorporated into a mathematical model to understand the extent to which allergic diseases prevalence has been changing over time and to forecast future changes.

The model

A standard *S* (Susceptible), *E* (Exposed), *I* (infected), *R* (Recovered) infectious disease model (SEIR)[6] was modified and used to illustrate the progression

of allergic diseases. It is important to stress here that modelling allergies is not equivalent to modelling infectious diseases but rather diseases with environmental transmission pathways (Lanzas, 2020).[7] A nonlinear mathematical model to study the spread of asthma, with a similar framework, due to inhaled pollutants from industry as well as tobacco smoke from smokers in a variable size population is presented in Naresha and Tripathi.[8] In this model, the entire population is considered to be susceptible to allergens, and after contact with a specific allergen, one is sensitised to the specific allergen and in this case only 2 allergen groups, food and inhalants, are considered. Once sensitised, an individual may become tolerant.

The SEIR model was adopted since we are focusing mainly on the population level spread of allergies through the interaction of the human population with allergens. This is a new method being used to understand the dynamics between the human population and allergens and to predict the prevalence of allergies in the Zimbabwean population.

In our model, the susceptible class (S) refers to the healthy individuals that have not been exposed to an allergen. The exposed individuals are those who ingest food, become sick, and get sensitised to a particular food allergen and join the class (X_f). The food allergens, A_f, grow at a rate $\wedge_f$ in the environment and are consumed at a rate $\nu_f A_f$. Inhalant allergens A_i, grow at a rate $\wedge_i$ and they decrease at a rate $\nu_i A_i$. When susceptible individuals get exposed and interact with inhalant allergens, they move into the class X_i which is the class of individuals that are sensitised to a particular inhalant allergen. The tolerant T_t are individuals who were once sensitised to either food or inhalant allergens and can later be exposed to either food or inhalant without getting affected. We assume that once individuals are tolerant to an allergen, then they belong to a single class irrespective of what they are tolerant to. The description of the model is depicted in Fig. 1.

The model was built under the following assumptions.

1. The rate of exposure to the allergens is assumed to be different for food and inhalant allergens.
2. The population in each class is assumed to be homogeneous for the allergen they are allergic to.
3. The interactions of the susceptible individuals and the allergens are assumed to be homogenous.
4. We assume a mass action form of interaction as humans and the allergens are assumed to have an equal chance of coming into contact.
5. We assume that tolerance is the final state any individual who develops allergies will get to.

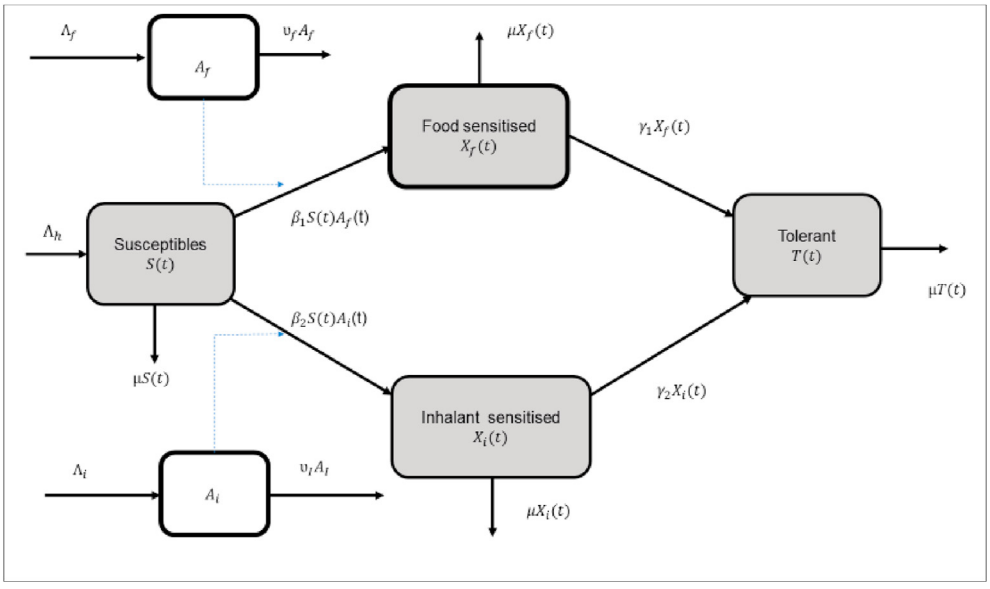

Fig. 1 Model framework

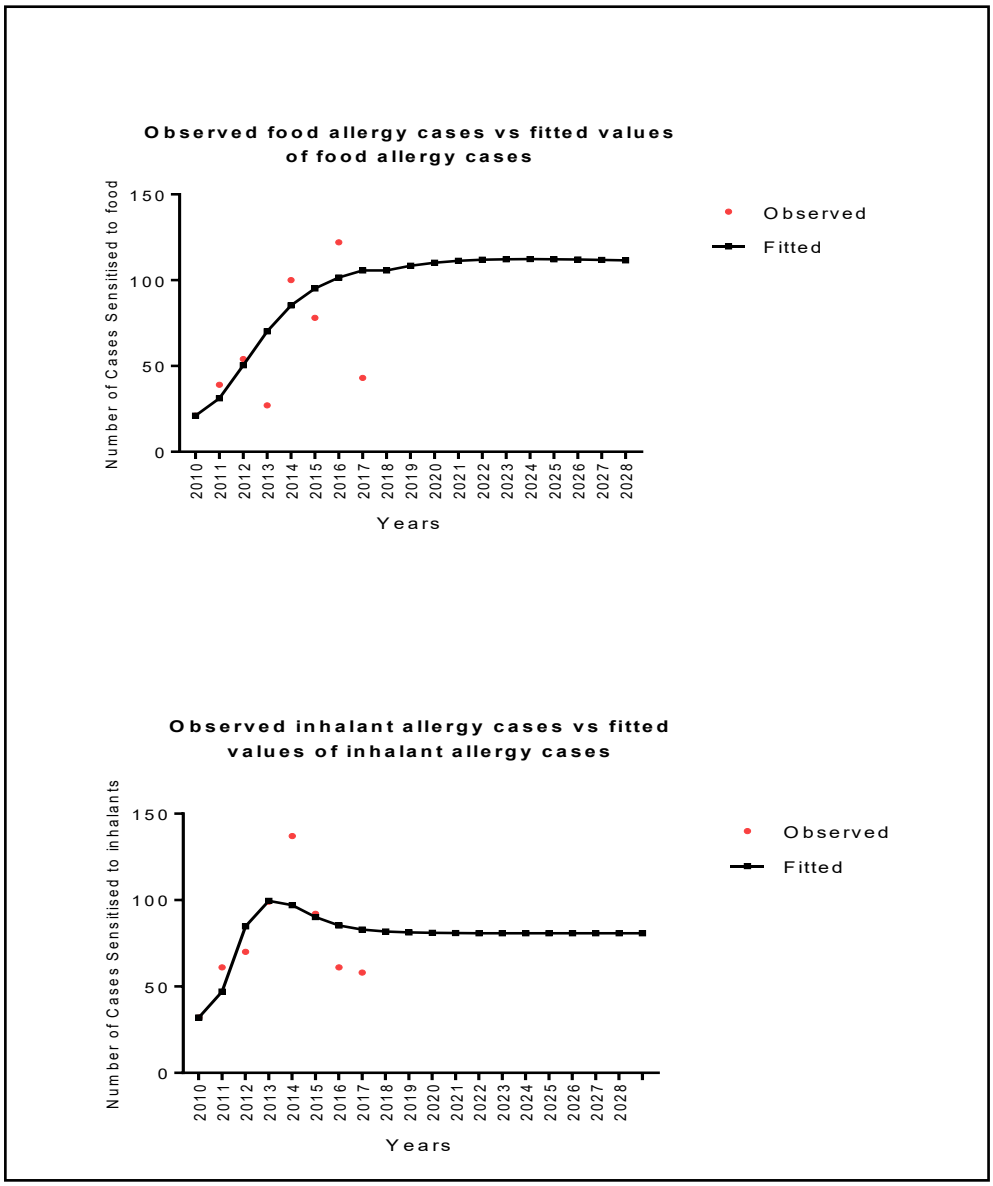

Fig. 2 Trends of food and inhalant allergies recorded in Zimbabwe from 2010 to 2017

6. The rate at which individuals develop tolerance for allergens is assumed to be different for food and inhalant allergens.
7. Allergy is not fatal; hence there are not disease-related parameters for the sensitised.

Model equations

When individuals get exposed to allergens, some of them interact with the allergens and develop an immunological response resulting in the production of IgE antibodies. They show allergy symptoms and are referred to the physician where they get tested for allergy. They may express antibodies specific to some allergen(s) or they may not be sensitised to an allergen.

The rate of change of the susceptible population is increased through births at a rate, Λ_h. This population is reduced by the sensitisation to food and inhalant allergens at rates β_1 and β_2, respectively and through natural mortality (assumed for all the human compartments) that occurs at a rate μ.

Hence the rate of change of the susceptible population is represented by the ordinary differential equation below.

$$\frac{dS}{dt} = \wedge_h - \beta_1 SA_f - \beta_2 SA_i - \mu S, \tag{3.1}$$

The rate at which individuals leave the susceptible population is equal to the rate at which they enter the sensitised classes. The proportion of individuals in the sensitised population increase since those in the susceptible class become exposed and some get sensitised to the allergens over time.

Let γ_1 and γ_2 be the rates at which sensitised individuals become tolerant to the food and inhalant allergens, respectively. Then, the rate of change of the population sensitised to only food allergens is given by

$$\frac{dX_f}{dt} = \beta_1 SA_f - (\mu + \gamma_1)X_f. \tag{3.2}$$

The rate of change of population sensitised to only inhalant allergens is given by

$$\frac{dX_i}{dt} = \beta_2 SX_i - (\mu + \gamma_2)X_i, \tag{3.3}$$

and the rate of change of the tolerant population is given by

$$\frac{dX_T}{dt} = \gamma_1 X_f + \gamma_2 X_i - \mu X_T. \tag{3.4}$$

The amounts of the allergens also vary with time depending on the availability and interaction of the available allergens with humans. The growth rates of food and inhalant allergens in the environment are respectively given by $\wedge_f$ and $\wedge_i$. On the other hand, the depletion rates of food and inhalant allergens are given by υ_f and υ_i respectively.

The rate of change of the amount of food allergens is given by

$$\frac{dA_f}{dt} = \wedge_f - \upsilon_f A_f, \tag{3.5}$$

while the rate of change of the amount inhalant allergens is given by

$$\frac{dA_i}{dt} = \wedge_i - \upsilon_i A_i. \tag{3.6}$$

We thus have the following model:

Human population

$$\frac{dS}{dt} = \wedge_h - \beta_1 SA_f + \beta_2 SA_i - \mu S, \tag{3.7}$$

$$\frac{dX_f}{dt} = \beta_1 SA_f - (\mu + \gamma_1)X_f. \tag{3.8}$$

$$\frac{dX_i}{dt} = \beta_2 SX_i - (\mu + \gamma_2)X_i, \tag{3.9}$$

$$\frac{dX_T}{dt} = \gamma_1 X_f + \gamma_2 X_i - \mu X_T \tag{3.10}$$

Allergens

$$\frac{dA_f}{dt} = \wedge_f - \upsilon_f A_f, \tag{3.11}$$

$$\frac{dA_i}{dt} = \wedge_i - \upsilon_i A_i. \tag{3.12}$$

The sensitisation rates, tolerance rates, birth rate/death, growth rate of allergens, and depletion rate of allergens are all non-negative. We summarise the descriptions of the state variable and parameters in the following tables.

Before we carry out any analysis of the model, it is important to look at the model properties to guarantee the existence and uniqueness of solutions, which in turn guarantees that our model is biologically feasible.

Model properties

Given that the summation of the equations for the human population N, is

$$\frac{dN}{dt} = \wedge_h - \mu N, \tag{3.13}$$

then, when we separate variables we have

$$\frac{dN}{\wedge_h - \mu N} = dt. \tag{3.14}$$

Integrating and solving for N gives

$$N(t) = \frac{\wedge_h}{\mu} + N_0 e^{-\mu t} \quad where \quad N_0 = N(0). \tag{3.15}$$

We observe that $lim_{t\to\infty} N(t) = \frac{\Lambda_h}{\mu}$. The human population will thus not exceed $\frac{\Lambda_h}{\mu}$. We thus mathematically say the population is bounded by the

expression $\frac{\Lambda_h}{\mu}$.

Solving the equations of the allergens we have, $A_f \to \frac{\wedge_h}{\upsilon_f}$ as $t \to \infty$ and $A_i \to \frac{\wedge_h}{\upsilon_i}$.

Therefore the state variables remain biologically meaningful in the set $\Omega = \left\{(S, X_f, X_i, T, A_f, A_i) \in \mathbb{R}_+^6 \middle| 0 < N \leq \frac{\wedge_f}{\mu}, \quad 0 < A_f \leq \frac{\wedge_f}{\upsilon_f}, 0 < A_i \leq \frac{n_i}{\upsilon_i}\right\}$ for all positive initial conditions in $\mathbb{R}^6$. The set Ω is said to be positively invariant and all solutions of system (3.1)–(3.6) with initial conditions $(S_0, X_{f0}, X_{i0}, A_{f0}, A_{i0}) \in \mathbb{R}^6$ remain in Ω for all $t > 0$.

The system is thus mathematically and epidemiologically well-posed, as the solutions are positive and bounded in Ω. We shall thus base our analysis on the solutions generated in Ω.

Model equilibria

By using the right-hand side of system (3.7)–(3.12) the model equilibria are obtained from

$$0 = \wedge_h - \beta_1 S A_f - \beta_2 S A_i - \mu S \tag{3.16}$$

$$0 = \beta_1 S A_f - (\mu + \gamma_1) X_f \tag{3.17}$$

$$0 = \beta_2 S A_i - (\mu + \gamma_2) X_i \tag{3.18}$$

$$0 = \gamma_1 X_f + \gamma_2 X_i - \mu X_T \tag{3.19}$$

$$0 = \wedge_f - \upsilon_f A_f \tag{3.20}$$

$$0 = \wedge_i - \upsilon_i A_i. \tag{3.21}$$

Using Mathematica, we establish that the model has one non-trivial equilibrium point given by

$$E = \left(S^*, X_f^*, X_i^*, T, A_f^*, A_i^*\right) \tag{3.22}$$

where:

$$S^* = \frac{\wedge_h \upsilon_f \upsilon_i}{\mu \upsilon_f \upsilon_i + \beta_1 \upsilon_i \wedge_f + \beta_2 \upsilon_f \wedge_i}. \tag{3.23}$$

$$X_f^* = \frac{\beta_1 \wedge_f \wedge_h \upsilon_i}{(\mu + \gamma_1)(\mu \upsilon_i \upsilon_f + \beta_1 \upsilon_i \wedge_f + \beta_2 \upsilon_f \wedge_i)} \tag{3.24}$$

$$X_i^* = \frac{\beta_2 \wedge_i \wedge_h \upsilon_f}{(\mu + \gamma_2)(\mu \upsilon_i \upsilon_f + \beta_1 \upsilon_i \wedge_f + \beta_2 \upsilon_f \wedge_i)} \tag{3.25}$$

$$X_T^* = \frac{\wedge_h (\beta_1 \gamma_1 \wedge_f \upsilon_i (\mu + \gamma_2) + \beta_2 \gamma_2 \wedge_i \upsilon_f (\mu + \gamma_1)}{\mu(\mu + \gamma_1)(\mu + \gamma_2)(\mu \upsilon_i \upsilon_f + \beta_1 \upsilon_i \wedge_f + \beta_2 \upsilon_f \wedge_i)} \tag{3.26}$$

$$A_f = \frac{\wedge_f}{\upsilon_f} \tag{3.27}$$

$$A_i = \frac{\wedge_i}{\upsilon_i} \tag{3.28}$$

The existence of non-trivial steady states suggests that there will never be an allergy-free population (see Table 1) (see Table 2).

Variable	Description
$S(t)$	The number of susceptible individuals in the population at time, *t*.
$X_f(t)$	The number of individuals sensitised to food allergens in the population at time, *t*.
$X_i(t)$	The number of individuals sensitised to inhalant allergens at time, *t*.
A_f	The amount of food allergens in the country at a time, *t*.
A_i	The amount of inhalant allergens in the country at a time, *t*.
$T(t)$	The proportion of individuals who develop tolerance to the allergens at time*t*.

Table 1. Variables and their descriptions

Parameter	Description
β_1	The rate sensitisation of the susceptible population when they interact with the food allergens.
β_2	The rate of sensitisation of the susceptible population when they interact with inhalant allergens.
μ	Natural birth/death rate.
γ_1	The rate of developing tolerance.
γ_2	The rate of developing tolerance.
$\wedge_h$	The rate of growth of the susceptible population.
$\wedge_f$	The growth rate of food allergens in the environment.
$\wedge_i$	The growth rate of inhalant allergens in the environment.
υ_f	The rate of depletion of food allergens.
υ_i	The rate of depletion of inhalant allergens.

Table 2. Parameter symbols and their description

NUMERICAL SIMULATIONS

Numerical simulations on the allergies model were done by fitting the model to the data. Matlab was used, and the values of the parameters obtained are in Table 3. The effects and changes that occur in the model after changing the values of some compartments in the model were investigated. Time was measured in years.

Records form the Asthma Allergy and Immune Dysfunction clinic show that allergies are endemic in Zimbabwe.[1,9] For example, from 2010 to 2014, there was a general increase in the number of both food and inhalant allergy cases, though a drop in the food allergy cases was seen in 2013.

Using the formulated model and fitting the observed data to the model and the results are depicted in Fig. 2. The sensitisation rate of inhalant allergens was observed to be higher than the sensitisation rate to food allergens, 0.066 and 0.2363, respectively. Food allergens, depletion and growth rates were observed to be higher than those of inhalant allergens. The population develops a tolerance to food allergens at a faster rate than inhalant allergens.

A susceptible population growth rate of 50, food and inhalant allergen availability increasing at a rate of 0.053 and 0.071, respectively, the food allergen sensitised compartment increased gradually each year and the inhalant allergen sensitised increase between 2010 and 2011 and remained constant from 2011 to around 2015 then began to decrease as shown in Fig. 3. The susceptible population decreased sharply because of the interaction of the susceptible population with allergens and moving to the food and inhalant allergies compartments.

Fig. 4 shows that doubling the growth rate of the susceptible population and the growth rates of the allergens resulted in an increase in the numbers in both the food allergy and inhalant allergy compartments with the rate of increase of the food allergy compartment being faster than that of inhalant allergens. The susceptible population increased in the first 4 years and began to decrease as more and more individuals continuously move to the food and inhalant sensitised compartments due to increased rates of interaction with the allergens. The number of individuals in the inhalant allergies compartment will decrease in the future while that in the food allergies compartment is seen to increase.

DISCUSSION AND CONCLUSION

In this paper, a mathematical model of the prevalence and acquisition of food and inhalant allergies was studied. A standard SEIR model was adopted and modified to predict the prevalence of food and inhalant allergies as well as determining the parameter estimates for acquiring allergy after exposure to allergens.

By analysing the model, we found that the prevalence of food allergy will increase in the next 2 decades and the prevalence of inhalant allergies will decrease. The existence of non-trivial steady states suggested that allergy will be endemic in the Zimbabwean population. Incidentally, when individuals are exposed to food allergens and interact with them, there is a 0.066 probability that the individuals get sensitised to the food allergens. Whereas, if individuals get exposed to inhalant

Parameter	Symbol	Value
The sensitisation rate of the susceptible population when they interact with food allergens	β_1	0.066
The sensitisation rate of the susceptible population when they interact with inhalant allergens	β_2	0.2363
The depletion rate of food allergens	υ_f	0.347
The depletion rate of inhalant allergens	υ_i	0.2124
Natural birth/death rate.	μ	0.0342
Rate of developing tolerance.	γ_1	0.0116
Rate of developing tolerance.	γ_2	0.005
Rate of growth of the susceptible population.	$\wedge_h$	1.00
The growth rate of food allergens in the country.	$\wedge_f$	0.8945
The growth rate of inhalant allergens in the country.	$\wedge_i$	0.7608

Table 3. Parameter estimates

allergens and interact with them, there is 0.2363 probability of getting sensitised to the inhalant allergens.

The model also showed that individuals develop tolerance to food allergens at a faster rate than the rate at which they develop tolerance to inhalant allergens with rates and γ_2 (0.01164 and 0.005), respectively. This means most people outgrow food allergies and most people live with inhalant allergies.

It was also seen that increasing the growth rate of the susceptible individuals, food allergens availability and inhalant allergen availability, increases the rate at which individuals interact with the allergens thereby increasing the number of individuals

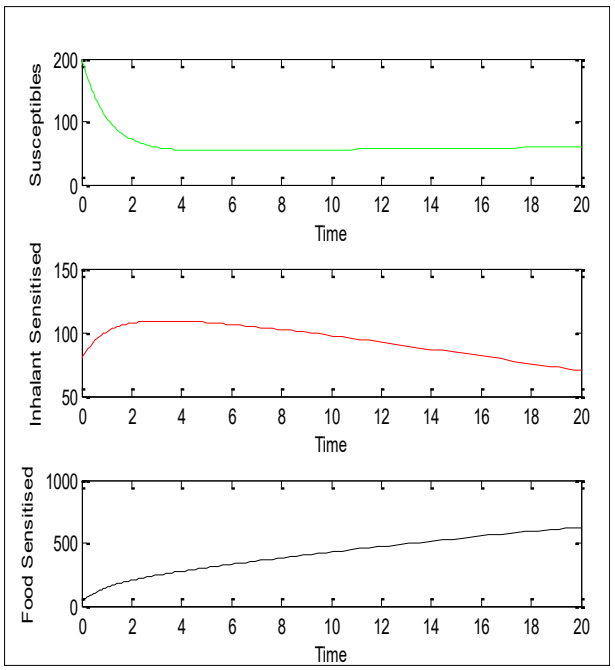

Fig. 3 Simulations over 20 year period with $\wedge_h = 50$, $\wedge_f = 0.053$, $\wedge_i = 0.071$

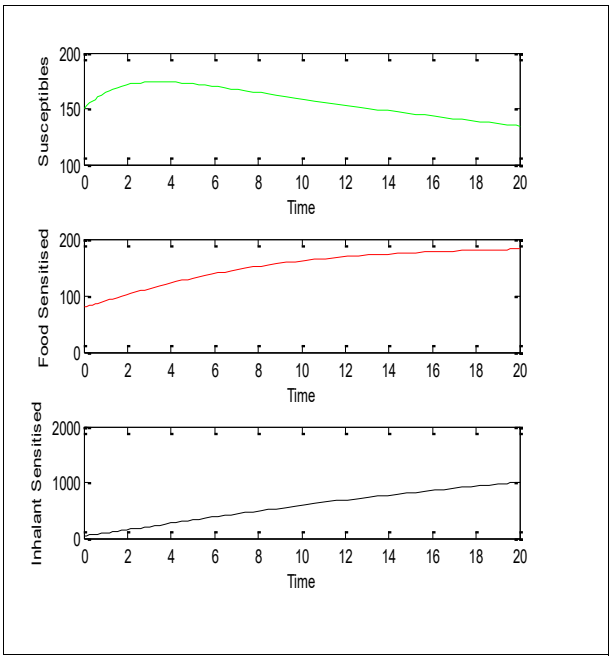

Fig. 4 Simulations over 20 year period with $\wedge_h = 100$, $\wedge_f = 0.106$, $\wedge_i = 0.142$

who get sensitised to the allergens. This means that stakeholders need to put in place measures to manage the disease for it will affect a greater proportion of the population in the near future.

In order to understand the trajectory of food and inhalant allergies, there is need to understand the mechanism of acquiring allergies. Many non-communicable diseases have been modelled using differential equations. The purpose of this study was to examine the mechanism for acquiring allergies and the trend of the prevalence of allergies and solve using differential equations.

The model presented in this paper is unquestionably very simple and can be improved in many ways. First, the model only focusses on 2 types of allergen groups when in actual fact there are more than 2. Second, the model fitting can be expanded to include the goodness of fit as observed in Fig. 2; the data have high statistical variance. The paper could be improved by including statistical measures reflecting the degree of fit. Despite these shortcomings, the model presents some interesting results on how mathematical models can be used to track the changes in the prevalence of allergens.

The model pointed out that allergy will be endemic in Zimbabwe with the prevalence of food allergy increasing and the prevalence of inhalant allergies tending to decrease in the next 2 decades. People should be educated to create awareness of allergy so that the community is cognisant of this chronic disease.

Abbreviations

SER; Susceptible, Exposed, Recovered, SEIR; Susceptible, Exposed, Infected, Recovered, IgE; immunoglobulin E.

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Authors' consent for publication

All authors consent to the publication of this manuscript.

Author contributions

MC, FN and EC were involved in the generation of the model extensively contributed to writing the paper, reviewed and approved the final draft for publication. SR supervised the student, guided statistical analyses, read and approved the manuscript for publication. LP and HP were involved in the data collection, reviewed the manuscript and approved the submission of the manuscript. ES was responsible for the clinical care of all the patients included in the model, supervised the laboratory testing, revised, reviewed and approved the submission of the manuscript.

Availability of data and materials

All data and materials from which the data are derived are available from the corresponding author and are accessible on request.

Ethics approval

Institutional approval was obtained. The anonymised analysis of these data is not applicable.

Declaration of competing interest

The authors declare no competing interests with respect to this manuscript.

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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Assessing early child development and its association with stunting and schistosome infections in rural Zimbabwean children using the Griffiths Scales of Child Development

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Abstract

There is a paucity of reference early childhood development (ECD) data at community level in rural Africa. Our objective was to conduct a comprehensive assessment of ECD in rural Zimbabwe and determine the impact of stunting and schistosome infections on ECD. Using the Griffiths Scales of Child Development, we conducted a cross sectional assessment of Eye and Hand Coordination (EHC), Personal-Social-Emotional (PSE), Language and Communication (LC), Foundations of Learning (FL) and Gross Motor (GM) domains and the summary General Development (GD) in 166 children aged 6–72 months. The effects of stunting, malnutrition and *Schistosoma haematobium* infection on ECD was determined. The impact of praziquantel curative treatment of schistosome infection on the developmental scores was determined through a longitudinal follow up at 6 and 12 months. From an initial 166 children, 11 were found to have developmental deficits warranting further investigation. Of the remaining 155, 58.7% recorded a good ($\geq$ average) score for the overall General Development (GD). Proportions of children scoring above the cut-off ($\geq$ average) for each domain were GM (84.5%), PSE (80.6%), EHC (61.9%), FL (43.9%) and LC (44.5%). The prevalence of stunting was 26.8% (95% CI = 20.1%–34.8%) Scores for stunted children were significantly lower for EHC ($p = 0.0042$), GM ($p = 0.0099$), and GD ($p = 0.0014$) with the fraction of lower scores attributable to stunting being GM = 63.4%, GD = 46.6%, EHC = 45%, and LC = 21%. *S. haematobium* infection prevalence was 39.7% and mean infection intensity was 5.4 eggs/10 ml urine. Infected children had poorer cognitive performance scores for the FL ($p = 0.0005$) with 30.8% of poor FL attributable to the infection. Performance in all domains improved to the expected normal or above reference levels at 6 and

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12 months post curative treatment of schistosome infections. Our study documented reference values for ECD in rural Zimbabwean children. The study detected deficiencies in the FL domain, which were more pronounced in children, infected with schistosomes, highlighting the need for provision of cognitive stimulation tools and access to early childhood foundation education. There is also need for improved child nutrition and treatment of schistosome infections to improve child development outcomes.

Author summary

There is a paucity of comprehensive early childhood development data at community level in rural Africa. We assessed the development of rural Zimbabwean children aged 6–72 months and determined the impact of stunting and schistosome infections on their 5 developmental domains; Eye and Hand Coordination, Personal-Social-Emotional, Language and Communication, Foundations of Learning and Gross Motor domains and the summary General Development. We have demonstrated that just over 50% of Zimbabwean children in rural areas are on course for normal child development; with many being more advanced for their age in Gross Motor and Personal-Social-Emotional domains. We also demonstrate that the children face developmental challenges in early childhood, particularly in Foundations for Learning which represents critical psychometric constructs and cognitive skills for learning, executive function, ways of thinking, problem solving, organizing and information planning, analytic thought and memory. We further demonstrate that the poor development scores especially in Foundations for Learning were also attributable to stunting and schistosome infection, with the impact of the latter being reversed by curative antihelminthic treatment. Taken together, the findings strengthen the call for the treatment of paediatric schistosomiasis, accessibility to cognitive stimulation tools and improved nutrition to improve childhood health outcomes.

Introduction

The early childhood period is considered the most important developmental phase throughout the lifespan of a child with events occurring in these first few years being critical for the child's developmental trajectory and life course. https://www.who.int/social_determinants/themes/earlychilddevelopment/en/). These events influence the child's mental and physical health, education and future economic participation.

In most African countries, child development in rural areas is monitored as part of the monthly growth monitoring in child health programs. However, these programs mostly consist of weighing, height measuring, and nutrition advice to caregivers. In Zimbabwe, the growth-monitoring program is part of the child health surveillance system, which uses basic measures such as weight-for-age, height-for-age and mid-upper arm circumference (MUAC) to chart child development. This is conducted at primary health centres with input from village health workers who monitor the number of eligible children in their communities. Nonetheless, a study in Mutasa District in Zimbabwe in 2015 indicated that 70% of children below 5 years of age missed their monthly weighing, meaning that for most children, subtle changes and malnutrition went undetected [1].

In addition to challenges arising from the low uptake of growth monitoring appointments, there is little provision of tools to detect cognitive development. This is due to a shortage of a trained workforce and tool kits for the direct assessment of child development. Community level data is often obtained using screening tools. Screening is conducted via interview of caregivers or through observation of limited sets of actions/behaviours representing a domain of development; they are reliant on predetermined cut-off points to identify children requiring a comprehensive assessment. Tools for comprehensive child development assessment such as the Griffiths Assessment Tool, the Cognitive Adaptive Test/Clinical Linguistic, and Auditory Milestone Scale (CAT/CLAMS) and Ages and Stages Questionnaire (ASQ) that have been assessed in South Africa [2] require trained personal and the assessment of each child is time-consuming. Thus, there is a paucity of comprehensive early childhood development (ECD) data at community level.

Our first aim was to conduct a comprehensive assessment of ECD in rural Zimbabwe to provide, for the first time, data on delays and changes that may have a significant impact on subsequent development in the children. The latest UNICEF Country Profiles for Early Childhood Development report (https://nurturing-care.org/resources/country-profiles/) indicates that 46% of Zimbabwe's children are at risk of poor development. Therefore, there is a need to identify these children and the causes of their poor development to implement context-specific interventions.

There is also a need to identify local factors contributing to poor child development. A recent analysis of indicators of early childhood experiences and outcomes in low and middle-income countries comprised of 135 national household surveys, collected between 2010 and 2018 from 94 countries indicated that children in sub-Saharan Africa were exposed to stunting or extreme poverty [3]. The study also highlighted that the children had less home stimulation and low attendance at early childcare education; indicators associated with a poorer status in subsequent school learning, labour market productivity and health [3]. Furthermore, the analysis also showed that children in urban areas or those in the richest household scored better on all indicators of child development than those in rural areas or those from poorer households. The authors of this study correctly highlight that these studies were handicapped by the lack of reliable data from several low-income countries and in cases where there were data available; there was poor representation of rural communities [3]. Our current study added data to the poorly represented rural populations.

The detrimental impact of helminth infections on childhood development has been highlighted in previous studies. In a study in Peru, infection with soil-transmitted helminths (STH) has been shown to reduce verbal IQ scores [4]. Furthermore, a meta-analysis of 36 studies of 12,920 children [5] indicated that STH infection compromised learning, memory and intelligence [5]. This study also demonstrated that children successfully treated for the STH infections scored better in these domains than untreated children. Quantitative studies have suggested that schistosome infections are associated with stunting and undernutrition and that both these effects can be reversed through antihelminthic treatment [6], while meta-analysis has indicated that schistosome infections were significantly associated with educational, learning, and memory deficits in school-aged children [7]. However, to date, there has been no longitudinal study investigating the impact of schistosome infection on ECD, focusing on children aged 6 years and below. Therefore, our second aim was to determine if *Schistosoma haematobium* infection compromised any of the child development domains. We further determined if the curative antihelminthic effect improved the performance of previously infected children as determined by their performance in child development assessments.

Methods

Ethical approval and consent

This study was part of a larger study investigating the overall health impact of paediatric schistosomiasis in children aged 6 years and below, from 2017 to 2019. The study received institutional approval from the University of Edinburgh and Ethical approval from the Medical Research Council of Zimbabwe (MRCZ/A/2246 and MRCZ/A/2573). Permission to conduct the study in the province was obtained from the Provincial Medical Director. Prior to enrolment, the study aims, and procedures were explained to all participants and their parents/guardians in English or the local language, Shona. Written informed consent was obtained from the participants' parents/guardians as appropriate. Recruitment into the study was voluntary and parents/guardians were free to withdraw the participants at any time with no further obligation.

Study area

The study was conducted in Murewa district located in Mashonaland East province of Zimbabwe (17˚38′49″S 31˚46′39″E). Murewa District is one of seven districts in the Mashonaland East province of Zimbabwe, whose people are primarily subsistence farmers producing maize and vegetables which form the stable diet. This is an area we have previously worked in and reported on water contact and usage [8]. The area was selected for this study because the prevalence of *S. haematobium* is high (>50%) while the prevalence of *S. mansoni* and soil transmitted helminths (STH) is low (<15%) [9]. As we have previously reported [10], this area was included in Zimbabwe's helminth control program running annually from September 2012 to November 2017 where schistosomiasis was treated via mass drug administration of praziquantel to all school children. In this area there was mopping up treatment exercise in January—February 2018. During the MDA only school children aged 6 years and above are treated, meaning that preschool children are excluded from MDA, a health inequality we have previously highlighted [11]. Since 2018, there has not been an MDA exercise in this area and the children recruited in this study had all never been treated for schistosomiasis.

Study design

The study comprised of a cross sectional study design followed by a 1-year longitudinal study (see S1 Fig). The longitudinal study had an intervention aspect; schistosome-infected children were treated with praziquantel and followed up at 6 weeks to check treatment efficacy. Children were then followed up at 6 months and 12 months to determine the impact of treatment on their performances in the child development assessments. Children were recruited from crèches and early child development centres. Parents/guardians of children not attending any of the educational programmes brought the children to meeting points used by the community for the Expanded Program for Immunisation (e.g., local school, or primary health centre) for enrolment into the project. A questionnaire designed in English and translated into the local dialect (Shona) was administered to gather demographic data and establish exposure behaviour to schistosome infective freshwater sources.

Study inclusion and exclusion criteria and study population

At baseline, the study enrolled children aged 6 months to 72 months who met the following inclusion criteria. The children had to, i) be lifelong residents of the study area, ii) not have previously received antihelminthic treatment, iii) be negative for *S. mansoni* and STH (in practical terms, as the prevalence of these is very low in the area, no children were excluded on this

criteria), and iv) guardian/career had given consent for them to participate in the study. Following these inclusion criteria, 166 children were included in the cross-sectional study.

To be included in the longitudinal cohort, children who fulfilled the inclusion criteria described above had to meet the additional criteria of; v) having full ECD assessment and; vi) not be suffering from any severe developmental disorder as diagnosed from the baseline assessments. Children with Development Quotient (DQ) scores below 50, were excluded from the longitudinal study as this score and below indicate a level of development impairment [12] requiring further clinical investigation or intervention. Following these inclusion criteria, 79 children were included in the longitudinal study.

Anthropometry

Weight (nearest 0.1 kg) and height (nearest 0.1 cm) without shoes and in light clothing were measured using an electronic scale and a stadiometer respectively. For babies, height was measured with an infantometer baby board, and weight with a baby scale. The Mid-Upper Arm Circumference (MUAC) was measured (nearest 1mm) using a child MUAC tape on the left arm, midpoint between the shoulder and the tip of the elbow, with the arm relaxed and hanging down the body. Growth and nutritional status were assessed using the WHO Anthro software, version 3.0.1 (http://www.who.int/childgrowth/en/) [13]. This generated Z- scores for specific measures of nutrition and growth, i.e. stunting by Height-for-Age (HAZ), underweight by Weight-for-Age (WAZ) and BMI-for-Age (BAZ), and malnutrition by MUAC and Weight-for-Height (WHZ). Measures were considered abnormal when Z scores were < -2 Z–scores [14].

Parasitological diagnosis

A sample of urine, about 50ml volume, was collected from each participant on three consecutive days and a stool specimen was collected on a single day from each participant. Samples were collected between 10:00h and 14:00h and processed within 2 hours of collection. Urine samples were examined microscopically for *S. haematobium* infection following the standard urine filtration method [15] and the number of eggs was reported per 10 ml of urine. Stool samples were processed using the Kato–Katz method [16] and parasite eggs also enumerated under light microscope for *S. mansoni* (in duplicates) and results reported per gram of stool.

Children were diagnosed positive for helminth infection if at least one parasite egg was detected in their urine or stool samples. All children who were positive for *S. haematobium* infection were treated with a single dose of praziquantel at the standard 40 mg/kg body weight after their early child development assessments. Tablets were crushed and administered with squash and sliced bread [17] by local nurses. A post-treatment efficacy check (egg count) was carried out for all treated participants at 6 weeks post treatment.

Developmental assessment

The child development assessment was conducted using the Griffiths Mental Developmental Scales III for children aged 72 months (6 years) and below [12]. Children were assessed at baseline and at 6 and 12 months post-treatment. The Griffiths Mental Developmental Scales III tool was selected, as it is comprehensive in assessing all developmental domains. The domains measured by this tool are Eye and Hand Coordination, (EHC), Personal-Social-Emotional (PSE), Language and Communication (LC), Foundations of Learning (FL), Gross Motor Function (GM). This assessment gives standardized sub-quotient scores for each domain. The Griffiths Mental Developmental Scales allow for the calculation of a summary General Development (GD) quotient which is derived using each of the measures for the 5 individual

domains. It is an indicator of a child's growth and development across the range of psychosocial competencies. This tool has been validated in South Africa [18] and prior to our study, we validated the tool to ensure relevant contextual language and phrases were used during the assessment. Clinical psychologists who had all completed an accredited training course on the Griffiths Mental Developmental Scales III (led by AM) assessed the children. Data were captured electronically and underwent quality control checks on the day.

Data handling and statistical analysis

Growth and nutritional indices adjusted for age and expressed as Z-scores [13], were calculated using the WHO Anthro software, version 3.0.1 for children ≤60 months (http://www.who.int/childgrowth/en/) and the WHO Anthro plus software for children ≥60 months (https://www.who.int/growthref/tools/en/). Based on raw anthropometric (including weight and height) and demographic data (including age and sex), anthropometric estimates based on length/height–for–age, weight–for–age, and body mass index (BMI)–for–age were used to generate Z-scores from which growth and nutritional status was measured. Stunting was determined by height–for–age Z-scores (HAZ), and malnutrition by weight–for–age Z-scores (WAZ) and BMI–for–age Z-scores (BAZ). Measures with Z-scores < -2 were considered abnormal, and children with MUAC measurements <12.5 were considered malnourished [14].

The data was analysed using SPSS version 22 (IBM Corp.), and graphs were plotted with GraphPad Prism version 8.4.2 (GraphPad Software, Inc.). The data was summarised using descriptive statistics. Continuous data is presented as mean ± standard deviation (SD), and the categorical data is presented as absolute numbers and percentages. At individual level all Griffiths subscale scores for each domain were assessed and a cut-off score of < 50 for the domain's development quotient (DQ) was applied to identify developmental challenges within each domain [12]. For the population analyses, in line with standard practise [19], a population reference DQ score of 50–99 was considered low to low average (i.e. poor) and score of ≥100 is considered average to high, indicating typical development for the chronological age (i.e good).

For all statistical analyses, data was checked for the assumptions of parametric tests. Thus, the data was checked for normality, using frequency distribution plots, in addition to the Shapiro Wilkes normality test to determine if parametric or non-parametric statistics were to be used. To determine differences between continuous data (between two groups or between one group and a reference mean), the t-test or the Wilcoxon Sign test (non-parametric) was used. To determine differences in categorical variables between two groups, the Fisher's exact test was used. For all analyses, approximate 95% confidence intervals (CI) were calculated using the modified Wald method [20], and p-values <0.05 were considered significant. For univariate analyses comparing DQ subscale scores between groups, it was hypothesised a priorly that schistosome-infected/stunted/malnourished individuals will have poorer scores than uninfected/healthy individuals, hence statistical tests were one-tailed.

The risk of associated poor cognitive performance scores in relation to *S. haematobium* infection or to stunting and malnutrition was calculated using prevalence ratios (PR). The PR was calculated as a ratio of the proportion of infected/stunted/malnourished individuals with the associated poor DQ scores (e.g. for General Development) to the proportion of uninfected/healthy individuals with the same poor DQ scores (e.g. for General Development). A subscale indicator with a PR >1, suggested an increased risk of the associated poor DQ scores from schistosome infection/stunting/malnutrition [21]. The method of attributable fraction (AF) was then used to estimate the proportion of poor DQ scores for each subscale that could be

attributed to *S. haematobium* infection/stunting/malnutrition. The population attributable fraction (AFp) and attributable fraction in the exposed (AFe) were used to estimate the attributable fractions in the total study population and among the exposed (i.e. schistosome-infected/stunted/malnourished children) respectively, according to Miettinen's formula [22].

The AF in the exposed population was calculated as:

$$AFe = \frac{(RR - 1)}{RR} \quad (1)$$

The AF in the total population was calculated as:

$$AFp = Pe \times AFe \quad (2)$$

Where RR is the risk ratio of poor DQ scores associated with infection/stunting/malnutrition, and Pe is the prevalence of poor DQ scores among the infected/stunted/malnourished population. As all AFs were estimated at the cross-sectional level (i.e. at the baseline survey), the RR was substituted with the PR [21] and AFs were only estimated for morbidity indicators with PR >1.

Results

Population and survey characteristics

Out of a total of 166 children recruited into the study aged 6–72 months, 11 (6.6%) were excluded from the study based on low DQ scores (i.e. DQ scores below 50) as well as observations/interactions by/with the psychologists. These children were referred for further clinical attention. Suspected diagnosis of these 11 children included Anxiety, Attention Deficient Disorder (ADD), Attention Deficient Hyperactivity Disorder (ADDH), Seizure Disorder and Intellectual Disabilities (ID).

Of the155 children included in the final analysis, 83 (53.5%) were male and 72 female (46.4%). Age range was 9–72 months (mean± SD = 45.5 ± 15.4 months; 95% CI = 43–48). Anthropometric measures were taken at baseline, from which standardised indices of malnutrition and stunting were determined; mean weight 14.8 ± 3.0 kg (95% CI = 14.3–15.3), mean height 95.7 ± 11.3 cm (95% CI = 93.8–97.6) and mean MUAC 15.8 ± 1.3 cm (95% CI = 15.6–16.0). Prevalence of stunting based on HAZ was 26.8% (37/138; 95% CI = 20.1–34.8), and malnutrition as measured by different indices were 0% (0/139) based on MUAC, 6.6% (9/137; 95% CI = 3.5–12) based on WAZ, and 3.6% based on BAZ.

Parasitology data was available for 141/155 (91%) children; *S. haematobium* infection prevalence was 39.7% (56/141; 95% CI = 32.0–48.0) and overall mean infection intensity was 5.4 eggs/10 ml urine (SEM = 1.6; 95% CI = 2.3–8.6). Follow up rates at subsequent surveys were 122/155 (78.7%) at 6 months (survey S1) and 61/122 (50%) at 12 months (survey S2). *S. haematobium* prevalence was 8.3% (8/96; 4.1–15.8) at survey S1, and 8.9% (5/56; 3.5–19.7) at survey S2.

Cognitive performance at baseline

Table 1 summarises the cognitive performance assessment of all children at baseline, based on the Developmental Quotient (DQ) for all six subscales. Using the population reference cut off DQ = 100, the mean DQ for the subscales (i.e. Eye and Hand Coordination EHC, PSE, GM,) and General Development (GD)) were good and so was the GD quotient. The mean population DQ for the FL and LC subscales were poor (i.e. <100) with majority of children (>50%) falling below the reference cut off.

Table 1. Summary baseline cognitive assessment based on the developmental quotient.

Subscale	Mean developmental age (SD; 95% CI)	Mean developmental quotient (SD; 95% CI)	Good score n (%)	Poor score n (%)
Foundations of Learning (FL)	43.5 (20.0; 40.3–46.7)	96.25 (21.6; 91.82–98.7)	68 (43.9)	87 (56.1)
Language and Communication (LC)	45.1 (20.0; 41.9–48.3)	96.9 (19.0; 93.8–99.9)	69 (44.5)	86 (55.5)
Eye and Hand Coordination (EHC)	46.7 (19.2; 43.6–49.7)	102.7 (15.9; 100.1–105.2)	96 (61.9)	59 (38.1)
Personal Social and Emotional (PSE)	49.4 (17.9; 46.5–52.2)	110.6 (14.7; 108.3–112.9)	125 (80.6)	30 (19.4)
Gross Motor (GM)	50.9 (18.7; 48.0–53.9)	120.3 (99.4; 104.5–136.0)	131 (84.5)	24 (15.5)
General Development (GD)	47.5 (21.5; 44.1–50.9)	104.0 (17.5; 101.3–106.8)	91 (58.7)	64 (41.3)

Children were classified based on developmental quotient into good (equal to, or above average) and poor scores (below average) using a population reference cut off ≥100 [19].

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The developmental age derived based on the Griffiths assessment was compared to the chronological age (actual ages) of children to determine how their normal physical and mental developments deviate or parallel to normal development milestones (Fig 1). Results showed that the children's developmental age was lower than expected for the FL subscale ($p < 0.0009$),

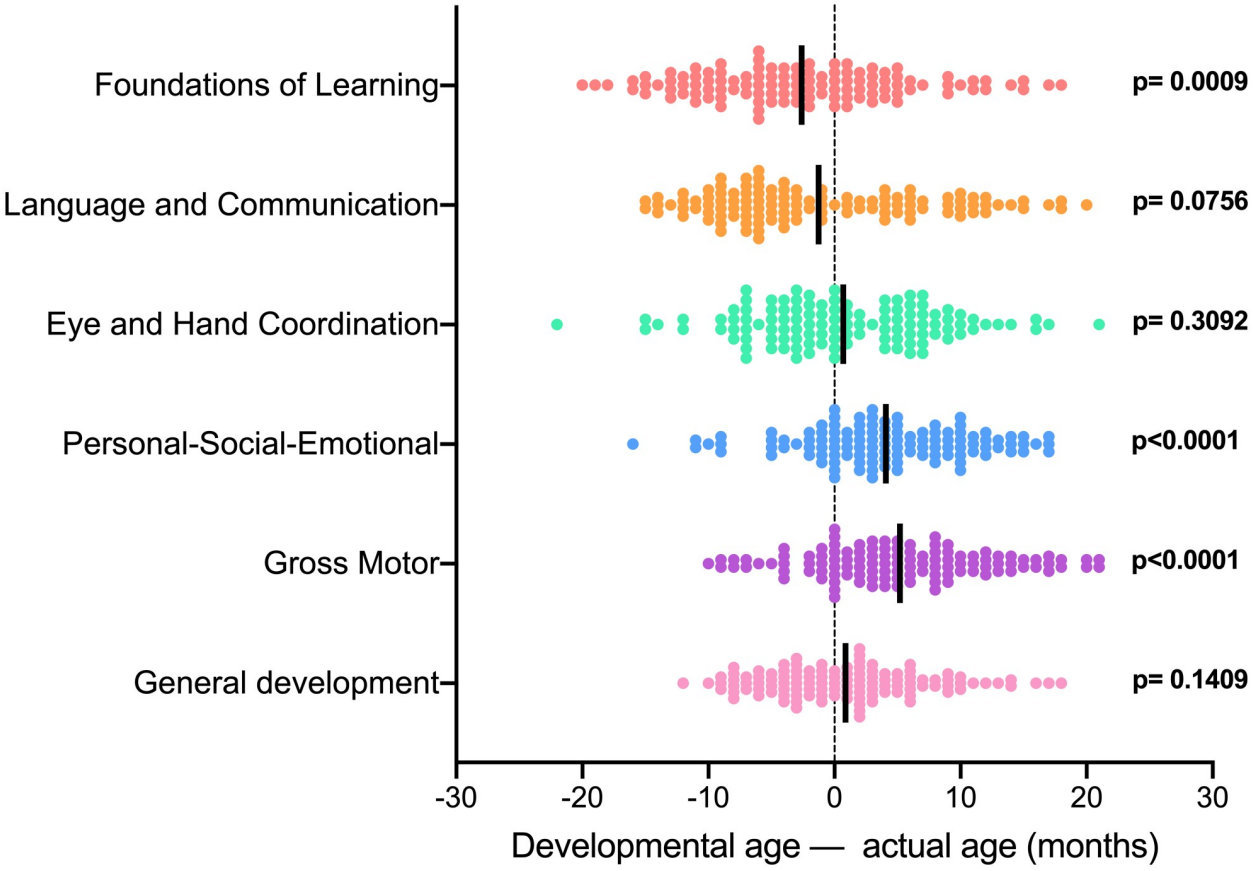

Fig 1. Difference between the developmental and chronological/actual ages for the six subscales. Dotted line represents children performing as expected for their chronological age as per Griffiths test, i.e. the difference between the chronical and developmental age performance. P-values indicate statistical tests comparing the difference between the mean developmental age as indicated by the Griffiths test performance and the mean chronological age. Solid lines = mean.

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and higher for the PSE ($p < 0.0001$) and GM ($p < 0.0001$) subscales. Developmental versus chronological age for the LC ($p = 0.0756$) and EHC ($p = 0.3092$) subscales were comparable.

S. haematobium infection and cognitive performance

To determine if any relationship exists between schistosome infection and poor cognitive performance, children were partitioned based on *S. haematobium* infection status and their cognitive performance scores. As shown in Fig 2, children positive for *S. haematobium* infection had poorer cognitive performance scores for the FL subscale ($p = 0.0005$), but this was not the case for uninfected children ($p = 0.2636$). Higher scores were recorded for the GD subscale in the uninfected children, and higher scores recorded for the PSE ($p < 0.0001$) and GM ($p < 0.0001$) subscales for both infected and uninfected children. In both groups, subscales for LC and EHC were comparable to the reference mean ($p > 0.05$).

Table 2 compares cognitive performance of children at baseline, stratified by *S. haematobium* infection status. Schistosome infection was significantly associated with FL ($p = 0.0068$), and this association was true for both younger (9–36 months; $p = 0.0305$) and older (37–72 months; $p = 0.0365$) children. There was a significant association between schistosome infection and GD amongst the older group of children (37–72 months; $p = 0.0476$).

Poor cognitive performance attributable to *S. haematobium* infection

Since cognitive performance scores in children may relate to different factors inherent to specific populations, we determined how much of poor cognitive performance was attributable to schistosome infection, focusing on the subscales that showed a significant association with *S. haematobium* infection (i.e. FL and GD subscales). As shown in Fig 3, 30.8% (AFe) of poor FL was attributable to schistosome infection in the infected population, and in the total population (AFp), 12.1%. When children were grouped into age categories, the proportion of poor FL attributable to schistosome infection was higher for children $\leq$36 months (AFe = 42.8%) and 28.9% for children >36 months. Similarly, poor GD was attributable to schistosome infection in the infected population (AFe = 26.7%) and in the total population but lower (AFp = 10.5%). The proportion of poor GD attributable to schistosome infection was higher for children >36 months (AFe = 40%) and was 32% for children $\leq$36 months.

Effects of praziquantel treatment on cognitive performance

To determine the effect of treatment on cognitive performance, a subset of children were followed up. These were children with full ECD assessments from the baseline survey as well as full *S. haematobium* parasitology data. This gave a total of 79 children, made up of 29 children who had been positive for *S. haematobium* infection at baseline and had been successfully treated for the infected as confirmed at by the treatment efficacy check and 50 children who had been free of schistosome infection at baseline. Due to the heterogeneity in the children's ages and sexes, statistical analyses were conducted for infected and uninfected children relative to the reference data for their age groups.

Thus the cognitive performance subscale scores (based on DQ) for the schistosome-positive children at baseline who were treated, confirmed negative at post-treatment follow-up, were compared to the normal population reference mean ($\geq$100) (Fig 4). The sample size at S1(6 months) was 29. At survey S2 (12 months), a few children from this subset were lost to follow up or were reinfected (n = 2), with data for 18 individuals available for analysis. Compared to the population reference mean, subscales FFL and LC were below normal at baseline (pre-treatment), although the difference was significant only for FL ($p = 0.0420$) (Fig 4a).

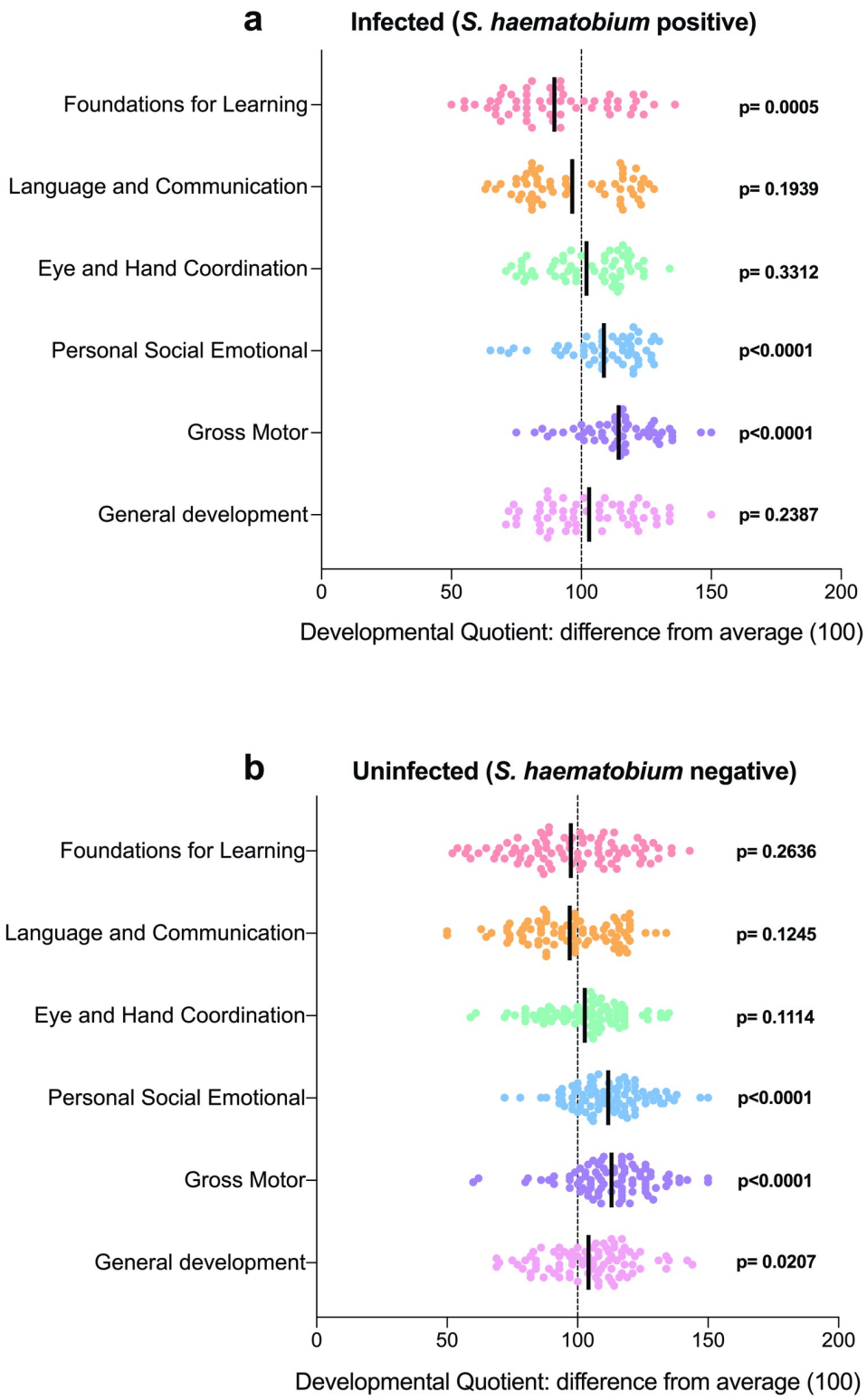

Fig 2. Scatterplot comparing the mean of developmental quotient (DQ) scores to the expected mean reference average for the six subscales. Graph shows baseline DQ scores for a) children positive for *S. haematobium* infection, and b) children negative for *S. haematobium* infection. Dotted lines represent the mean reference average for DQ (100) [19]. p-values indicate statistical tests for the scores of the children compared to the reference mean of 100. Solid lines = mean.

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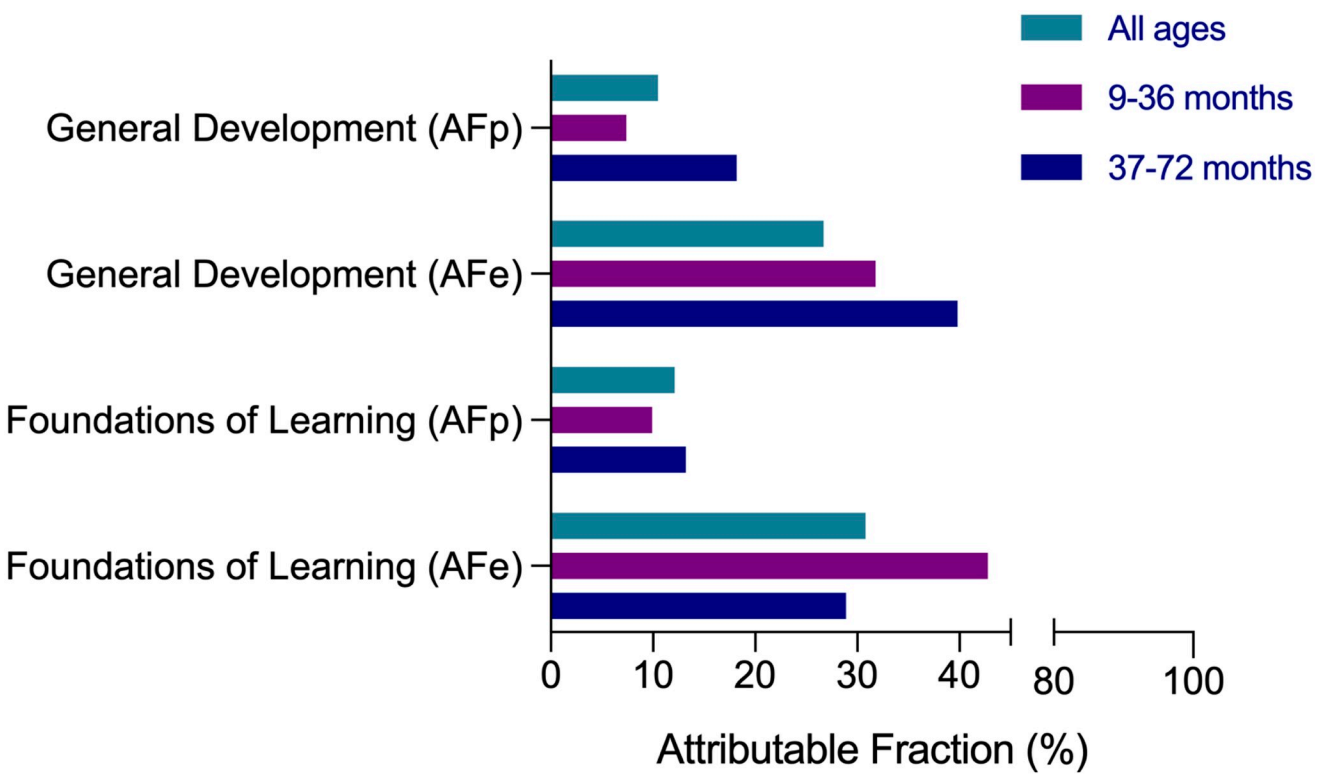
Table 2. Cognitive performance classification based on schistosome infection status.

Age category (months)	Subscale classification	*S. h.* positive	*S. h.* negative	p-value
	Foundations of Learning			
All (9–72)	Below for age	41 (70.7%)	44 (48.9%)	**0.0068**
	Normal for age	17 (29.3%)	46 (51.1%)	
9–36	Below for age	9 (90%)	17 (51.5%)	**0.0305**
	Normal for age	1 (10%)	16 (48.5%)	
37–72	Below for age	32 (66.7%)	27 (47.4%)	**0.0365**
	Normal for age	16 (33.3)	30 (52.6%)	
	Language and Communication			
All (9–72)	Below for age	32 (55.2%)	52 (57.8%)	0.4428
	Normal for age	26 (44.8%)	38 (42.2%)	
9–36	Below for age	9 (90%)	27 (69.7%)	0.4763
	Normal for age	1 (10%)	6 (30.3%)	
37–72	Below for age	23 (47.9%)	25 (43.9%)	0.4132
	Normal for age	25 (52.1%)	32 (56.1%)	
	Eye and Hand Coordination			
All (9–72)	Below for age	26 (44.8%)	32 (35.6%)	0.1696
	Normal for age	32 (55.2%)	58 (64.4%)	
9–36	Below for age	8 (80.0%)	18 (54.5%)	0.1413
	Normal for age	2 (20.0%)	15 (45.5%)	
37–72	Below for age	18 (37.5%)	14 (24.6%)	0.1109
	Normal for age	30 (62.5%)	43 (75.4%)	
	Personal-Social and Emotional			
All (9–72)	Below for age	12 (20.7%)	16 (17.8%)	0.4069
	Normal for age	46 (79.3%)	74 (82.2%)	
9–36	Below for age	4 (40.0%)	11 (33.3%)	0.4879
	Normal for age	6 (60.0%)	22 (66.7%)	
37–72	Below for age	8 (16.7%)	5 (8.8%)	0.1772
	Normal for age	40 (83.3%)	52 (91.2%)	
	Gross Motor			
All (9–72)	Below for age	10 (17.2%)	14 (15.6%)	0.4783
	Normal for age	48 (82.8%)	76 (84.4%)	
9–36	Below for age	4 (40.0%)	7 (21.2%)	0.2141
	Normal for age	6 (60.0%)	26 (78.8%)	
37–72	Below for age	6 (12.5%)	7 (12.3%)	0.6010
	Normal for age	42 (87.5%)	50 (87.7%)	
	General development			
All (9–72)	Below for age	29 (50.0%)	33 (36.7%)	0.0759
	Normal for age	29 (50.0%)	57 (63.3%)	
9–36	Below for age	8 (80.0%)	18 (54.5%)	0.1413
	Normal for age	2 (20.0%)	15 (45.5%)	
37–72	Below for age	21 (43.8%)	15 (26.3%)	**0.0476**
	Normal for age	27 (56.2%)	42 (73.7%)	

Data are presented as n (%), and p-values indicate Fishers exact tests for indices. S.h., S haematobium

https://doi.org/10.1371/journal.pntd.0009660.t002



Subscale (based on development quotient)	Prevalence ratio (PR)
Foundations of Learning	
All ages	1.5
9-36 months	1.8
37-72 months	1.4
General Development	
All ages	1.4
9-36 months	1.5
37-72 months	1.7

Fig 3. Estimated proportion of poor cognitive performance (based on development quotient) attributable to *S. haematobium* infection.
AFe = Attributable fraction in the infected population, AFp = attributable fraction in the total population. Attributable fractions were estimated for indices with PR >1.

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Nonetheless, these improved at 6 months post-treatment so that the mean vales for FL were just above the normal level of 100 although not significant (p = 0.7052) while LC mean values were significantly higher that the population reference of 100 (p = 0.0043) (Fig 4b). EHC (p = 0.0105), PSE (p <0.0001), GM (p <0.0001), and GD (p = 0.0030) were all significantly higher than the normal population reference (Fig 4b). Similarly, scores for all six subscales

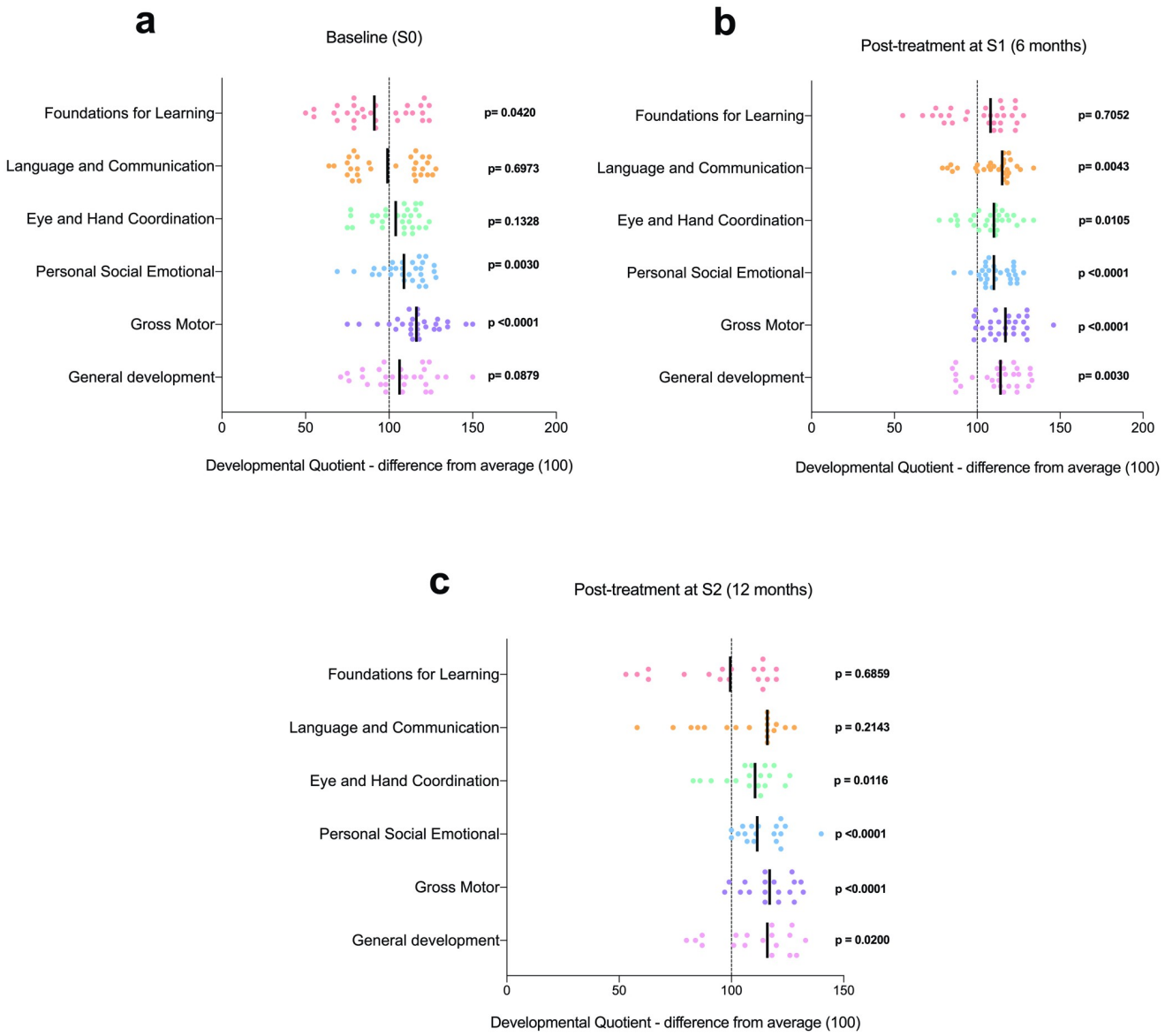

Fig 4. Six- and 12-month time course of developmental quotient (DQ) scores for treated *S. haematobium* positive children. Scatterplots show DQ scores for a) children positive and treated for *S. haematobium* infection at baseline (n = 29), b) children present at S1 survey (6 months later) and tested negative for *S. haematobium* infection (n = 29), and c) children present at S2 survey (12 months later) who remained negative for *S. haematobium* infection (n = 18). Dotted lines represent the mean reference average for DQ (100). p-values indicate statistical tests for either a one sample t-test or Wilcoxon sign test (based on normality) comparing to the hypothetical mean of 100. Solid lines = mean.

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were near or above normal at 12 months post-treatment within the same subset of children (Fig 4c).

The analysis was repeated for a subset of children who were uninfected at baseline and remained uninfected (confirmed by egg count) through from surveys S1 (6 months) to S2 (12 months) (n = 50). Similarly, there were some lost to follow up for this subset of children at survey S2, with data for 29 individuals available for analysis. Compared to baseline subscale scores for schistosome-positive children (see Fig 4a), all six subscale scores were near or above normal for uninfected children and this was similar for scores at 6 and 12 months. However, for both

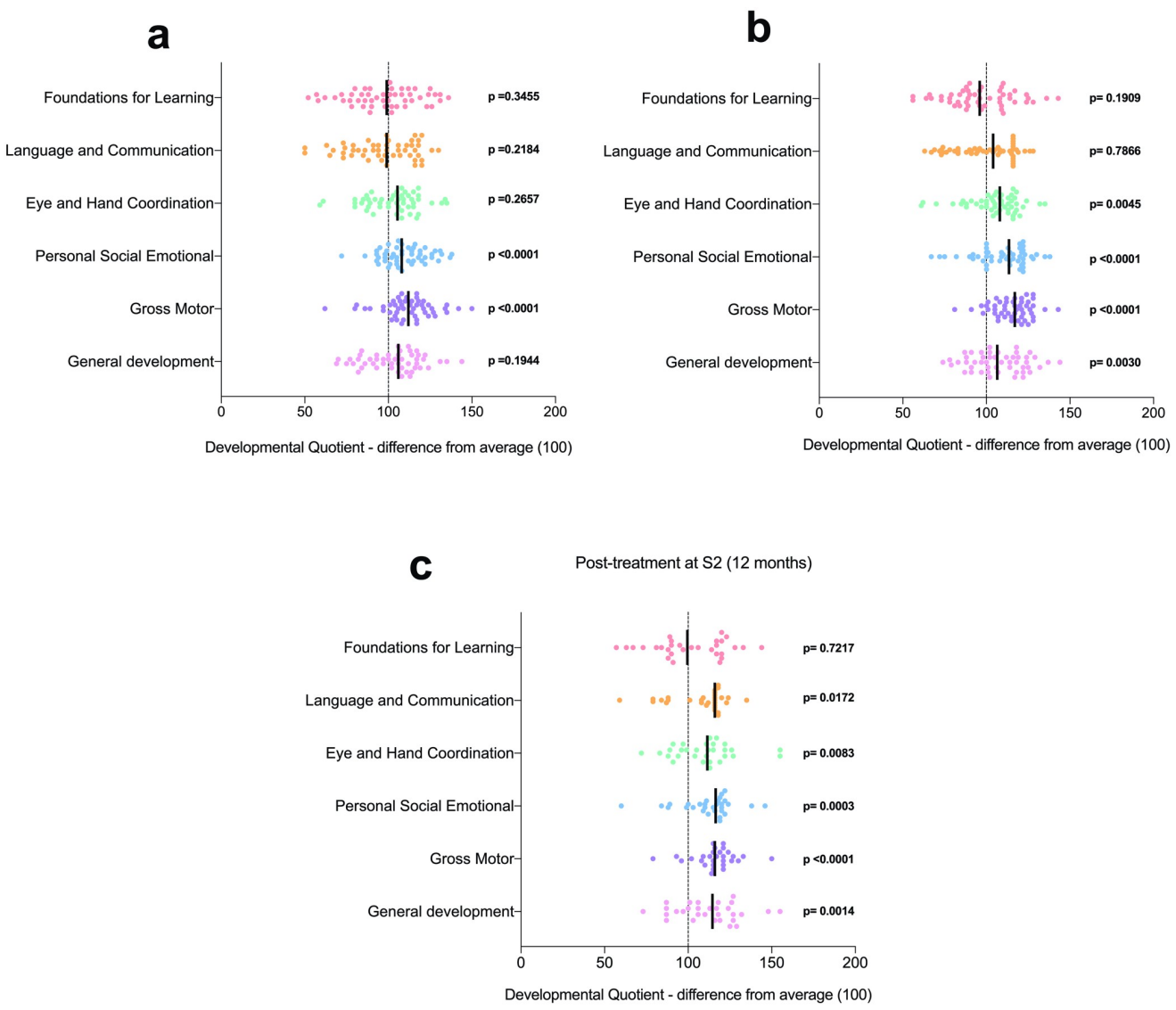

Fig 5. Six- and 12-month time course of developmental quotient (DQ) scores for untreated/uninfected children. Scatterplots show DQ scores for a) children negative for *S. haematobium* infection at baseline (n = 50), b) children present at S1 survey (6 months later) and remained negative for *S. haematobium* infection (n = 50), and c) children present at S2 survey (12 months later) who remained negative for *S. haematobium* infection (n = 29). Dotted lines represent the mean reference average for DQ (100). p-values indicate statistical tests for either a one sample t-test or Wilcoxon sign test (based on normality) comparing to the hypothetical mean of 100. Solid lines = mean.

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infected/treated and uninfected children, subscale scores for PSE and GM were significantly higher than the expected population reference mean at all three time points (see Figs 4 and 5).

Poor cognitive performance attributable to stunting

We went on to determine the impact of growth and nutritional status on the development measures among the children. As shown in Table 3, development scores showed that stunting was significantly associated with EHC (p = 0.0042), GM (p = 0.0099), and GD (p = 0.0014) subscale DQ scores. When grouped according to age category, stunting was significantly associated with DQ scores for LC (p = 0.0351) for younger children (≤36 months) and with GD

Table 3. Cognitive performance classification based on stunting status.

Age category (months)	Subscale Classification	Stunted	Normal	P value
	Foundations of Learning			
All (9–72)	Below for age	25 (67.6%)	53 (52.5%)	0.0814
	Normal for age	12 (32.4%)	48 (47.5%)	
9–36	Below for age	10 (62.5%)	15 (62.5%)	0.6282
	Normal for age	6 (37.5%)	9 (37.5%)	
37–72	Below for age	15 (71.4%)	38 (49.4%)	0.0589
	Normal for age	6 (28.6%)	39 (50.6%)	
	Language and Communication			
All (9–72)	Below for age	25 (67.6%)	54 (53.5%)	0.0980
	Normal for age	12 (32.4%)	47 (46.5%)	
9–36	Below for age	16 (100%)	18 (75.0%)	**0.0351**
	Normal for age	0 (0.0%)	6 (25.0%)	
37–72	Below for age	9 (42.9%)	36 (46.8%)	0.4735
	Normal for age	12 (57.1%)	41 (53.2%)	
	Eye and Hand Coordination			
All (9–72)	Below for age	22 (59.5%)	33 (32.7%)	**0.0042**
	Normal for age	15 (40.5%)	68 (67.3%)	
9–36	Below for age	12 (75.0%)	12 (50.0%)	0.1046
	Normal for age	4 (25.0%)	1250.0%)	
37–72	Below for age	10 (47.6%)	21 (27.3%)	0.0674
	Normal for age	11 (52.4%)	56 (72.7%)	
	Personal-Social and Emotional			
All (9–72)	Below for age	11 (29.7%)	16 (15.8%)	0.0601
	Normal for age	26 (70.3%)	85 (84.2%)	
9–36	Below for age	8 (50.0%)	7 (29.2%)	0.1587
	Normal for age	8 (50.0%)	17 (70.8%)	
37–72	Below for age	3 (14.3%)	9 (11.7%)	0.4984
	Normal for age	18 (85.7%)	68 (88.3%)	
	Gross Motor			
All (9–72)	Below for age	11 (29.7%)	11 (10.9%)	**0.0099**
	Normal for age	26 (70.3%)	90 (89.1%)	
9–36	Below for age	6 (37.5%)	4 (16.7%)	0.1322
	Normal for age	10 (62.5%)	20 (83.3%)	
37–72	Below for age	5 (23.8%)	7 (9.1%)	0.0793
	Normal for age	16 (76.2%)	70 (90.9%)	
	General Development			
All (9–72)	Below for age	24 (64.9%)	35 (34.7%)	**0.0014**
	Normal for age	13 (35.1%)	66 (65.3%)	
9–36	Below for age	12 (75.0%)	12 (50.0%)	0.1046
	Normal for age	4 (25.0%)	12 (50.0%)	
37–72	Below for age	12 (57.1%)	23 (29.9%)	**0.0212**
	Normal for age	9 (42.9%)	54 (70.1%)	

Data are presented as n (%), and p-values indicate Fishers exact tests for indices.

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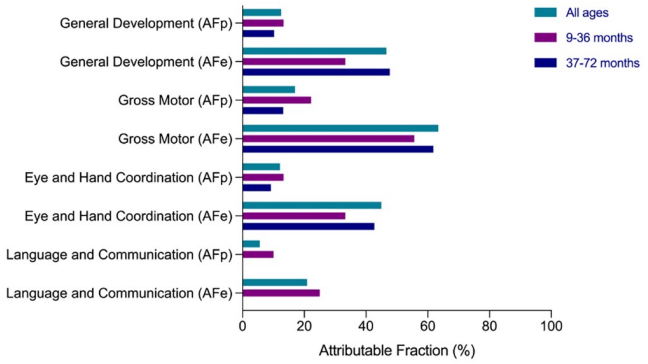

Subscale (based on development quotient)	Prevalence ratio (PR)
Language and Communication	
All ages	1.3
9-36 months	1.3
37-72 months	0.9
Eye and Hand Coordination	
All ages	1.8
9-36 months	1.5
37-72 months	1.8
Gross Motor	
All ages	2.7
9-36 months	2.3
37-72 months	2.6
General Development	
All ages	1.9
9-36 months	1.5
37-72 months	1.9

Fig 6. Estimated proportion of poor cognitive performance (based on development quotient) attributable to stunting. AFe = Attributable fraction in the stunted population, AFp = attributable fraction in the total population. Attributable fractions were estimated for indices with PR >1.

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(p = 0.0014) in older children (>36 months). Association between all six subscale DQ scores and malnutrition as determined by HAZ and BAZ were not significant (p>0.05).

Based on the initial analysis, we then determined how much of poor development scores were attributable to stunting (Fig 6). Among the stunted population, poor scores for the four subscales were highly attributable to stunting (AFe: GM = 63.4%, GD = 46.6%, EHC = 45%, and LC = 21%). When children were grouped according to age category, the proportion of poor scores attributable to stunting among older children (i.e. >36 months) was higher (AFe: GM = 61.8%, GD = 47.7%, and EHC = 42.7%) than that in younger children (i.e. ≤36 months; AFe: GM = 55.6%, GD = 33.3%, and EHC = 33.3%). In the total population, poor subscale scores were attributable to stunting although lower (AFp: GM = 17.0%, GD = 12.5%, EHC = 12.1%, and LC = 5.6%).

Discussion

Indexes monitoring early child development such as The Sustainable Child Development Index [23] and Early Child Development Index, (https://www.unicef.org/socialpolicy/files/child-development-index.pdf) have indicated that children in sub-Saharan Africa are doing worse than those from any other region in terms of reaching developmental milestones. However, there is a paucity of comprehensive child development data from African children, particularly those in rural areas to fully inform such indices and to identify locally relevant interventions. Therefore, we conducted a comprehensive assessment of child development at community level to provide baseline characterisation of child development patterns in rural Zimbabwe.

Our analysis considered development in children aged 6 to 72 months in five domains: Eye and Hand Coordination (EHC), Personal-Social-Emotional (PSE), Language and Communication (LC), Foundations of Learning (FL) and Gross Motor (GM). We also combined the scores from these domains to calculate a score for General Development as per protocol) [12].

Our study detected that 6.6% of the children faced developmental or neurological challenges/disorders that warranted further clinical attention. Suspected diagnosis among these children included Anxiety, Attention Deficient Disorder (ADD), Attention Deficient Hyperactivity Disorder (ADDH), seizure disorder and intellectual disabilities (ID). Our study sample size meant that informative comparisons on these suspected disorders could not be made with findings other African communities. Nonetheless, studies in Africa focusing on single developmental challenges have indicated higher prevalences of such disorders. For example, a study in rural Uganda [24] reported up to 25% prevalence of anxiety disorders in children less than 5 years old. Separate studies have reported lower prevalences of ADHD e.g. 1.5% in the general population in Nigeria [25] but higher prevalences e.g. 7% of children aged below 10 years in paediatric neurology and psychiatry clinics in Uganda reported [26]. Children within communities especially rural communities, are not routinely screened for cognitive or neurodevelopmental development disorders and subtle indicators that can be detected by comprehensive child development assessment are not detected in the normal baby growth monitoring activities (see e.g. [27]). Our identification of the 11 children requiring further clinical investigation and intervention indicates a need for more studies and screening programs to document and diagnose these disorders that are going untreated in Africa children [28].

Our findings that the Zimbabwean children performed better than average in the GM domain are consistent with finding from other studies in Africa. A study in South African children compared gross motor skills in 3–5 year old children from rural low-income backgrounds to those from urban backgrounds (both low and high income) [29]. In our study, just over half of the children performed as expected for their age or better for the overall general development score (GD). This is lower that what has been reported in the South African study where the overall majority of the children (93%) performed well for their age being classified as having average or better scores. This was also the case in Nigerian children [30]. Interestingly, in the South African study, rural children performed better that urban children [29] although the reasons for this finding were not explored. In the Zimbabwean study, it is likely that the GD score was brought down by poor performances in some of the subscales. The Griffiths Developmental Scales compare a child's chronological age to a developmental age derived from his/her performance on test items in the subscales. An analysis of this cohort's performance showed their developmental level as lower than the chronological age in the FL domain, but higher for PSE and GM. Developmental ages for LC and EHC were comparable.

There is a paucity of studies in children aged below 5 years characterising the PSE development of children independent of confounders such as infection and non-infectious conditions. Nonetheless, a large, study comparing the performance of 7 year olds from the Millennium Cohort [31] investigating ethnic differences in children's socioemotional difficulties indicated that after accounting for maternal and family environment factors Black African children had significantly fewer socioemotional difficulties. This is consistent with our finding that the majority of children in our study performed well (or above expectation) for their age in the PSE domain.

The cognitive FL assesses skills and abilities essential for early childhood learning that set the pace and trajectory for future educational achievement. FL measures critical psychometric constructs including cognitive skills for learning, executive function, ways of thinking, problem solving, organizing and information planning, analytical thought, memory and play [32]. Data from this study shows children's lowest performance was in this domain. The FL domain would be improved by cognitive simulation tools and activities. According to the UNICEF Country Profiles for Early Childhood Development report (https://nurturing-care.org/resources/country-profiles/) only 37% of Zimbabwean children received early stimulation at home, 28% were in attendance of early childhood education and only 3% have books in the

home. Persistent gaps in children's development in cognitive and non-cognitive domains have been noted between children from disadvantaged backgrounds and peers from more advantaged backgrounds. Enriching caregiving practices are essential for normal child development. Children with responsive caregivers, and those who are in more stimulating, environments, are reported to be more cognitively advanced at the start of school than children from less stimulating homes. In addition frequent interaction between parent/caregiver through e.g. play, has been reported to promote the children's cognitive, social and emotional development [33].

A study on the challenges faced by children aged 5 years and below conducted in all the provinces of Zimbabwe [34], showed that the majority of children attending ECD classes came from backgrounds fraught with challenges such as hunger, economic hardships, poor mobility of parents, lack of infrastructure and low parental knowledge about the importance of ECD education. Hunger and poor nutrition contribute to stunting and several studies [35,36] have shown a link between stunting and poor child development in low- and middle-income countries. We detected a 26.8% prevalence of stunting, similar to the prevalence of 26% reported in Zimbabwean children aged 5 years and below in 2018 (http://fnc.org.zw/documents/). In this study, stunting was significantly associated with lower scores for EHC, GM, and GD domains across all age groups. Stunting in the first 5 years of life has both immediate and long-term effects in children [37]. For example, work in Benin has shown that stunting compromises cognitive development in children [35], while a cohort study in 8 Low-Middle Income Countries including those in Africa demonstrated that stunting within the first 6 months which persisted for 60 months was associated with lower cognitive development in children at 5 years old [36]. UNICEF recognizes malnutrition as having potential adverse consequences on child survival and long-term well-being with far reaching consequences for economic productivity and human capital development. This increases the urgency to accelerate progress towards Sustainable Development Goal 2 to end hunger, achieve food security and improved nutrition, to also attain the other SDGs.

In addition to nutrition, other factors such as childhood infections [38] also affect early child development. We have recently reported that early childhood infections with schistosomes affect metabolism in children aged 5 years and below, [39], which may have a subsequent impact on ECD. Whilst the detrimental effects of helminth infections (both STH [4] and schistosomes [7]) have been previously reported, to date there has been no study investigating the impact of schistosome infection on ECD focusing on children aged 5 years and below. In this study we showed that children infected with schistosomes scored significantly lower in the FL domain across all ages while older children (aged 37–72 months) scored significantly lower in GD. Overall, in the schistosome positive children, 30.8% of the poor FL scores were attributable to schistosome infection. Thus, it is not surprising that 6 months following treatment, these scores increased; and by 12 months post treatment, the scores for schistosome infected children improved across all domains, to be similar to those of previously uninfected children. Smaller sample sizes for the cohort studies mean more detailed subgroup analyses could not be conducted. Nonetheless, our findings are consistent with findings from quantitative studies in older children [6].

In conclusion, we have demonstrated that just over 50% of Zimbabwean children in rural areas are on course for normal child development; with many being more advanced for their age in GM and PSE domains. Nevertheless, the children do face developmental challenges in early childhood, particularly in FL. This reflects several factors including those identified by the WHO, including lack of early stimulation at home, lack of access to early childhood education and lack of books in the home. Poor development scores were also attributable to stunting and schistosome infection, with the impact of the latter being reversed by curative

antihelminthic treatment. The children's low FL scores and the impact of S *haematobium* infection, stunting and malnutrition among other factors should be of concern, as they can be a predictor of future educational and occupational challenges. Taken together, the findings strengthen the call for the treatment of paediatric schistosomiasis, accessibility to cognitive stimulation tools and improved nutrition to improve childhood health outcomes and accelerate progress towards the SDGs.

Supporting information

S1 Fig. Study design.
(TIF)

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Writing – review & editing: Francisca Mutapi, Derick Osakunor, Rivka Lim, Maritha Kasambala, Arnold Mutemeri, Simbarashe Rusakaniko, Dixon Chibanda, Takafira Mduluza.

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