Immunological Evaluation of HIV-negative Invasive Fungal Disease at Groote Schuur Hospital; Cape Town, South Africa.

ONYANGO VONWICKS CZELESTAKOV

ONYVON001

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Supervisor: Prof. Jonathan Peter[1]

Co-supervisor: Prof. Sipho Dlamini[1]

[1]Department of Medicine

University of Cape Town

DECLARATION

I, Dr. Vonwicks Czelestakov Onyango, do hereby declare that the study on which this thesis/dissertation is based, as well as the entire thesis/dissertation itself, is my original work (except where citations and acknowledgements indicate otherwise); and that neither the whole work, nor any part of it whatsoever, has been, is being, or will be submitted for another degree in this, or any other university.

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Abstract

Background

The majority of invasive fungal disease in South African hospitals is HIV-related or associated with another secondary immunodeficiency e.g. haematopoietic stem cell transplant. After excluding secondary immunodeficiency, a detailed immune work-up can lead to a diagnosis of primary immunodeficiency.

Objective

To detail an appropriate step-wise immunological work-up for a series of patients with invasive fungal diseases and possible underlying primary immune deficiency.

Methods

Detailed review of all culture- or histologically confirmed cases of invasive fungal disease (IFD) at Groote Schuur Hospital between 2007-2017. Step-wise immunological work-up of IFD patients with no secondary immunodeficiency. Clinical characteristics and step-wise immunological profiles were evaluated.

Results

Sixty-seven adults with IFD were identified; 72% (48/67) were HIV-related. 8/19 HIV-negative cases were either deceased (4) or lost-to-follow-up (4). Work-up of the remaining 11 cases found five with non-HIV secondary immunodeficiencies (Lupus, liver transplant, end-stage renal failure and haematological malignancy). A primary immunodeficiency was suspected in six cases, but 1 case of cutaneous sporotrichosis was excluded; with five cases (4 with disseminated *Cryptococcus neoformans* and 1 with cerebral aspergillosis) undergoing detailed immune work-up. A case of idiopathic CD4 lymphopenia was diagnosed; but all other cases had no evidence of neutrophil or a cell-mediated immune defect; including investigations of naïve and memory T-cell subsets and cytokine responses to PHA and candida. All cases were noted to have low baseline vaccine responses and Vitamin D deficiency.

Conclusion

Invasive fungal disease is predominantly associated with HIV and secondary immunodeficiency in South Africa. Known primary immunodeficiencies can be identified with basic immune work-up; but no obvious functional immune defect is evident in the majority of these cases.

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DEDICATION

I dedicate this dissertation to my parents, Mr. and Mrs. Robert Onyango, and my spouse, Joan; for their continued positive influence on the direction of my professional and personal choices.

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List of acronyms and abbreviations

AD	autosomal dominant
ADHIES	autosomal dominant hyper-IgE syndrome
ANA	antinuclear antibodies
AR	autosomal recessive
BMAT	bone marrow aspiration and trephine biopsy
CARD9	caspase recruitment domain-containing protein 9
CD	cluster of differentiation e.g. CD4, CD8
CLAT	cryptococcal latex agglutination test
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease
CSF	cerebrospinal fluid
CT	computer tomography
CXR	chest x-ray
DM	diabetes mellitus
GM-CSF	granulocyte monocyte colony stimulating factor
GOF	gain of function mutation
HbA1c	glycosylated haemoglobin A1c
HSCT	hematopoietic stem cell transplantation
ID	infectious disease
IFD	invasive fungal disease
IFN-γ	interferon gamma
IL	interleukin e.g. IL-17
LOF	loss of function mutation
NTM	non-tuberculous mycobacteria
PID	primary immune deficiency/primary immunodeficiency
SLE	systemic lupus erythematosus
STAT	signal transducer and activator of transcription e.g. STAT1
VZV	varicella zoster virus

CHAPTER 1: BACKGROUND AND LITERATURE REVIEW

Background

Introduction

Whereas fungi are ubiquitous, only a minority cause infection in humans. Innate and adaptive immune responses are usually effective in preventing disease or restricting to non-invasive infections. Invasive fungal infections occur whenever there's failure of these immune mechanisms to contain invading fungal elements; often leading to severe and disseminated disease. Secondary immunodeficiencies, especially HIV/AIDS; account for most patients presenting with invasive fungal disease. However, a small number of patients present with invasive fungal disease (IFD) in the absence of HIV, or any other common secondary immunodeficiency. In this literature review, we explore the epidemiology and general management of invasive fungal diseases, while also reviewing the clinical and immunological characteristics of patients diagnosed with IFD, but without any secondary causes of immunosuppression.

Definition of invasive fungal disease (IFD)

Standard definitions for invasive fungal infections were published in 2002, by a consensus group of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC) and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG)(1).

Invasive fungal disease defines a disease process caused by fungal infection, following the exclusion of an alternative etiology. This definition may include: (See tables 1-3 in Appendix at the end of Literature Review)

Proven IFD: - this requires demonstration of fungal elements in diseased tissue, either directly (by culture or histology), or indirectly by use of assays that are highly specific for the infection being detected (e.g. presence of coccidiodal antibodies in CSF proves coccidioidomycosis; whereas the presence of cryptococcal capsular antigen in CSF proves disseminated cryptococcosis).

Probable IFD: - this requires that a host factor, clinical features, and mycological evidence be present, as outlined in tables 2 and 3.

Possible IFD: - this includes only those cases with the appropriate host factors and with enough clinical evidence consistent with IFD but for which there was no mycological support, as outlined in table 2.

All the cases evaluated in this study were of proven IFD.

Besides, chronic mucocutaneous candidiasis (CMC) is defined by recurrent or persistent infections affecting the nails, skin and oral and genital mucosae caused by *Candida spp*(2, 3). This entity was excluded in the study.

Epidemiology of invasive fungal diseases

The Leading International Fungal Education (LIFE) portal has been facilitating the estimation of the global burden of serious fungal infections within regions of the same country and between at-risk populations(4). Between 2013 and 2017, they estimated that more than 150 million people have serious fungal diseases, which have a major impact on their lives or are fatal (4). There are about 3,000,000 cases of chronic pulmonary aspergillosis; 223,100 cases of cryptococcal meningitis complicating HIV/AIDs; 700,000 cases of invasive candidiasis;

500,000 cases of *Pneumocystis jirovecii* pneumonia; 250,000 cases of invasive aspergillosis; 100,000 cases of disseminated histoplasmosis; over 10,000,000 cases of fungal asthma; and 1,000,000 cases of fungal keratitis occurring annually (4-8). The HIV/AIDS pandemic, TB, COPD, asthma and the increasing incidence of cancers are the major drivers of fungal infections in both developed and developing countries globally(4, 6-9).

Invasive candidiasis

In 2016, the annual incidence of invasive candidiasis in the UK was estimated to be 5142 cases; with up to 40% mortality (10). In the US, *Candida* species were the third most frequently isolated organisms in a nationwide surveillance for nosocomial sepsis, with the crude mortality rates of up to 50%(11). In the LIFE portal, countries with high burdens of candida peritonitis included Mexico (5596; 4.98 per 100,000), Nigeria (2321; 1.5 per 100,000) and Spain (668; 1.42 per 100,000)(4). The high morbidity and mortality of invasive candidiasis has been associated with the use of broad-spectrum antibiotics and corticosteroids, prolonged stay in ICUs, major intra-abdominal procedures, increased transplantations and cancers (10). Primary immune deficiencies (PIDs) associated with invasive candidiasis include abnormalities/deficiencies of CARD9, CD18, IL-12 and IL-17(12, 13). In these patients, PBMCs and neutrophils display impaired proinflammatory cytokine release and impaired killing function(12).

Invasive aspergillosis (IA)

Up to 81,927 cases of invasive aspergillosis had been observed by 2016; with the maximum number of cases in Vietnam (14,523/year) and the minimum in Trinidad and Tobago (8/year)(4). An annual incidence of 294 cases was estimated in Denmark, with 27 cases (9.1%) occurring in haematological malignancies; and 12 cases (4%) in transplant patients(14). COPD was the main driver for the burden and variability of IA in these cases(15). PID associated with IA include NADPH oxidase complex defect in phagocytes and granulocytes (leading to impaired respiratory burst activity and inability to kill fungal pathogens) and hyper IgE syndrome (HIES) (due to abnormal STAT-3 dependent epithelial immunity)(12). Egypt and Algeria reported cases of 10.7 and 7.1 per 100,000 persons annually, with the majority cases occurring in COPD. On the other hand, Nigeria and Kenya each had 0.6cases/100,000 persons with 10% of these in AML patients. In all cases, the mortality of IA has been reported to be ranging between 30-80%(4).

Chronic pulmonary aspergillosis (CPA) (aspergilloma, chronic cavitary pulmonary aspergillosis and chronic fibrosing pulmonary aspergillosis) is thought to affect about 3 million people worldwide and, if untreated 80% of patients with CPA will die within 5 years (16). Prior TB is perhaps the most common underlying condition, with post-tuberculous cases probably representing 50% of the total cases. In the LIFE portal, an annual incidence of CPA was found to be 22cases/100,000persons for all the countries included. The highest incidence was in Russia (126.9/100,000), while the lowest incidence was in Canada (1.38/100,000 (4).

Allergic bronchopulmonary aspergillosis (ABPA) is a common complication of asthma or cystic fibrosis (17, 18). These patients present with poorly controlled asthma, haemoptysis, fever and expectoration of mucus plugs. They display a positive skin test or high levels of *Aspergillus* specific IgE(17). Data from referrals to specialists in South Africa, New Zealand, Iran, Ireland, Saudi Arabia and China, translate to over 4.8 million cases of ABPA in adults (19). The prevalence of ABPA reflects adult asthma prevalence country by country, which has been estimated to be 2.5% in all the countries in the LIFE portal (4).

Cryptococcus neoformans

Cryptococcus neoformans is the most common fungal infection in HIV, where cryptococcal meningo-encephalitis is an AIDS-defining illness (20). Park et al estimated that one million cases are reported each year in Sub-Saharan Africa, with more than 600 000 deaths/year in HIV-infected patients. Cryptococcal meningitis accounts for 20 to 25% of AIDS-related deaths in Africa(21). A study done in Cape Town showed cryptococcal meningitis accounting for 63% of all cases of meningitis in HIV patients, with a mortality of almost 50% despite treatment(22). Disseminated cryptococcal infection can cause clinical manifestations in the skin, ocular, soft tissue or bones and joints. Large cohort studies in London had 5% prevalence of cryptococcosis among HIV positive patients (23).

Pneumocystis jirovecii (carinii)

Pneumocystis jirovecii (carinii) is an obligate extracellular fungus which exclusively infects humans, causing a life-threatening form of interstitial pneumonia. HIV is the most common risk factor, where it causes an AIDS-defining pneumonia, commonly in patients with CD4 counts <350 cells/µl. The global prevalence is thought to be >400,000 cases annually; with mortality ranging from 10-30% and even higher with delayed diagnosis(4, 8). The mortality has generally reduced in the era of highly active anti-retroviral therapy (HAART), but unacceptably high mortalities continue to be seen when diagnosis is not made in time, where drug shortages and stock outages limit access to HAART, and in those who default on HAART(4, 8). In 40 published papers within the LIFE program, 133,487 cases have been estimated; with 75% occurring in Sub-Saharan Africa (4). In a systematic review by Wasserman et al, the pooled prevalence of PJP from all clinical settings in Sub-Sahara Africa was 15. 4%. This was associated with a case fatality rate of 18.8% (24).

Deep dermatophytosis

Deep dermatophytosis is characterized by the invasion of the dermis and hypodermis by dermatophytes, sometimes associated with lymph node, brain, digestive tract or bone involvement(25). While secondary causes of immunosuppression are the common risk factors, large global cohort studies identified 69 cases without any known immune deficiencies, with 45 of these (65%) originating from North Africa(26). Twenty-four of these patients were from consanguineous families, with 19 familial cases from eight multiplex families. This suggests an autosomal recessive (AR) pattern of heredity to be a predisposing factor (26-29). Subsequent genetic studies have identified AR CARD9 deficiency among related family members with idiopathic deep dermatophytosis from North Africa (3, 13, 26).

Dimorphic fungal infections

Dimorphic fungal infections are endemic to certain geographical areas, including *Histoplasma* (Midwestern US), *Coccidioides* (South West US), and *Paracoccidioides* (Latin America)(30-32). Disseminated histoplasmosis occurs in patients with immunodeficiency, mostly AIDS, and involves the reticulo-endothelial system and brain (12). A 5-year multicentre review of 70 transplant patients (64 solid organ transplant recipients and 6 hematopoietic stem cell transplant [HSCT] recipients) with endemic mycoses showed 52 with histoplasmosis (74%), 9 with blastomycosis (12.8%), and 9 with coccidioidomycosis (12.8%) (33). Disseminated coccidioidomycosis involving the lungs, CNS and bones occurs in 55% of patients without

known risk factors. Genetic studies have revealed mutations affecting the interleukin-12/IFN-g circuit and STAT-1 GOF (12).

Diagnosis of invasive fungal disease

The gold standard for the diagnosis of IFD is by culturing clinical specimens (tissue, sputum, urine, wound, or blood) to isolate the etiologic fungal agent(s), provided that the samples are from sterile sites such as blood, tissue or cerebrospinal fluid, in which fungal infection can be documented. Histopathology examination with special stains confirms fungal tissue involvement (34-36).

Serological methods avail results sooner than culture. These may include skin tests and serum IgE for ABPA; cryptococcal antigen tests (CRAG) for cryptococcosis, fungal cell wall components e.g. galactomannan for Aspergillus spp. As per the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines, the combined detection of mannan and anti-mannan in blood/serum is considered specific (specificity up to 90% compared with blood culture=46%) for detection of *Candida spp*(36).

The polymerase chain reaction (PCR) assay may be used for the diagnosis of systemic fungal infection in high-risk patients. Quantitative methods include PCR ELISA, with a sensitivity of about 83.3% and a specificity of about 91.7%; and real-time PCR, with the sensitivity about 100% and a specificity about 97% (37, 38).

Radiological evidence from X-rays and HRCT are useful adjuncts in the diagnoses. Pulmonary fungal infections such as aspergillosis, fusariosis, scedosporiosis or zygomycosis are characterized by central cavitation of pulmonary lesions, infiltration, pulmonary nodules, and halo or air-crescent signs (39, 40).

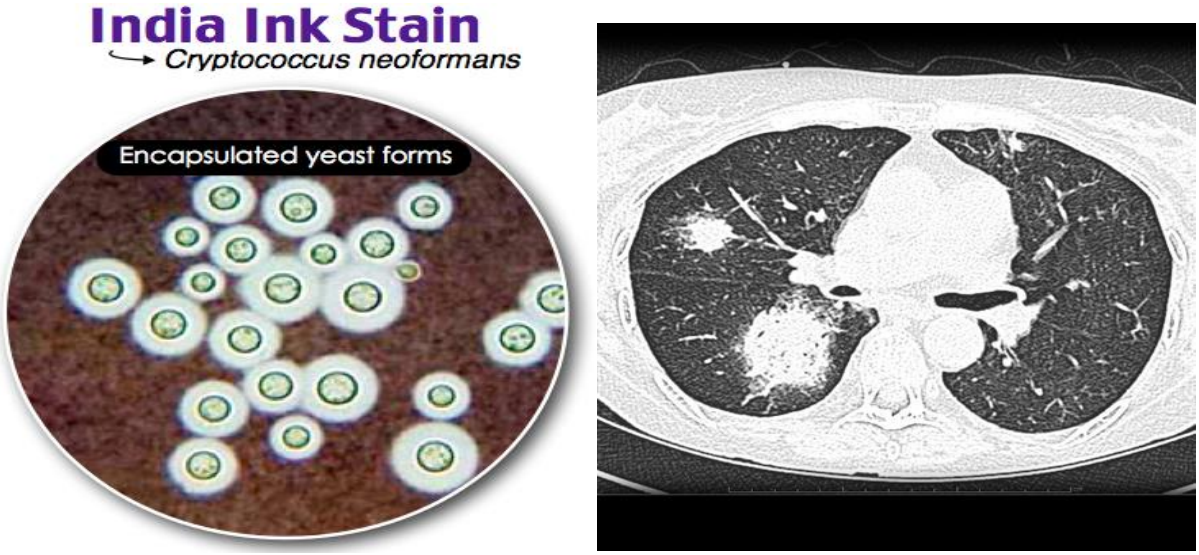


Figure 1 showing Indian ink stain for C.neoformans in CSF; and CT chest showing lung aspergillomas(41).

Management/treatment of patients with invasive fungal diseases

Timely initiation of systemic antifungals is critical to prevent significant morbidity and/or mortality. Mortality increases by 20% for each 12-hour delay in antifungal therapy following a positive fungal culture(39). Besides, source control is a critical component of good anti-

fungal stewardship, and may necessitate surgery debridement of infected tissues, or drainage of collections (39, 42).

Commonly used antifungal agents include the azoles, polyenes, allyamines and echinocandins. Various examples and their mechanisms of action are summarized in table 1 below(43). The choice depends on drug sensitivities, availability and co-morbidities. It may be necessary to monitor for drug toxicities and efficacy. Adjunctive therapy for co-morbidities is necessary, often with multi-disciplinary input.

Table 1:- Antifungal drug classes, mechanisms of actions and common examples.

ANTIFUNGAL DRUG CLASS	**MECHANISM OF ACTION**	**DRUG NAMES**
Azoles	Ergosterol synthesis inhibitors (cell membrane)	Ketoconazole, fluconazole, itraconazole, voriconazole, posaconazole, ravuconazole
Polyenes	Fungal membrane-disrupting agents (e.g. pores in membrane)	Amphotericin B, nystatin
Echinocandins	Glucan synthesis inhibitors (cell wall)	Caspofungin, anidulafungin, micafungin
Allylamines	Ergosterol synthesis inhibitors	Terbinafine, butenafine
Other	Anti-mitotic (spindle disruption)	Griseofulvin

Primary treatment often requires parenteral administration for 4-6 weeks, or longer, in consultation with ID specialists. Patients would then need secondary/preventive therapy, usually orally, for a specified duration, which may be life-long in selected cases. For patients with treatable or manageable secondary immunodeficiency, restoration of immune function is crucial. This may be in the form of anti-retroviral therapies (ARVs) or immune therapies (34, 44).

Literature Review

Objectives of literature review

This literature review sought to explore the clinical and immunological characteristics of adult patients with invasive fungal disease. This included mechanisms of antifungal immunity, primary and secondary immune defects leading to IFD, and site-specific disease susceptibilities. The scope of the search involved studies done locally and internationally.

Literature search strategy

Articles only in English were considered. PubMed and Google Scholar search engines were used to identify both full-text and abstract-type journals spanning through 1990 to 2017. The following key phrases were used for the search: "HIV-negative AND fungal diseases", "invasive fungal infections AND HIV-negative", "invasive fungal infections AND immunocompetent", "primary immune deficiency AND invasive fungal infections", "genetics AND invasive fungal infections", "risk factors AND invasive fungal infections", "clinical features AND invasive fungal infections', "immunology AND fungal infections", "management of invasive fungal infections". The articles were saved directly into EndNote version X8 for citation and referencing management. These were then imported to HP notebook 2013.

Results

The table below shows the number of articles identified and selected from the databases.

Articles identified and selected from the databases

Database searched	**Results****	**Used##**
PubMed	74	47
Google scholar	46	18
Total	**120**	**65**

******Articles of potential interest identified from the two databases

##Articles used after screening for relevance, duplication and citation permissions

Antifungal immunology and known defects

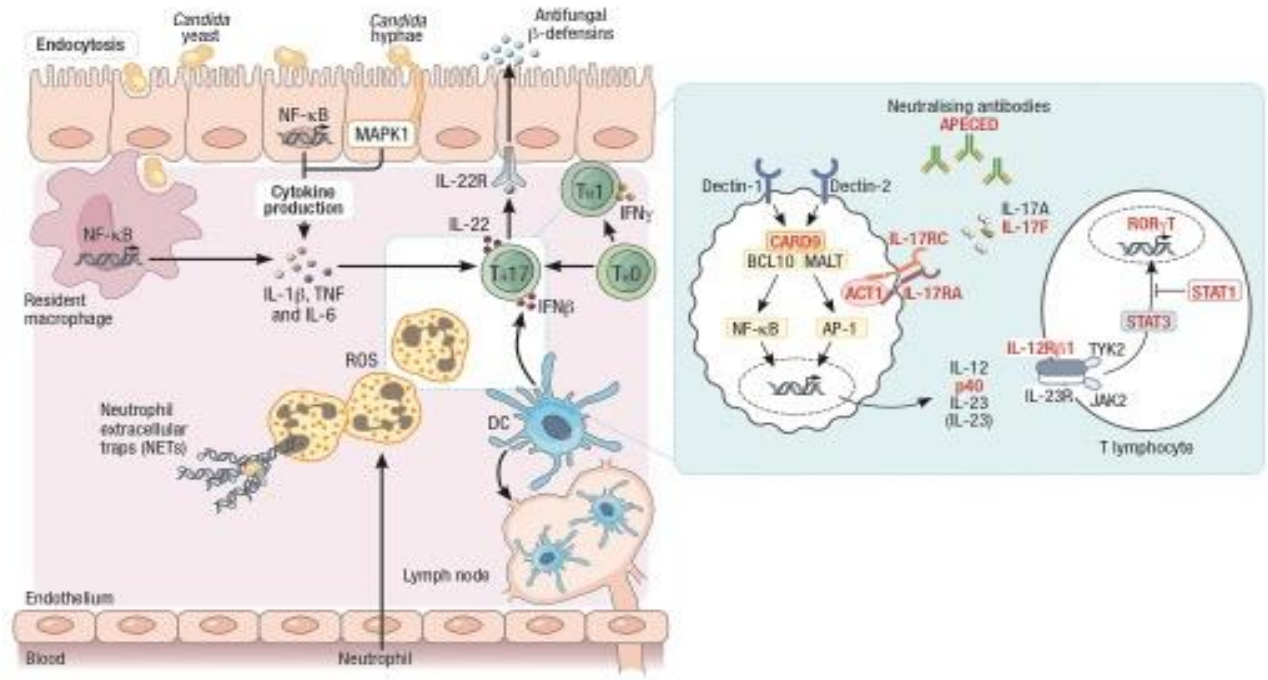
Introduction

Fungi are ubiquitous in the environment and have co-evolved with human beings over several millennia. Due to constant exposure to these fungi, and with the ever-present risk of serious infections, humans have developed complex mechanisms for immune surveillance, pathogen recognition, processing and elimination; while maintaining auto-tolerance. The fungi have over the years also acquired increasingly complex evasion strategies against host immunity(43). On the balance, a host-fungal homeostasis exists(43). Recent findings of certain monogenic disorders among individuals with primary immune deficiencies, e.g. STAT1 GOF mutations, have shown such to have unusual susceptibilities to chronic and/or invasive fungal infections (3). These findings continue to elucidate interesting aspects of antifungal immune responses.

Antifungal Immunity

Both innate and adaptive immune responses work in concert to eliminate or limit fungal infections.

Figure 2 Key immune players in normal fungal immunity:-



Abbreviations: ROS: Reactive oxygen species; DC: Dendritic cells; APECED: Autoimmune polyendocrinopathy candida and ectodermal dysplasia.

Adapted from CMC review article by Peter and Onyango(43).

The first line defence against fungal infections is the innate immunity; by way of physical barriers and cellular components. Intact skin and mucosal membranes deter fungal adhesion and entry. Therefore, any breaches in skin and mucosal integrity predisposes to fungal infections. Cells of the innate system e.g. Natural killer cells, neutrophils, dendritic cells (DC) and macrophages, bind components of fungal cell walls using pattern recognition receptors (PRR) and initiate killing by means of phagocytosis. C-type lectin receptors (CLR) such as Dectin-1 and Toll-like receptors (TLR) are particularly important for anti-fungal immunity. Recognition and ligation of fungal cell wall sugars and nucleic acids occurs by the evolutionarily conserved fungal pathogen associated molecular patterns (PAMPs); which activate TLR (TLR1-4, 6-9) and CLR signalling (45-47). This leads to the uptake, processing and presentation of the fungal components by antigen presenting cells (APCs), e.g. macrophages and dendritic cells. These APCs present the processed fungal antigens to naïve T cells, leading to their differentiation into Th-1 effector cells for fungal clearance. Due to their central role in fungal recognition and initiation of effective innate and adaptive immune

responses, individuals with certain genetic deficiencies of the PRRs e.g. CARD9 and Dectin-1 are highly susceptible to fungemia, especially candida infections(45).

Adaptive fungal immunity revolves around the balance between Th-17, Th-2 and Th-1 effectors, driven by the DC responses to TLR and CLR signals provided by the fungus. The signalling activities of TLR and CLR induce MHC class II, co-stimulatory molecules, and cytokine expression by antigen-presenting cells that dictate T cell differentiation. Th1 and Th17 cells are the principal T helper subsets that contribute to protective immunity to several pathogenic fungi. Th-2 responses favour fungal infections, fungus-associated allergic responses, and disease relapse. Activated Th-1 cells produce effector cytokines e.g. IFN-gamma and induces B-cell production of opsonizing antibodies (45, 48-50).

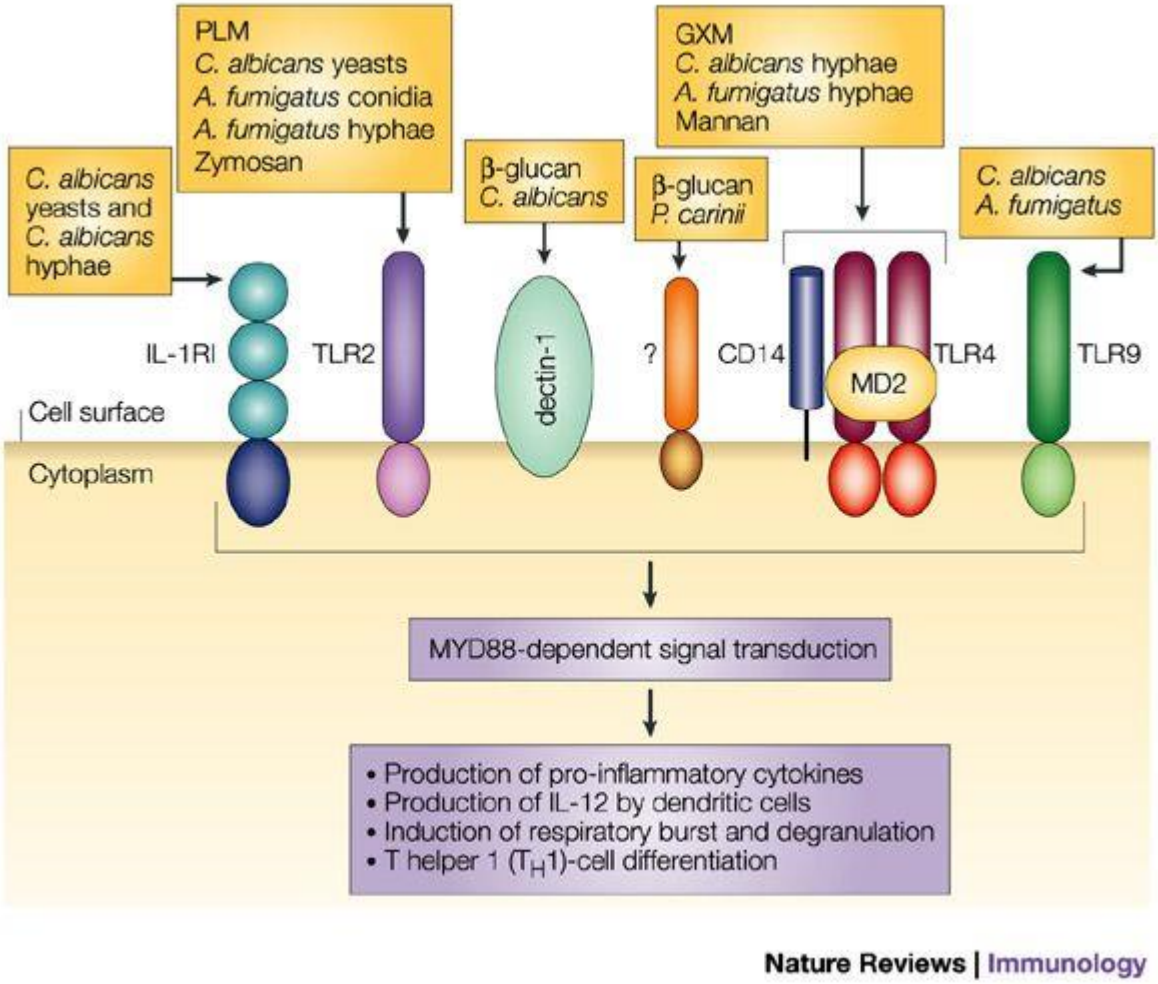
MyD88-dependent signals, a major downstream component of the TLR pathway, are responsible for CD4+ T cell priming and trafficking to infected airways during respiratory fungal infection. For example, they mediate the stepwise differentiation of *A. fumigatus*-specific CD4 T cells into IFN-γ-producing airway effector cells (51). IL-17 contributes to host resistance in oropharyngeal, cutaneous, and systemic fungemia, especially with *C. albicans*, and pulmonary infection with *H. capsulatum*. Th-17 cells are regulatory in fungal immunity by promoting Th-1 responses and restraining Th-2 responses (52, 53). These cells form part of the fungus-specific repertoire of memory cells.

In the absence of Th-1 and Th-17 cells, then IL-22 (part of the AHR-IL-22 pathway), provides protection against candida at mucosal sites. IL-22 is produced by numerous activated innate lymphoid cells, as well as Th-1 and Th-17 cells. This protection is particularly prominent in the intestinal mucosa, against candida overgrowth (49, 50).

Naturally acquired fungal infections do not induce protective antibodies against subsequent infections. Despite a growing medical need, no fungal vaccines are commercially available (54). One of the best ways to induce sterilizing immunity and achieve maximal protection is by using virulence-attenuated mutants. This has been under trials in experimental models of blastomycosis, histoplasmosis and coccidioidomycosis (55). Besides, vaccination with attenuated mutants induces antifungal memory CD8+ T cells that are maintained for at least 6 months. These vaccine strains are however not likely to be safe in immunocompromised individuals.

Figure 3 below, adapted from Romani et al(54), illustrates the interplay between innate and adaptive immune mechanisms activated by various fungal epitopes.

Figure 3: - Interplay between innate and adaptive immune mechanisms



The knowledge gained from experimental vaccine models is crucial for the rational design of the next generation of human vaccine putative candidates. Since the bulk of fungal protection centres on Th17 and Th1 cells, this is a novel paradigm for human vaccines (55-57). Essentially, these vaccines will confer protection via T-cell induced inflammatory cytokines e.g. IFN-γ, TNF, IL-17 and IL-22 that regulate the influx and killing functions of effector cells e.g. phagocytes; as well as by inducing memory antibodies that function by neutralizing virulence factors (e.g. adhesins), inhibiting fungal growth, and by stimulating direct killing, opsono-phagocytosis, and complement activation(58).

Secondary immunodeficiency leading to invasive fungal diseases

Secondary causes of immune deficiencies are associated with increased susceptibility and severity of various invasive fungal diseases. HIV/AIDS is the single most common secondary risk factor for invasive fungal infections; especially in high prevalence areas. Other secondary risk factors include solid and haematological cancers, chemotherapy, hematopoietic and solid organ transplantation, neutropenia, diabetes mellitus, prolonged corticosteroid use, prolonged stays in the ICU, and autoimmune diseases e.g. SLE and sarcoidosis.

The table below gives a summary of spectrum of some of the invasive fungal infections, the associated secondary co-morbidities, and some identified genetic susceptibilities (42). A more

comprehensive discussion of the known genetic/monogenic disorders associated with invasive fungal infections follows in the subsequent section.

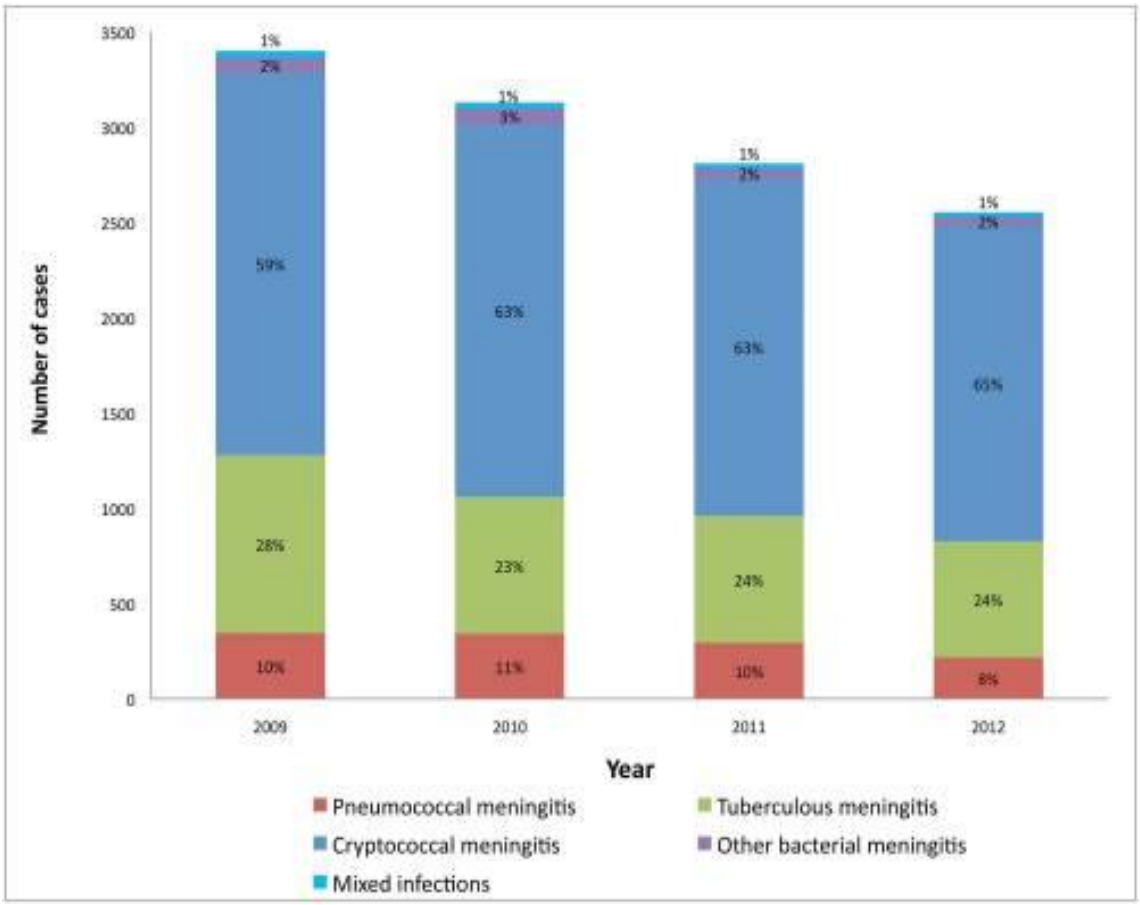
Table 2:- Spectrum of invasive fungal infections, associated secondary co-morbidities and associated genetic susceptibilities

SPECTRUM OF INVASIVE FUNGAL DISEASE, ASSOCIATED SECONDARY CO-MORBIDITIES AND GENETIC SUSCEPTIBILITIES		
Invasive candidiasis: - sepsis, meningitis, invasive organ disease	HIV, DM Solid tumor HSCT Immunosuppressive Rx Major organ failure	Severe combined immunodeficiency (SCID)
Invasive aspergillosis: - hypersensitivity pneumonitis, meningoencephalitis	Chronic steroid use DM HIV	Mutations in the NADPH oxidase complex
Cryptococcosis: - sepsis, meningitis, invasive solid organ disease	HIV DM Immunosuppressive Rx HSCT Solid organ transplant	IL-12R defect MonoMac, ADHIES, and HIGM syndromes
Mucormycosis/zygomycosis: - pulmonary, rhinocerebral, disseminated	HIV, HSCT, Solid organ transplant, Chronic steroid use, DM	IL-12R defect Severe combined immunodeficiency (SCID)

(DM=diabetes mellitus, HSCT=hematopoietic stem cell transplant, Rx=treatment, AD HIES=autosomal dominant hyper-IgE syndrome, HIGM=hyper-IgM syndrome)

Cryptococcus neoformans, as previously noted, is the most common fungal infection in HIV, where cryptococcal meningo-encephalitis is an AIDS-defining illness. An epidemiological review of 11891 HIV-positive adults over a 3-year period with meningitis in Gauteng province, South Africa; showed the majority to have cryptococcal meningitis; associated with advanced HIV (59).

Figure 4: - Incidence of meningitis in Gauteng province, South Africa



Cryptococcus gattii infection outbreak was first reported in 1999 in temperate British Columbia, Canada(60). A subsequent survey done between 1999-2007 showed up to 218 reported cases with predominant respiratory and CNS syndromes at presentation; with a case fatality rate of 8.7%(61). Up to 73.7% of case-patients who died had an underlying secondary immunodeficiency; including HIV, diabetes, liver failure and solid organ transplant(61). The organism is now endemic in northern America with considerable public health consequences(62, 63).

Surveillance studies done at Groote Schuur Hospital in Cape Town between 2008 to 2011 identified *Emmonsia species* as an emerging new species of dimorphic fungi causing disseminated fungemia among patients with advanced HIV; especially those with CD4 counts of under 50cells/ml. More cases have since been described in other parts of South Africa, as well as in non-HIV patients (64, 65).

In 2016, there were over 5000 cases of invasive candidiasis in the UK, with mortality rates of over 40% despite newer antifungals. This has been attributed to the use of broad-spectrum antibiotics and immunosuppressant agents, prolonged hospital and intensive care unit (ICU) stay, diabetes, nosocomial bacterial infection, recent surgery, pancreatitis, mechanical ventilation, total parenteral nutrition, use of medical devices such as central venous catheter and shunts, increased transplantation and more immune-compromising medical conditions such as malignancies. Abdominal surgery has been associated with increased risk of peritoneal candidiasis, as shown by data from Germany, Mexico and Nigeria (4). A retrospective hospital-based study in Soweto, South Africa; showed Candida albicans to be the most common species

of invasive candidiasis. Others are *C. tropicalis*, *C. glabrata* and *C. parapsilosis*. Major predisposing conditions were abdominal surgery, HIV infection, trauma, diabetes mellitus and cancer(66).

The highest risk populations for invasive aspergillosis comprise chronic granulomatous disease (especially TB, where aspergilloma are often found in old healed TB cavities and present with varying degrees of haemoptysis), prolonged neutropenia, HSCT and heart, lung and pancreas organ transplantation. In the UK, IA was found to complicate 9% of HSCT, 10% of, S.O.T.; 0.6% of H.I.V.; 15% in haematological disease, and 1.3% of COPD admissions. Kenya, Tanzania and Nigeria each reported IA complicating 10% of A.M.L. and 10% of non-AML haematological patients, without data on COPD cases (4).

Invasive histoplasmosis has been increasingly being reported in Africa, especially since the advent of HIV. Pulmonary histoplasmosis may be misdiagnosed as tuberculosis (TB). In the last six decades (1952–2017), 470 cases of histoplasmosis have been reported. HIV-infected patients accounted for 38% (178) of the cases. West Africa had the highest number of recorded cases with 179; the majority (162 cases) of which were caused by *Histoplasma capsulatum var. dubuosii* (Hcd). From the Southern African region, 150 cases have been reported, and the majority (119) was caused *by H. capsulatum var. capsulatum* (4).

Known monogenic diseases associated with IFD.

Defects of T-cell development and differentiation, phagocytic functions, and pathways involved in the innate recognition of pathogens and downstream signalling are associated with increased risk of fungal infections, the most common being candidiasis and aspergillosis(43). In South Africa, the bulk of these infections occur in the context of HIV/AIDS, as well as the aforementioned causes of secondary immune deficiency(43). Once these secondary risk factors have been excluded, a search for genetic factors should be made, especially where a family history exists(12).

CD40L is expressed on activated T-cells and signals through NEMO/NF-κB to induce IL-12 production(12). CD40L deficiency, also known as hyper-IgM syndrome, is inherited in an X-linked recessive manner(67). Patients have impaired interaction between T-cells and antigen-presenting cells (APCs)(68). Subsequently, they have low serum IgA & IgG; an elevated serum IgM; reduced IL-12 &IL-10, and are susceptible to opportunistic infections including PCP, cryptosporidiosis, and mycobacterial infections(12, 68). They are especially susceptible to disseminated histoplasmosis(69).

GATA2 is a member of the GATA family of transcription factors. It is widely expressed on early hematopoietic progenitors, megakaryocytes and in mast cell lineages(70). Patients with haploid insufficiency of the hematopoietic transcription factor GATA2 are recognized by a syndrome of monocytopenia with susceptibility to non-tuberculous mycobacterial (NTM) infections, often termed “MonoMAC”(71). These patients display monocytopenia; NK, B cell &CD4 lymphocytopenia, and neutropenia(71). A cohort of 57 patients with GATA2 mutations evaluated in the US had 16% with serious fungal infections, including invasive aspergillosis, disseminated histoplasmosis and recalcitrant mucosal candidiasis(72). Other significant findings in these patients include congenital lymphedema, pulmonary alveolar proteinosis, and predisposition to myelodysplastic syndrome or acute myeloid leukaemia(70). The table below is an adaptation from the excellent review by Lanternier et al, which highlights the main known genetic defects underlying some invasive mycoses (12).

Table 3: - Genetic defects underlying IFD

MONOGENIC DISORDERS ASSOCIATED WITH INVASIVE FUNGAL DISEASES				
GENETIC DEFECT	***INHERITANCE***	***IMMUNOLOGICAL PHENOTYPE***	***FUNGAL INFECTION***	***CLINICAL AND ASSOCIATED FEATURES***
CD40 Ligand deficiency (Hyper IgM syndrome)	X-linked recessive	Low s-IgA & IgG; elevated serum IgM; reduced IL-12 &IL-10	Histoplasmosis	Disseminated histoplasmosis with esophageal ulcers, lymphadenitis, pulmonary, and bone marrow involvement, CGD
GATA2 Deficiency	Heterozygous familial/sporadic with multiple hit sites	Monocytopenia, NK, B cell &CD4 lymphocytopenia, and neutropenia	Histoplasmosis Aspergillosis Candidemia	Disseminated histoplasmosis & aspergillosis, Recalcitrant candidemia, MonoMac, MDS, AML, viral infections
IL12Rβ1 Deficiency	Autosomal recessive	No response to IL-12, IL-17 and IL-23. Impaired IFN-γ production by T and NK cells	Candida Histoplasma Coccidioides Paracoccidioides	MSMD, non-typhoidal salmonellosis of unusual severity, Klebsiella, Nocardia, Leishmania, Klebsiella, Nocardia, Leishmania
IFNγ Receptor Deficiency	Autosomal recessive; or Autosomal dominant	IFNγR1 or R2 deficiency, both quantitative and qualitative	Histoplasmosis Candidemia Coccidioidomycosis	Disseminated fungemia, listeriosis, CMV, VZV, NTM
STAT1	AD GOF mutation	Decreased IL-17-producing T-cells decreased switched memory B-cells	Candidiasis (CMC) Histoplasmosis Cryptococcus Coccicioides	Aneurysm, autoimmune diseases, endocrine diseases, susceptibility to mycobacterial, viral, and bacterial infections
STAT3	AD LOF	Increased serum IgE, eosinophilia decreased IL-17-producing T-cells	Aspergillosis	Hyper IgE synd, disseminated fungemia, Eczema, scoliosis, pneumatocele, hyperextensibility, dysmorphic

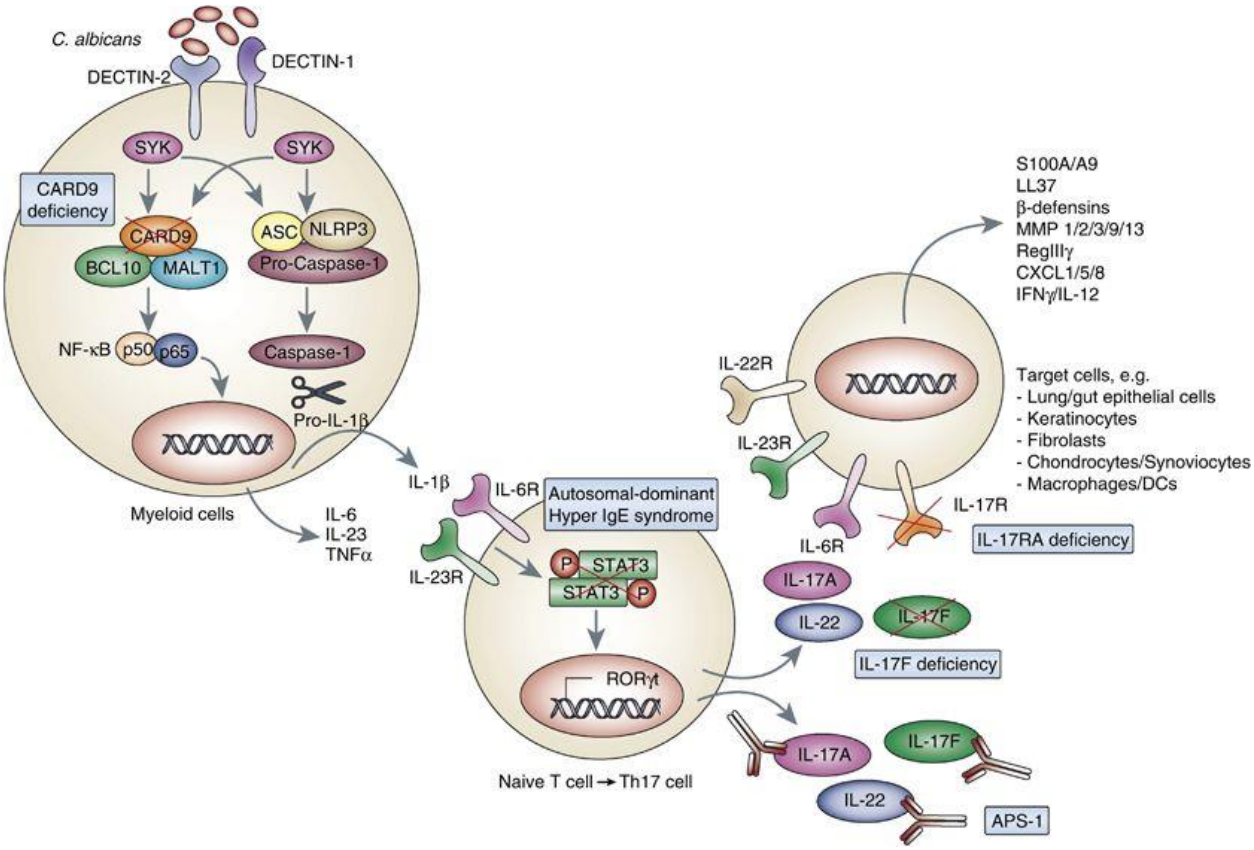
				facial features, retention of primary teeth
CARD9	AR	Decreased IL-17-producing T-cells, impairment of Candida killing by neutrophils	Candidemia	Dermatophytes, candida brain abscess

(CGD=chronic granulomatous disease, GATA2= a zinc finger transcription factor, MDS=myelodysplastic syndrome, AML=acute myeloid leukaemia, MSMD=mendelian susceptibility to mycobacterial diseases, CMV=cytomegalovirus, VZV=varicella zoster virus, NTM=non-tuberculous mycobacteria, STAT=signal transducer and activator of transcription, AD LOF=autosomal dominant loss of function gene, CARD9=caspase recruitment domain-containing protein 9, AR=autosomal recessive)

Inborn errors of immunity associated with impaired Th-17-mediated immunity are many. For example, in STAT1 AD GOF mutations, there is impaired dephosphorylation of STAT1, leading to a persistent 'on' switch after stimulation with IFN-γ, IFN-α/β, and IL-27(3). This, in turn, inhibits TH-17 cells and IL-17A, IL-17F and IL-22 production and leads to increased fungal susceptibility(3). This is thus associated with both minor and invasive forms of *Candidiasis* (CMC), *Histoplasmosis, Cryptococcosis* and *Coccicioides* infections(26). STAT3 AD LOF mutations have impaired RORγt induction in response to IL-1β, IL-6, and IL-23, causing severe deficiency of IL-17-producing effector cells(73). These are associated with increased serum IgE levels (hyper IgE syndrome), eczema, disseminated Aspergillosis, recurrent staphylococcal cold abscesses, pneumonia, joint hyperextensibility, skeletal abnormalities and pathological fractures, delayed dental deciduation, coronary artery aneurysms and several congenital dysmorphic features(73). CARD9 is a member of the CARD protein family, which is defined by the presence of a characteristic caspase-associated recruitment domain (CARD)(74). CARD9 mediates signals from pattern recognition receptors (PRRs), e.g. Dectin 1; to downstream signalling pathways, thus activating appropriate pro- and anti-inflammatory pathways to induce effective clearance of infections(74). CARD9 AR deficiency has been associated to both chronic mucocutaneous and disseminated candidiasis, including candida brain abscesses; as well as inflammatory diseases e.g., Ankylosing spondylitis and inflammatory bowel disease(12, 13, 74).

Figure 5 below serves to highlight some of the known molecular defects underlying invasive fungal diseases(75).

Figure 5:-Molecular defects underlying invasive fungal disease



(CARD9= caspase recruitment domain-containing protein 9, STAT3= signal transducer and activator of transcription)

Site specific disease susceptibilities

Certain monogenic abnormalities are associated with site-specific invasive fungal diseases. The table below is an adaptation from Lanternier et al, who looked at various examples of such site-specific invasive fungal diseases, with their corresponding primary immune aberrations.

Table 4:- Site-specific fungal disease and associated monogenic disorders

INVASIVE FUNGUS	***SITE OF INFECTION***	***MONOGENIC DISORDER(S)***	***CLINICAL ASSOCIATIONS***
Invasive aspergillosis	Pulmonary disease	X-linked CYBB mutations AD HIES (STAT3 def), SCN, CD18 def AD GATA2 deficiency	CGD, pneumatocysts, recurrent bacterial & mycobacterial infections
Invasive candidiasis	Cerebrovascular disease Mucocutaneous disease	AR CD18 deficiency CARD9 deficiency	Candida meningitis, CGD, CMC
Deep dermatophytosis	Invasion of the dermis and hypodermis	CARD9 deficiency	Idiopathic deep dermatophytosis

Invasive cryptococcosis	Cerebrovascular disease	Autoantibodies against GM-CSF or against IFN-γ X-linked CD40L deficiency AD GATA2 deficiency	Meningo-encephalitis disseminated NTM
Pneumocystosis	Pulmonary disease	X-linked CD40L def SCID, AR MHC class II deficiency, AR DOCK8 deficiency X-linked NF-kB Essential Modulator (NEMO) deficiency AR CARD11 deficiency	Pneumocystis jirovecii pneumonia
Disseminated histoplasmosis	Reticulo-endothelial system, and brain	Interleukin-12Rb1 deficiency, IFN-gR1 deficiency, STAT1 GOF mutation	Severe infections of lymph nodes, liver, spleen, mucosae, bone marrow and brain
Disseminated coccidioidomycosis	Pulmonary and cerebrovascular disease	Interleukin-12Rb1 deficiency, IFN-gR1 deficiency, STAT1 GOF mutation	Severe infections of lungs, CNS and bones

(CGD=chronic granulomatous disease, AD HIES=autosomal dominant hyper-IgE syndrome, CARD=caspase recruitment domain-containing protein, AR=autosomal recessive, CMC=chronic mucocutaneous candidiasis, MHC=major histocompatibility complex)

Patients with the pulmonary form of invasive aspergillosis have an NADPH oxidase complex defect in phagocytes, mostly due to X-linked CYBB mutations(76). This leads to defects in reactive oxygen species production and effectively, impaired respiratory burst function necessary for effective fungal killing by effector cells(77). In CARD9 deficiency, patients have increased susceptibility for disseminated candida (including candida meningitis) and deep dermatophytosis due to impaired production of pro-inflammatory cytokines, especially IL17(74). Cases of disseminated coccidioidomycosis involving the lungs, CNS and bones; and disseminated histoplasmosis involving the lymph nodes, liver, spleen, mucosae, bone marrow and brain; have been described in patients with STAT1 GOF mutations; GATA2, DOCK8, XL CD40L deficiencies(12). In these patients, there's impaired pro-inflammatory signalling related to IF deficiencies in the IFN -gamma/ interleukin-12 axis(12).

Polygenic susceptibility to IFD

Complex interplay between gene polymorphisms and genetic errors have been implicated in an immunodeficiency phenotype and an increased incidence of opportunistic fungal diseases. These genetic variations, known as single-nucleotide polymorphisms (SNPs), have been proposed to affect both innate and adaptive immunity to IFD such as Candida and Aspergillus(78). For example, IFN-γ 874T>A polymorphism as a single SNP or as a combined

deficiency with an SNP in Toll-the like receptor (TLR)-4 gene (1063A>G) has shown a trend to cause increased susceptibility to invasive aspergillosis(79). SNPs affecting IL-10 and IL-12B genes are significantly associated with persistent candidemia, owing to decreased proinflammatory cytokine concentrations(80).

Future areas of research

Whereas cases of invasive fungal infections abound in many population groups, the vast majority of the cases are found in the context of secondary immune deficiency. The bulk of research into these infections has been done outside of Africa. African populations have marked ethnic and genetic diversity. This is an area of potential active studies, in order to unravel possible genetic aberrations that may predispose to chronic or recurrent severe infections.

Conclusion

Isolated cases of monogenic disorders predisposing to recurrent fungal and other infections, have also recently been published in Sub-Saharan Africa (43). The possibility of new discoveries in the African populations, of novel genes and their variants as the key determinants of recurrent or severe disease, should inspire more active scientific interest in the various African populations. This is especially important for invasive fungal diseases, as these are associated with significant morbidity and mortality. This case series serves as a starting point in response to this need.

APPENDIX

Table 1

Criteria for proven invasive fungal disease except for endemic mycoses

Analysis and specimen	Mould[a]	Yeasts
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy from a normally sterile site (other than mucous membranes) showing yeast cells—for example, *Cryptococcus* species indicated by encapsulated budding yeasts or *Candida* species showing pseudo hyphae or true hyphae[c]
Culture		
Sterile material	Recovery of a mould or "black yeast" by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding bronchoalveolar lavage fluid, a cranial sinus cavity specimen, and urine	Recovery of a yeast by culture of a sample obtained by a sterile procedure (including a freshly placed [<24 h ago] drain) from a normally sterile site showing a clinical or radiological abnormality consistent with an infectious disease process
Blood	Blood culture that yields a moul[d] (e.g., *Fusarium* species) in the context of a compatible infectious disease process	Blood culture that yields yeast (e.g., *Cryptococcus* or *Candida* species) or yeast-like fungi (e.g., *Trichosporon* species)

Analysis and specimen	Mould[a]	Yeasts
Serological analysis: CSF	Not applicable	Cryptococcal antigen in CSF indicates disseminated cryptococcosis

[a]If culture is available, append the identification at the genus or species level from the culture results. [b]Tissue and cells submitted for histopathologic or cytopathologic studies should be stained by Grocott-Gomorri methenamine silver stain or by periodic acid Schiff stain, to facilitate inspection of fungal structures. Whenever possible, wet mounts of specimens from foci related to invasive fungal disease should be stained with a fluorescent dye (e.g., calcofluor or blankophor). [c]Candida, Trichosporon, and yeast-like Geotrichum species and Blastoschizomyces capitatus may also form pseudohyphae or true hyphae. [d]Recovery of Aspergillus species from blood cultures invariably represents contamination.

Table 2

Criteria for probable invasive fungal disease except for endemic mycoses

Host factors[a]
Recent history of neutropenia (<0.5 × 10^9 neutrophils/L [<500 neutrophils/mm^3] for >10 days) temporally related to the onset of fungal disease
Receipt of an allogeneic stem cell transplant
Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks
Treatment with other recognized T cell immunosuppressants, such as cyclosporine, TNF-α blockers, specific monoclonal antibodies (such as alemtuzumab), or nucleoside analogues during the past 90 days
Inherited severe immunodeficiency (such as chronic granulomatous disease or severe combined immunodeficiency)
Clinical criteria
Lower respiratory tract fungal disease[c]
The presence of 1 of the following 3 signs on CT:
Dense, well-circumscribed lesions(s) with or without a halo sign

Air-crescent sign

Cavity

Tracheobronchitis

Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis

Sinonasal infection

Imaging showing sinusitis plus at least 1 of the following 3 signs:

Acute localized pain (including pain radiating to the eye)

Nasal ulcer with black eschar

Extension from the paranasal sinus across bony barriers, including into the orbit

CNS infection

1 of the following 2 signs:

Focal lesions on imaging

Meningeal enhancement on MRI or CT

Disseminated candidiasis

At least 1 of the following 2 entities after an episode of candidemia within the previous 2 weeks:

Small, target-like abscesses (bull's-eye lesions) in liver or spleen

Progressive retinal exudates on ophthalmologic examination

Mycological criteria

Direct test (cytology, direct microscopy, or culture)

Mold in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:

Presence of fungal elements indicating a mold

Recovery by culture of a mold (e.g., *Aspergillus, Fusarium*, Zygomycetes, or *Scedosporium* species)

Indirect tests (detection of antigen or cell-wall constituents)[e]

Aspergillosis

Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF

Invasive fungal disease other than cryptococcosis and zygomycoses

β-d-glucan detected in serum

NOTE. Probable IFD requires the presence of a host factor, a clinical criterion, and a mycological criterion. Cases that meet the criteria for a host factor and a clinical criterion but for which mycological criteria are absent are considered possible IFD.

[a]*Host factors are not synonymous with risk factors and are characteristics by which individuals predisposed to invasive fungal diseases can be recognized. They are intended primarily to apply to patients given treatment for malignant disease and to recipients of*

allogeneic hematopoietic stem cell and solid-organ transplants. These host factors are also applicable to patients who receive corticosteroids and other T cell suppressants as well as to patients with primary immunodeficiencies.
[b]*Must be consistent with the mycological findings, if any, and must be temporally related to current episode.*
[c]*Every reasonable attempt should be made to exclude an alternative etiology.*
[d]*The presence of signs and symptoms consistent with sepsis syndrome indicates acute disseminated disease, whereas their absence denotes chronic disseminated disease.*
[e] *These tests are primarily applicable to aspergillosis and candidiasis and are not useful in diagnosing infections due to Cryptococcus species or Zygomycetes (e.g., Rhizopus, Mucor, or Absidia species). Detection of nucleic acid is not included, because there are as yet no validated or standardized methods.*

Table 3

Criteria for the diagnosis of endemic mycoses

Diagnosis and criteria
Proven endemic mycosis
In a host with an illness consistent with an endemic mycosis, 1 of the following:
Recovery in culture from a specimen obtained from the affected site or from blood
Histopathologic or direct microscopic demonstration of appropriate morphologic forms with a truly distinctive appearance characteristic of dimorphic fungi, such as *Coccidioides* species spherules, *Blastomyces dermatitidis (*thick-walled broad-based budding yeasts), *Paracoccidioides brasiliensis* (multiple budding yeast cells), and, in the case of histoplasmosis, the presence of characteristic intracellular yeast forms in a phagocyte in a peripheral blood smear or in tissue macrophages
For coccidioidomycosis, demonstration of coccidioidal antibody in CSF, or a 2-dilution rise measured in 2 consecutive blood samples tested concurrently in the setting of an ongoing infectious disease process
For paracoccidioidomycosis, demonstration in 2 consecutive serum samples of a precipitin band to paracoccidioidin concurrently in the setting of an ongoing infectious disease process

Diagnosis and criteria

Probable endemic mycosis

Presence of a host factor, including but not limited to those specified in table 2, plus a clinical picture consistent with endemic mycosis and mycological evidence, such as a positive *Histoplasma* antigen test result from urine, blood, or CSF

NOTE. *Endemic mycoses include histoplasmosis, blastomycosis, coccidioidomycosis, paracoccidioidomycosis, sporotrichosis, and infection due to Penicillium marneffei. Onset within 3 months after presentation defines a primary pulmonary infection. There is no category of possible endemic mycosis, as such, because neither host factors nor clinical features are sufficiently specific; such cases are considered to be of value too limited to include in clinical trials, epidemiological studies, or evaluations of diagnostic tests.*

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CHAPTER 2: PUBLICATION-READY MANUSCRIPT

Invasive Fungal Disease in HIV-negative South African Patients – functional immune work-up in search of primary immunodeficiency

[1]Onyango Vonwicks Czelestakov, [1]Kathryn Manning, [2]Steven Makatsa Mohau, [2]Claire Hoving, [2]Wendy Burgers, [3]Sipho Dlamini, [4]Jonny Peter

Abstract

Background

The majority of invasive fungal disease in South African hospitals is HIV-related or associated with another secondary immunodeficiency e.g. haematopoietic stem cell transplant. After excluding secondary immunodeficiency, a detailed immune work-up can lead to a diagnosis of primary immunodeficiency.

Objective

To detail an appropriate step-wise immunological work-up for a series of patients with invasive fungal diseases and possible underlying primary immune deficiency.

Methods

Detailed review of all culture- or histologically confirmed cases of invasive fungal disease (IFD) at Groote Schuur Hospital between 2007-2017. Step-wise immunological work-up of IFD patients with no secondary immunodeficiency. Clinical characteristics and step-wise immunological profiles were evaluated.

Results

Sixty-seven adults with IFD were identified; 72% (48/67) were HIV-related. 8/19 HIV-negative cases were either deceased (4) or lost-to-follow-up (4). Work-up of the remaining 11 cases found five with non-HIV secondary immunodeficiencies (Lupus, liver transplant, end-stage renal failure and haematological malignancy). A primary immunodeficiency was suspected in six cases, but 1 case of cutaneous sporotrichosis was excluded; with five cases (4 with disseminated *Cryptococcus neoformans* and 1 with cerebral aspergillosis) undergoing detailed immune work-up. A case of idiopathic CD4 lymphopenia was diagnosed; but all other cases had no evidence of neutrophil or a cell-mediated immune defect; including investigations of naïve and memory T-cell subsets and cytokine responses to PHA and candida. All cases were noted to have low baseline vaccine responses and Vitamin D deficiency.

Conclusion

Invasive fungal disease is predominantly associated with HIV and secondary immunodeficiency in South Africa. Known primary immunodeficiencies can be identified with basic immune work-up; but no obvious functional immune defect is evident in the majority of these cases.

Key words: invasive fungal disease, primary immune deficiency, secondary immune suppression

Word count: 3633

Correspondence: Jonny.Peter@uct.ac.za

Division of Allergology and Immunology; Department of Medicine, University of Cape Town, Groote Schuur Hospital, Observatory, Cape Town, 7925

Full author details

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[1]Onyango Vonwicks Czelestakov, [1]Kathryn Manning, [2]Steven Makatsa Mohau, [2]Claire Hoving, [2]Wendy Burgers, [3]Sipho Dlamini, [4]Jonny Peter

[1]OV: Department of Medicine, University of Cape Town, Old Main Building, J-Floor; Groote Schuur Hospital, Observatory, Cape Town, South Africa, 7925

drvonczelo@gmail.com

[1]KM: Department of Medicine, University of Cape Town, Old Main Building, J-Floor; Groote Schuur Hospital, Observatory, Cape Town, South Africa, 7925

kathryn.manning@uct.ac.za

[2]SM: Division of Virology, Department of Pathology, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory, Cape Town, South Africa, 7925

MKTMOH003@myuct.ac.za

[2]CH: Division of Virology, Department of Pathology, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory, Cape Town, South Africa, 7925

Clare.Hoving@uct.ac.za

[2]WB: Division of Virology, Department of Pathology, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory, Cape Town, South Africa, 7925

wendy.burgers@uct.ac.za

[3]SD: Division of Infectious Diseases and HIV Medicine; Department of Medicine, University of Cape Town, Groote Schuur Hospital, Observatory, Cape Town, 7925

sk.dlamini@uct.ac.za

Corresponding Author: [4]JP: Division of Allergology and Immunology; Department of Medicine, University of Cape Town, Groote Schuur Hospital, Observatory, Cape Town, 7925

Jonny.Peter@uct.ac.za

Introduction

Innate and adaptive immune mechanisms often restrict fungal infections to the skin and mucosa. Invasive fungal infections occur whenever there is failure of these immune mechanisms to contain invading fungal elements; often leading to severe and disseminated disease. The main causes of invasive fungal disease (IFD) are secondary immunodeficiency; particularly HIV/AIDS. Disseminated mycoses are AIDS-defining opportunistic illnesses. Cryptococcal meningo-encephalitis is the most common fungal infection in HIV[1]. Other causes of secondary immunodeficiency associated with severe, recurrent or persistent fungal infections include diabetes, cancers, chemotherapy, bone marrow and solid organ transplant and immunosuppressive drug regimens, as well as auto-immune diseases, e.g. . systemic lupus erythromatosus [SLE][2].

A small number of patients present with IFD in the absence of HIV, or any other common secondary immunodeficiency. These patients display widely variable clinical manifestations of IFD, with different degrees of morbidity and mortality. Whether these patients have an undiagnosed secondary immune deficiency, or an already described primary immunodeficiency, or novel genetic mutations with an increased susceptibility to invasive fungal disease, remains unanswered. Several primary immune deficiencies have been described in patients with IFD including: homozygous mutations in caspase recruitment domain-containing protein 9 (CARD9) with deep dermatophytosis of central nervous system (CNS) and gastrointestinal (GIT) system[3]; isolated CD4 lymphopenia[4]; severe congenital neutropenia; leucocyte adhesion deficiency, and inborn errors of the phagocytic respiratory burst activity, necessary for fungal killing. Endemic mycoses have also been linked to congenital errors of IFN-γ/IL-12 signalling pathway and acquired auto-antibodies to both IFN-γ and GM-CSF[5].

To date, we have not found any African studies that have conducted in-depth clinical and immunological assessments in HIV-negative cohorts of IFD without an obvious underlying secondary immunodeficiency. This case series is the result of our study to systematically review the immunological basis of cases of microbiological or histology-proven IFD in adults (>18 years) diagnosed at Groote Schuur Hospital - a tertiary hospital in Cape Town, South Africa, between 2007-2017. To the best of our knowledge, this is the first such study conducted on a series of adult African patients.

Methods

Work-up of invasive fungal disease: Secondary to primary immunodeficiency

The infectious diseases clinic maintains an electronic database of all patients treated for IFD at Groote Schuur hospital, thereby allowing us to identify all patients with culture and/or histologically proven IFD treated between 31st August 2007 to 30th September 2017. Adult patients (>18 years) from this database were systematically reviewed for confirmed secondary immunodeficiency e.g. HIV status. All patients without a known secondary immunodeficiency were contacted by phone or community health worker. The study was approved by the University of Cape Town Human Research Ethics Committee (HREC 010/2017). Traceable patients were investigated for a previously unidentified secondary immunodeficiency; followed by a detailed immune work-up (see online appendix for details of laboratory and immunological work-up)

Non-routine flow cytometry investigations

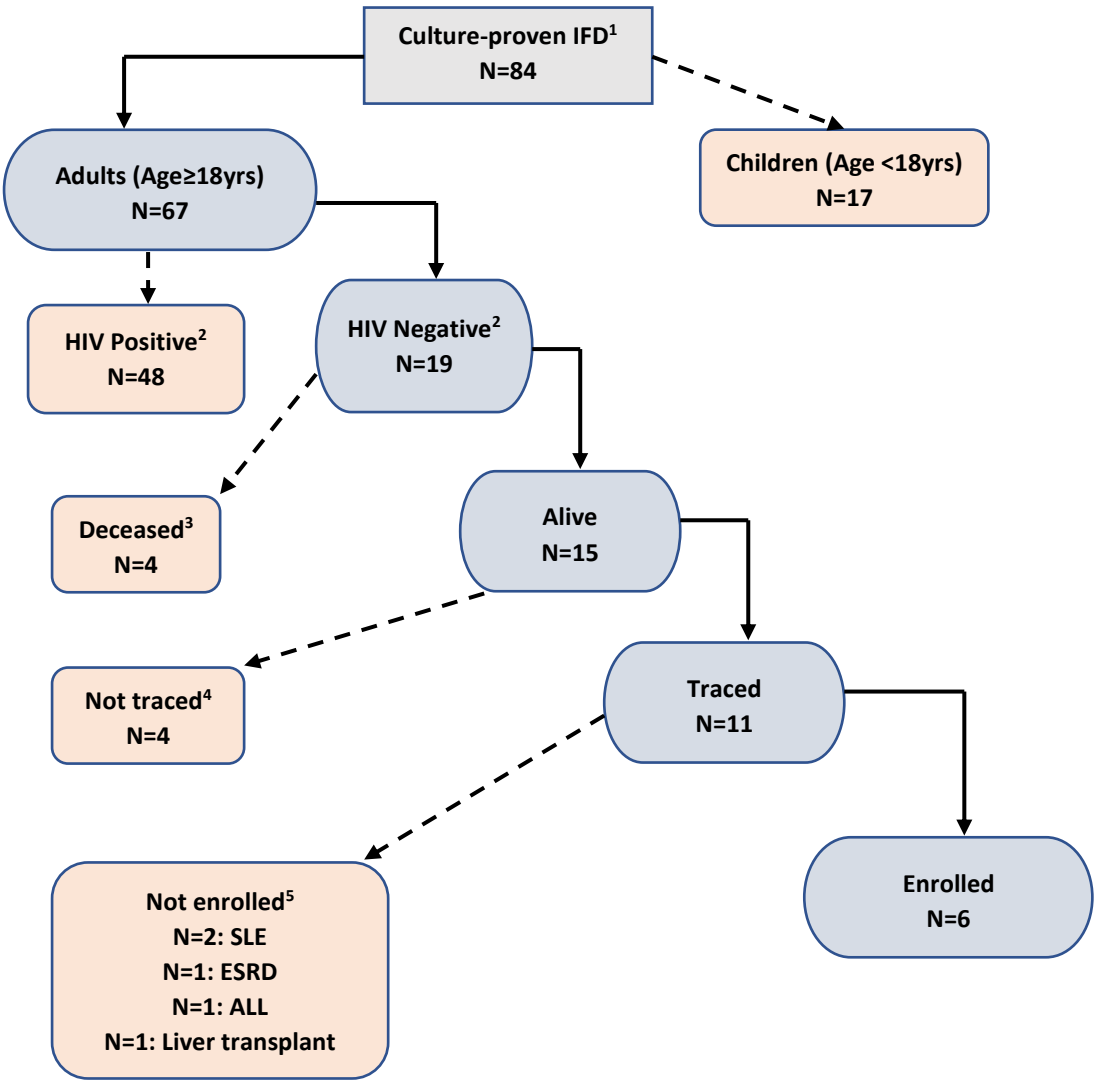
Peripheral blood mononuclear cells (PBMCs) from each patient was subjected to cytokine stimulation assays using mycobacterium tuberculin (MTB) lysate and mitogens phytohemagglutinin (PHA) and phorbol myristate acetate (PMA). These were then run by flow cytometry. 4 healthy control samples were included, while non-stimulated blood was used as negative control. Analyses included T cell subsets, cytokines, and T cell memory phenotype.

Results

Figure 1 shows a flow diagram of the 84 IFD cases in the electronic database. Of these, 79.7% (67/84) were adults (≥18 years); and 72% (48/84) were HIV-positive. Of the remaining 19 HIV-negative patients; four were deceased and four were not traceable. We investigated 13% (11/84) patients in further detail and found that five had a previously unidentified secondary immunodeficiency including: two had systemic lupus erythematosus (SLE), one had end stage renal disease (ESRD), one had acute lymphoblastic leukaemia (ALL), and one was a post liver transplant patient on immunosuppressants. Hence, only six patients were considered to warrant detailed immunological work-up for a possible underlying primary immunodeficiency. One case of cutaneous sporotrichosis was excluded from this cases series, and further detailed case vignettes and laboratory data are presented on five cases.

(See appendix 1, online supplement, showing the infectious work-up at the time of IFD presentation.)

Figure 1: Flow chart showing patient enrolment



KEY 1
SLE: Systemic lupus erythromatosus
ESRD: End-stage renal disease
ALL: Acute lymphoblastic leukaemia

KEY 2
1: Diagnosis of IFD made on fungal cultures (blood and tissue) and/or histology
2: Diagnosis of HIV made on ELISA tests
3: Patients already deceased before study enrolment
4: Patients not traceable
5: Patients found to have secondary immune deficiency on further review

Clinical case vignettes

Case 1

A 49-year-old male security guard, who presented to hospital with 4-months of recurrent headaches and 3rd, 4th, and 6th right-sided cranial nerves palsies. He had no relevant past medical history. Family history included deceased father and mother from pulmonary TB and suspected meningitis respectively; his teenage son also has a pulmonary TB history. MRI brain showed a left cavernous sinus mass, that progressed to a left temporal lobe abscess communicating with the para-cavernous space *(See the corresponding images in Figure 2).* The abscess was debrided and drained, with both histology and culture showing *Aspergillus fumigatus*. He was treated with induction intravenous voriconazole (2 weeks), with maintenance oral voriconazole. An incidental diagnosis of neurosyphilis was also made and treated with intravenous penicillin. He has had no further IFD in the subsequent four years, and only a mild residual 6th cranial nerve paresis.

Case 2

A 68-year old male pensioner, who presented to hospital with a 3-month history of dysphagia, hoarse voice and weight loss. His past medical history included recurrent tinea corporis and herpetic shingles in puberty; and hypertension. Family history includes a grandchild who is asthmatic, and another with eczema. Chest x-ray and chest CT scans showed a well-circumscribed mass in his left upper lobe *(See the corresponding images in Figure 2)*. He had partial pneumonectomy of the left upper lobe, and the culture and histopathology of the tissue confirmed *Cryptococcus neoformans*. The peri-bronchial lymph node tissue was also positive for *M. tuberculosis* on histology. Given his repeatedly negative serum CLAT, a clinical decision was made not to treat him with antifungals following the operation. He was fully treated for the TB, and mild anaemia of chronic illness. He has had no further IFD in the subsequent four years.

Case 3

A 36-year old female call centre employee, who presented with a 3-week history of left breast swelling with inflammatory features. This was found to be an abscess *(See the corresponding images in Figure 2).* She had incision and drainage with debridement of the infected breast, and tissue samples confirmed *Cryptococcus neoformans* on culture and histopathology. Her serum CLAT was positive. Moderate eczema was her only comorbidity; family history includes atopy, and her 4-year old son with recurrent tinea corporis. She was treated with 8 weeks of intravenous Amphotericin B, and subsequently maintained on oral Fluconazole. During longstanding azole therapy, she developed a normocytic anaemia, leukopenia ($3.0x10^9$/L), neutropenia($1.4x10^9$/L) and lymphopenia($0.9x10^9$/L), which have resolved without treatment on follow-up. She has had no further IFD in the subsequent three years.

Case 4

A 41-year old male casual labourer; whom presented with a 3-month history of right breast swelling and recurrent headaches, with no constitutional symptoms and normal neurological exam. His past medical history includes recurrent tinea corporis in childhood; and polysubstance abuse (smoking of methamphetamine, mandrax and tobacco). Family history includes a brother who had recurrent tinea corporis, and a daughter with eczema and asthma. He was found to have a persistent low CD4 count of 218 cells/µl, absolute lymphopenia, with positive serum and CSF CLAT. He had a normal chest x-ray and brain CT scan. The breast

swelling was an abscess, and he had diagnostic aspiration and subsequent incision and drainage with debridement of infected tissue. All these confirmed *Cryptococcus neoformans* on culture and histopathology. He was started on intravenous Amphotericin B but left the hospital against medical advice after 10 days of treatment. He has since been on maintenance high dose oral fluconazole. His absolute lymphopenia resolved on follow-up blood tests. He has remained stable during two years of follow-up, but his CD4 counts have remained <300 cells/µl.

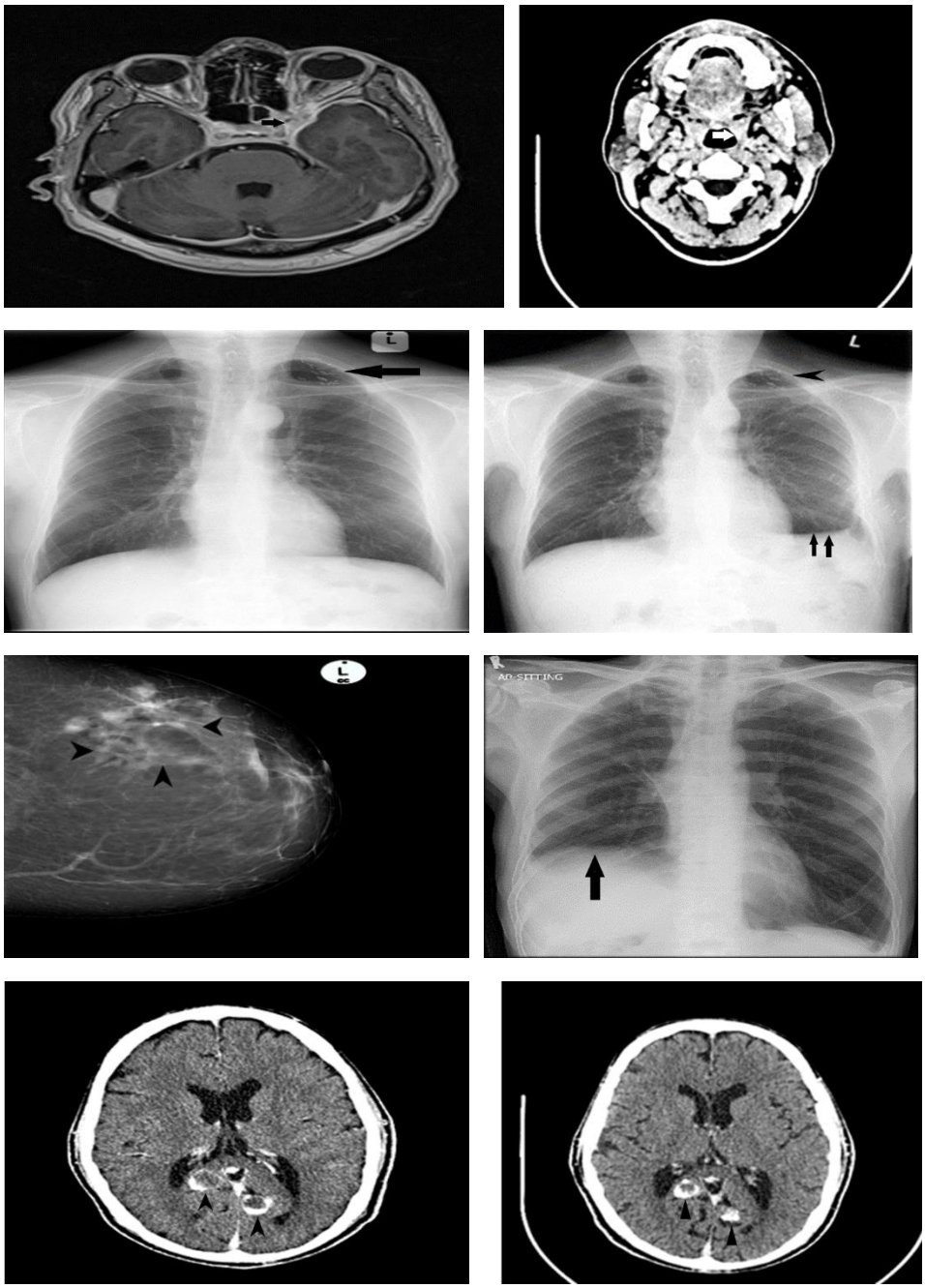
Case 5

A 44-year old male laboratory technician; whom presented in 2007 with a 2-week history of cough with haemoptysis, and no constitutional symptoms. His past medical history included chronic tobacco smoking and a spontaneous pneumothorax at age 39. Family history included a sister who died of unknown cause at age 2-3 years; and a daughter with penicillin allergy. TB work up was negative. His CXR and CT chest showed a well-circumscribed mass in his right lung hilar *(See the corresponding images in Figure 2)*. He had excision of the lung mass (partial pneumonectomy), with samples confirming *Cryptococcus neoformans* on culture and histopathology. He was treated with 6 weeks of intravenous Amphotericin B and subsequently has been on long term oral fluconazole. He developed focal seizures in 2012, and a CT brain showed cerebral parenchymal lesions, which have remained static on serial scans. His serum and CSF CLAT were both positive then. His cerebral lesions have been presumed to be cryptococcomas and deemed to be the cause of his seizures; and he's on long-term antiepileptic therapy, plus ongoing oral fluconazole. He had also been managed with tapering doses of oral steroids for about 2 years following the diagnosis of the cerebral lesions; but this was stopped when he developed avascular necrosis of the left femur head, with total hip replacement in 2014. Now, age 55, he is stable, with subsequent CSF and serum CLAT negative on serial reviews; and is on regular follow up at the ID clinic.

Figure 2 below shows a collage of the image series for the case vignettes

(See appendix 2, online supplement, for comprehensive case vignettes with expanded image series.)

Figure 2: Collage of Image series



KEY: IMAGING STUDIES OF THE VARIOUS PATIENTS

Row 1: **Case 1** (L) *MRI brain showing enhancing left cavernous sinus mass* ***(black arrow)*** *;(R) CT showing near complete resolution- normal left cavernous sinus with mild residual expansion only* ***(white arrow).***

Row 2: **Case 2** (L)CXR *showing spiculated left upper lobe mass (black arrow) ;(R) CXR post excision of the left upper lobe mass* ***(black arrow-head),*** *with loss of lung volume* ***(double black arrows)***

Row 3: **Case 3** (L) *Mammogram showing increased architectural distortion and density in the upper outer quadrant* ***(black arrow-heads).*** *Sonography confirmed a sinus with a linear fluid collection with reactive lymph node. No masses.* **Case 5** *(R) CXR done in 2012 following right partial pneumonectomy with volume loss* ***(black arrow)***

Row 4: **Case 5** (L) *CT brain in 2012 and (R) in 2018 showing unchanged cerebral parenchymal lesions with peripheral enhancement* ***(black arrow-heads)***

Patient Clinical Characteristics

At the time of IFD diagnosis, two patients had no significant medical comorbidities. However, one patient (case 2) had hypertension, one (case 3) had eczema, and one (case 5) a history of active polysubstance abuse. All the diagnoses of IFD were made based on fungal culture and/or histopathology of body fluids and/or tissue specimens; 80% (4/5) of cases had invasive cryptococcal disease, caused by *Cryptococcus neoformans.* Sites of IFD included: i) Case 1 presented with intracerebral abscesses secondary to aspergillosis (organism was *Aspergillus fumigatus*); ii) Case 2 had a left lung upper lobe cryptococcoma; iii) Cases 3, 4 and 5 had disseminated cryptococcosis, involving the breast tissue, lung, cerebrum and isolated from blood.

(See appendix 2, online supplement, for comprehensive case vignettes with expanded image series.)

Baseline basic laboratory tests

Laboratory tests were done, on average, 2 years (for 3 patients) and 10 years (for 1 patient), since the completion of induction intravenous antifungal therapy; 4/5 patients were on maintenance oral fungal therapy at the time of the tests.

All the patients had normal renal and liver functions. Whereas one patient (case 3), had an absolute leukopenia of 3000 cells/µl (Reference ranges: 4000-1000 cells/µl), this was noted to have recovered in follow-up blood tests. Significantly, one patient (case 4) had persistent absolute lymphopenia of 620 cells/µl. (Reference ranges: Total lymphocytes=1000-4200 cells/µl). The rest of the basic baseline laboratory tests were unremarkable. (NB: All reference ranges as per South Africa National Health laboratory System).

(See appendix 3, online supplement, for comprehensive basic laboratory results)

Important Baseline Immunological Tests.

All the 5 patients had normal levels of total serum immunoglobulins (IgG, IgA and IgM), B-cells, NK cells, total complement levels and T-cell subsets (CD3 and CD8). One patient (case 4) had a low CD4 count of 218 cells/µl and inverted CD4 to CD8 ratio; CD4 lymphopenia (<300 cells/µl) persisted during two years of follow-up.

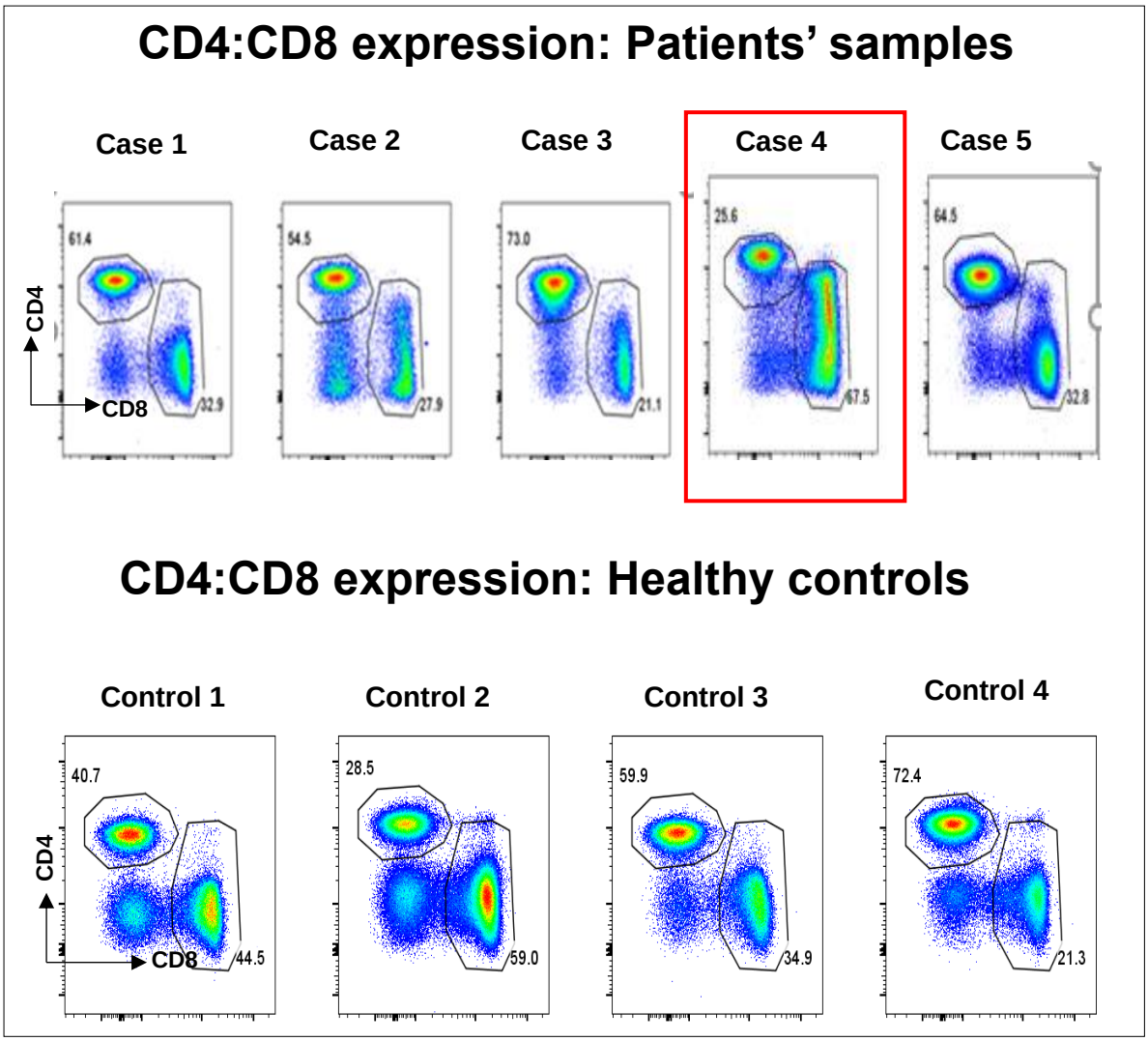
Whereas one patient (case 3) had normal expression of CD27 and CCR7 (markers of naïve and memory T cells, respectively), three other patients (case 1, 2 and 5)) had low levels; while one patient (case 4) had no expression of CCR7 at all. Nonetheless, all patients had normal stimulated and unstimulated CD4+ cytokine expression for IFN-γ, TNF-α, IL-22 and IL-17. When compared to normal transport controls, all patients had normal neutrophil oxidative burst results for the PMA and *E. coli* stimuli.

All the patients had low levels of recall antibody titers to childhood vaccines given as part of SA expanded program of immunisation (EPI): diphtheria (cases 3 and 5), tetanus (cases 1 and 2), and *Hemophilus influenza B* (cases 2, 3 and 5). Additionally, all the patients had low serum Vitamin D3 levels (median level of 52 nmol/L), with patients 2 and 4 having significant deficiency (mean level of 46.15 nmol/L).

Figure 3 shows flow cytometry plots of the CD4 cell deficiency in case 4.

(See appendix 4, online supplement, showing summaries of the important baseline immunological test results; and further flow cytometry plots for various cytokine assays)

Figure 3: Flow cytometry plots showing CD4 deficiency in case 4.



KEY: FLOW CYTOMETRY PLOTS

In healthy subjects, the ratio of CD4 to CD8 is at least 2:1; meaning that there are at least twice as many CD4 cells as there are CD8 cells. This is true for the healthy controls and Cases 1, 2, 3 and 5. However, Case 4 has an inverted CD4 to CD8 ratio. This is consistent with severe CD4 deficiency

Discussion

This is the first African study to evaluate a cohort of patients with IFD, with stratified basic and advanced immunology investigations. The key findings of our study include: i) the majority of IFD have an easily identifiable secondary immunodeficiency, most commonly advanced HIV/AIDS; ii) detailed immunological work-up can identify known primary immunodeficiencies e.g. idiopathic CD4 lymphopenia; iii) possible secondary contributing factors e.g. vitamin D3 insufficiency or suboptimal vaccine antibody titres that may contribute to increased fungal susceptibility could be detected and treated; and iv) the majority of cases had normal immune cell numbers and fungal-stimulated adaptive cytokine production. A whole genome sequencing approach is underway to screen for known or unknown monogenic primary immune deficiency.

In high prevalence settings, advanced HIV/AIDs is the single most common risk factor, and *Cryptococcus neoformans* is the most common organism[6]. In Brazil, for example, Prado et al report about 51% of deaths from systemic mycosis in HIV positive patients was due to invasive cryptococcosis[7]. In Sub-Saharan Africa, one million cases of disseminated cryptococcosis are reported annually, with over 600,000 deaths per year among HIV infected patients(1). In South Africa, Jarvis et al showed cryptococcal meningitis accounting for 63% of all cases of meningitis in HIV patients, with a mortality of almost 50% despite treatment[8].

Despite low CD4 count being the main risk of disease, not all HIV patients with low CD4 counts develop cryptococcal disease, or IFD. In fact, infection rates differ between patients with similar levels and durations of CD4 lymphocytopenia[9]; reflecting the complexity of anti-fungal immunity, and possible compensation of barrier and innate immunity, as well as differential depletion of TH1 and TH17 subsets with advancing HIV. Other causes of secondary immune deficiency identified among IFD patients in this study included: diabetes, autoimmune diseases (e.g. SLE); major organ failure, malignancy (solid organ and haematological), and organ transplant. These diagnoses highlight the need for thorough work-up that should include mandatory HIV testing, fasting blood sugars and $HBA1_C$, ANA, possible BMAT, and organ-function tests e.g. renal and liver function.

Our detailed immune work-up identify partial to severe vitamin D deficiency and poor specific vaccine titres in all cases. Vitamin D modulates both innate and adaptive immunity via complex transcriptional mechanisms[10]. The development of active TB has been linked to vitamin D deficiency and seasonal changes in Vitamin D levels[11,12]. No studies have demonstrated a link with increased susceptibility to fungal disease; but there are *in vitro* studies that demonstrate beneficial effects of Vitamin D3 (active form) on Th1 cytokines production[13,14]. Thus, it is reasonable to suggest this may have a small contributory role for the association noted in this study. However, we are unable to say whether this association is causal or a consequence of infection. Further studies are needed to answer this. Furthermore, vitamin D deficiency is easily amenable to treatment.

Antibody deficiency is not known to increase risk of fungal disease, however measurement of recall antibody titres to various childhood vaccines may be a marker for impaired humoral immune function. None of our cases had an increased burden of bacterial infections, but 80% had low specific antibody titres. Cases will receive vaccine boosters with measurement of subsequent humoral responses, confirming an intact endogenous antibody response. In addition, we noted that 80% of IFD cases were atopic; higher than the general population prevalence. Hyper IgE syndrome is well-described to link severe allergic disease and fungal

susceptibility. Although none of the cases had this syndrome, several genetic pathways overlap between allergic and fungal diseases[15].

Several primary immunodeficiencies (PIDs) have been described to underpin (increased) susceptibility to IFD. In North Africa, Lanternier et al reported on a series of 17 IFD patients, ages 25 to 75 years; all of whom were diagnosed with autosomal recessive CARD 9 deficiency with deep dermatophytosis; 15 of these patients shared a common ancestral gene[3]. There are no studies from sub-Saharan Africa detailing PIDs underlying IFD or endemic mycosis such as *Emmonsia spp*. We identified one patient with idiopathic CD4 lymphocytopenia – a known PID associated with disseminated cryptococcal disease[4]. Several gene mutations including hypomorphic RAG1, Heterozygous UNC119 and MAGT1 mutations have been found as the cause for a CD4 lymphopenia immune phenotype[16-18]. We are pursuing a genetic diagnosis for this case. An interesting additional contributing factor in this case is the role of substance abuse. Animal studies have demonstrated that recreational drugs can alter T-cell function[19]. However, Chao et al conducted a longitudinal study whose results indicated that use of these substances does not adversely affect the numbers and percentages of circulating CD4 or CD8 T cells in either HIV-uninfected or -infected patients[20]. Functional immune abnormalities, either of phagocyte or T-cell function (proliferation and cytokine responses), have been identified in patients with known PIDs associated with IFD, including CARD9, STAT1, STAT3, CD40L, IL12Rβ1 and IFNγRD[21-23]. Thus, the normal functional immune responses in our cases suggest that none of these patients have another a known monogenic PID.

This study is a case series of only five patients and thus has limitations. However, we have included all patients at a single centre with IFD over a ten-year period, thus providing practicing clinicians with a sense of the relative contributions of secondary immunodeficiency and appropriate investigations. The study relied on downstream contact of patients from a database, thus eight potential patients with an underlying immune defect were not included (4 dead, and 4 untraceable); these patients may have increased the detection of PID.

Which patients should be referred for Immunological work-up?

Patients who have individual or family history of **s**evere, **p**ersistent, **u**nusual, and **r**ecurrent infections (**SPUR** algorithm) should be referred for a full immunological work-up[24,25]. All patients with IFD should be investigated. This should include: i) a detailed family and clinical history; ii) exclusion of secondary immunodeficiencies e.g. HIV, DM, autoimmunity and malignancy, iii) step-wise immune work-up starting with immune cell numbers – the full blood count differential identified the case of idiopathic CD4 lymphopenia; proliferative T-cell and phagocyte function. Investigations for other possible contributing factors such as allergy and vitamin D status, allows optimisation of factors easily amendable to treatment. Patients should be referred to sub-specialist immunology or infectious disease centres for more specialised cytokine and genetic studies if warranted.

Conclusion

We have described the clinical and immunological characteristics of five South African patients with invasive fungal diseases, and no secondary immunodeficiency. We have identified one known PID phenotype and possible contributing factors amenable to intervention through detailed work-up. Further planned genetic study may identify novel gene defects associated with increased susceptibility to fungal disease in African populations. We also recommend further studies to look into possible relationship between vitamin D deficiency and IFD.

Declarations

Ethical approval

The study was approved by the University of Cape Town Human Research Ethics Committee ***(HREC protocol 010/2017).***

Consent for publication

None

Competing interests

None

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Author contributions

OV, SD, and JP designed the study, wrote the protocol, collected the data, analysed it, and prepared the manuscript. KM was involved in writing the case report form and in data analysis. SM, CH and WB were involved in designing the laboratory SOP, sample processing, and doing flow cytometry.

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None

Author's details

[4]Division of Allergology and Immunology; Department of Medicine, University of Cape Town, Groote Schuur Hospital, Cape Town, South Africa. [1]Department of Medicine, University of Cape Town, Old Main Building, J-Floor; Groote Schuur Hospital, Observatory, Cape Town, South Africa. [2]Division of Virology, Department of Pathology, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa. [3]Division of Infectious Diseases and HIV; Department of Medicine, University of Cape Town, Groote Schuur Hospital, Observatory, Cape Town, South Africa.

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ONLINE SUPPLEMENTS

Appendix 1: Infectious work-up at presentation with IFD

Table 1: infectious disease work-up during diagnosis of IFD

Case			**1**	**2**	**3**	**4**	**5**
INFX	HIV		Neg	Neg	Neg	Neg	Neg
	RPR		Positive	Neg	Neg	Neg	Neg
	HBsAg		Neg	Neg	Neg	Neg	Neg
	HCV Ab		Neg	Neg	Neg	Neg	Neg
	Blood culture		Neg	Neg	+MSSA	Neg	Neg
CSF	Polys	0-2	0	0	1	0	0
	Lymphs	0-2	4	1	16	0	0
	Erythro	0	0	125	1	1760	0
	Glucose	60% of serum glucose	4.1	4.8	3.3	3.5	4.1
	Proteins	<0.45g/dL	0.28	0.28	0.22	0.39	0.28
	CLAT	Neg	Neg	Neg	Neg	***Pos***	Neg
	Bacteria	None	None	None	None	None	None
	Culture	Neg	Neg	Neg	Neg	Neg	Neg
	Malign	Neg	Neg	Neg	Neg	Neg	Neg
URINE	Leuco		1+		3+	2+	
	Nitrites		Nil		Nil	Nil	
	Fungi		Nil		Nil	Nil	
	Culture		Neg		Neg	Neg	
SPUTUM	Bacteria		Pos	Neg		Neg	Pos
	Fungi		-	-		Yeasts	Nil
	AFBs		Nil	Nil		Nil	Nil
	GXP		Neg	Neg		Neg	Neg
	Culture		N. flora	Mix gr		Neg	S.pneum
	TB Culture		Neg	Neg		Neg	Neg
FLUID	Bacteria			Nil		Neg	
	Culture			Neg		Neg	
	Z-N stain for AFBs			Neg		Neg	
	TB culture			Neg		Neg	
	Cytology			Neg		+Fungi	
TISSUE	Bacteria		Nil	Nil	Nil	Nil	Nil
	Fungi		Neg	Neg	**Pos**	Neg	Neg
	Culture		**Pos**	Neg	**Pos**	Neg	Neg
	Z-N stain for AFBs		Neg	**Pos**	Neg	Neg	Neg
	TB culture		Neg	Neg	Neg	Neg	Neg
	Malignancy		Neg	Neg	Neg	Neg	Neg
	Histology		**+Fungi**	**+Fungi**	**+Fungi**	**+Fungi**	**+Fungi**

(Infx=infection screen, CSF=cerebrospinal fluid, RPR=rapid plasma regain test for syphilis, MSSA=methicillin sensitive Staphylococcus aureus, CLAT=cryptococcal latex agglutination test, Z-N=Ziehl-Neelsen test, GXP=gene expert test, TB=tuberculosis, AFBs=acid fast bacilli)

Appendix 2a: Case Summaries

Table 2: Patient characteristics

Case	**1**	**2**	**3**	**4**	**5**
Age at Dx (Yr)	49	68	36	41	44
Sex	Male	Male	Female	Male	Male
Self-reported ethnicity; birth place	Black,	Mixed	Black	Mixed	Mixed
Birth province	E. Cape	W. Cape	E. Cape	W. Cape	W. Cape
Parents related	No	No	No	No	No
Family history of fungal infection	Nil	Nil	Son with *Tinea corporis*	Brother with *Tinea corporis*	Nil
Comorbidity	Nil	Hypertension	Nil	Substance abuse	Nil
EPI Vaccination (BCG scar)	Yes	Yes	Yes	Yes	Yes
IFD diagnosis	Cerebral aspergillosis	Disseminated cryptococcosis	Disseminated cryptococcosis	Disseminated cryptococcosis	Disseminated cryptococcosis
Sites/organs involved	Brain	Lungs	Breast, Blood	Breast, CSF, Blood	Lungs, Blood
Fungus	*Aspergillus fumigatus*	*Cryptococcus neoformans*	*Cryptococcus neoformans*	*Cryptococcus neoformans*	*Cryptococcus neoformans*
CLAT	-	Negative	Positive	Positive	Positive

(Dx=diagnosis; EPI=Expanded program of immunization; BCG=Bacillus Calmette-Guerin; CLAT=cryptococcus latex agglutination test)

Table 3: Case 1 summary

Case 1	
Demographics	
Date of birth	19/12/1965
Sex	Male
Self-identified Ethnicity	Black African
Birth place	Eastern Cape
Are your parents related?	No
Medical History	
BCG scar present	Yes
Significant childhood &medical history	Nil
Significant Family medical history	Father=Pulmonary TB& Ca prostate; Mom died of meningitis; 1 son has been treated for pulmonary TB
Significant social history	Nil
Year of first diagnosis of IFD	2014
Presenting complaints	4months of Recurrent headaches with 3rd, 4th and 6th cranial nerve palsy
Date of first admission	1/10/2014
Date of discharge	1/11/2014
Number of admissions	4
Invasive Fungal Disease Diagnosis	
Genus	Aspergillus
Species	Fumigatus
Samples and sites for diagnosis	Brain abscess
Direct microscopy	Branching fungal hyphae
Fungal culture	Aspergillus fumigatus
Histopathology	Free lying aggregates of fungal hyphae showing septations and acute angle branching, consistent with Aspergillus spp.
Final clinical diagnosis	Cerebral aspergillosis
Management and follow-up	
First-line treatment	IV Voriconazole
Duration of first line therapy	2 weeks
Adjunctive treatment	Drainage of brain abscess; Steroids given (IV Dexamethasone followed by oral prednisone)
Secondary prophylaxis	Oral Voriconazole
Duration of secondary prophylaxis	Ongoing
Associated infections/conditions	Neurosyphilis, treated with IV penicillin
Follow-up	Ongoing at the ID clinic

Table 4: Case 2 summary

Case 2	
Demographics	
Date of birth	23/10/1946
Sex	Male
Self-identified Ethnicity	Coloured African
Birth place	Cape Town
Are your parents related?	No
Medical History	
BCG scar present	Yes
Significant childhood &medical history	Recurrent tinea corporis; shingles in puberty; diagnosed with HPTN and DM in Jan 2017
Significant Family medical history	1 grandchild has asthma, another grandchild has eczema
Significant social history	Nil
Year of first diagnosis of IFD	2014
Presenting complaints	3 months of dysphagia, hoarse voice and weight loss
Date of first admission	16/03/2015
Date of discharge	28/03/2015
Number of admissions	2
Invasive Fungal Disease Diagnosis	
Genus	Cryptococcus
Species	Neoformans
Samples and sites for diagnosis	Left upper lobe lung tissue
Direct microscopy	Budding yeast cells
Fungal culture	Cryptococcus neoformans
Histopathology	Scattered granulomas with big reactive follicular hyperplasia and some histiocytes. Budding yeast cells noted; consistent with a cryptococcoma
Final clinical diagnosis	Pulmonary cryptococcosis
Management and follow-up	
First-line treatment	No antifungals given
Duration of first line therapy	No antifungals given
Adjunctive treatment	Excision of the left upper lobe of lung (partial pneumonectomy)
Secondary prophylaxis	No antifungals given
Duration of secondary prophylaxis	No antifungals given
Associated infections/conditions	Pulmonary tuberculosis; treated for 6 months
Follow-up	Discharged from ID clinic after 1 more visit

Table 5: Case 3 summary

Case 3	
Demographics	
Date of birth	19/04/1978
Sex	Female
Self-identified Ethnicity	Black African
Birth place	Cape Town
Are your parents related?	No
Medical History	
BCG scar present	Yes
Significant childhood &medical history	Eczema
Significant Family medical history	Mother has Eczema and allergic rhinitis; 4yr-old son has recurrent skin rashes and rhinitis (HIV negative)
Significant social history	Nil
Year of first diagnosis of IFD	2015
Presenting complaints	3 weeks history of left breast swelling; found to be breast abscess
Date of first admission	15/08/2015
Date of discharge	14/09/2015
Number of admissions	3
Invasive Fungal Disease Diagnosis	
Genus	Cryptococcus
Species	Neoformans
Samples and sites for diagnosis	Serum CLAT=positive; Breast tissue
Direct microscopy	Numerous budding yeast cells
Fungal culture	Cryptococcus neoformans
Histopathology	Deep fungal infection with budding yeast cells
Final clinical diagnosis	Disseminated cryptococcosis with breast involvement
Management and follow-up	
First-line treatment	IV Amphotericin B
Duration of first line therapy	8 weeks
Adjunctive treatment	Incision and drainage of breast abscess; debridement of necrotic breast tissue
Secondary prophylaxis	Oral Fluconazole
Duration of secondary prophylaxis	Ongoing
Associated infections/conditions	MSSA septic shock due to J-line infection
Follow-up	Ongoing at the ID clinic

Table 6: Case 4 summary

Case 4	
Demographics	
Date of birth	18/10/1975
Sex	Male
Self-identified Ethnicity	Coloured African
Birth place	Cape Town
Are your parents related?	No
Medical History	
BCG scar present	Yes
Significant childhood &medical history	Recurrent tinea corporis in childhood
Significant Family medical history	Dad had pulmonary TB, 1 brother had recurrent tinea corporis and onychomycosis, 1 daughter has eczema and asthma
Significant social history	Longstanding smoking of methamphetamines, mandrax and tobacco
Year of first diagnosis of IFD	2016
Presenting complaints	3 months of right breast swelling with associated recurrent headaches
Date of first admission	24/07/2016
Date of discharge	4/08/2016
Number of admissions	1
Invasive Fungal Disease Diagnosis	
Genus	Cryptococcus
Species	Neoformans
Samples and sites for diagnosis	Serum CLAT=positive, CSF CLAT=positive; breast aspirate and breast tissue
Direct microscopy	Numerous budding yeast cells
Fungal culture	Cryptococcus neoformans
Histopathology	Scattered budding yeast cells seen with surrounding necrotizing inflammation; morphologically Cryptococcus spp.
Final clinical diagnosis	Disseminated cryptococcosis with cerebral and breast involvement
Management and follow-up	
First-line treatment	IV Amphotericin B
Duration of first line therapy	7 days. Patient left hospital AMA. Intended to be 6 weeks of IV Amphotericin B
Adjunctive treatment	Aspiration of breast abscess; followed by excision of breast mass.
Secondary prophylaxis	Fluconazole
Duration of secondary prophylaxis	Ongoing
Associated infections/conditions	Genital warts-cauterization done successfully
Follow-up	Ongoing follow up at the ID clinic

Table 7: Case 5 summary

Case 5	
Demographics	
Date of birth	10/11/1963
Sex	Male
Self-identified Ethnicity	Coloured African
Birth place	Cape Town
Are your parents related?	No
Medical History	
BCG scar present	Yes
Significant childhood &medical history	Spontaneous pneumothorax in 2002; Steroid-induced AVN of Left femur head, with THR in 2014
Significant Family medical history	1 sister died at age 2-3 years, of unknown cause, 1 daughter has Penicillin allergies
Significant social history	Chronic cigarette smoking
Year of first diagnosis of IFD	2007
Presenting complaints	2 weeks of cough with hemoptysis; with mass found on the Rt lung hilar region; subsequently had focal seizures, with cerebral mass noted
Date of first admission	12/12/2007
Date of discharge	22/02/2008
Number of admissions	3
Invasive Fungal Disease Diagnosis	
Genus	Cryptococcus
Species	Neoformans
Samples and sites for diagnosis	Serum CLAT=positive, CSF CLAT=positive, Lung tissue
Direct microscopy	Numerous budding yeast cells
Fungal culture	Cryptococcus neoformans
Histopathology	Numerous medium to large encapsulated yeasts of Cryptococcus spp., with associated granulomatous inflammation
Final clinical diagnosis	Disseminated cryptococcosis with pulmonary and cerebral involvement
Management and follow-up	
First-line treatment	IV Amphotericin B
Duration of first line therapy	6 weeks
Adjunctive treatment	Excision of lung mass (partial pneumonectomy); anticonvulsant therapy
Secondary prophylaxis	Oral Fluconazole
Duration of secondary prophylaxis	Ongoing
Associated infections/conditions	Focal seizures; treated for pneumonia
Follow-up	Ongoing follow-up at the ID clinic

Appendix 2b: Image series for case vignettes

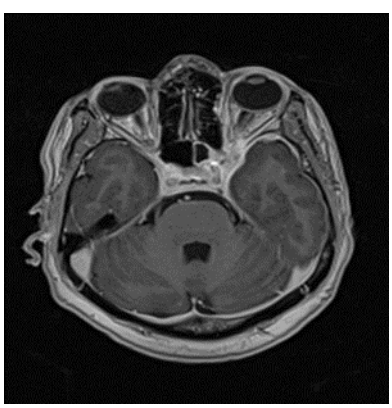
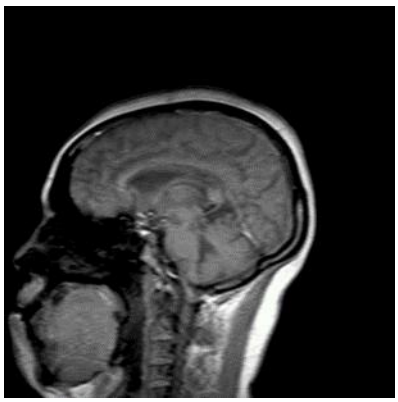
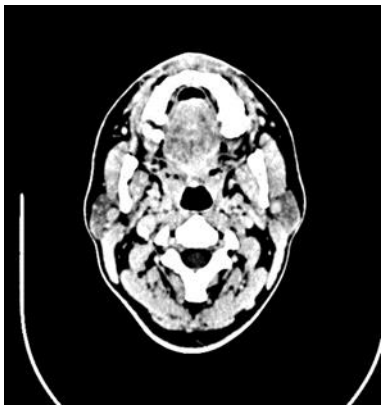




Figure 1 Figure 2 Figure 3

Case 1: Figure 1 MRI brain showing enhancing left cavernous sinus mass, Figure 2 MRI showing interval deterioration with a large left temporal lobe abscess communicating with the left para-cavernous sinus, and Figure 3 CT scan showing near complete resolution to normal left cavernous sinus morphology with mild residual expansion only.

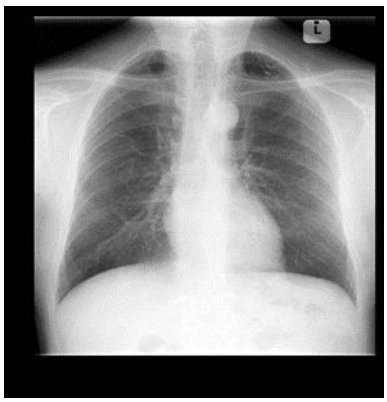
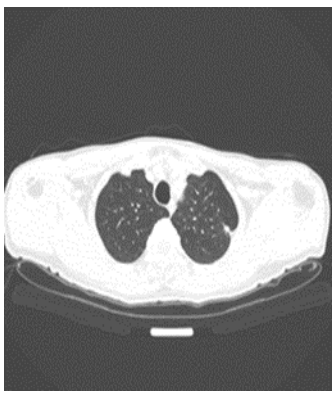
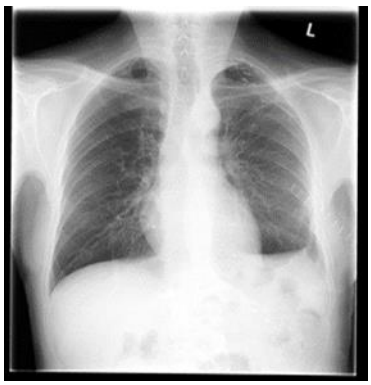
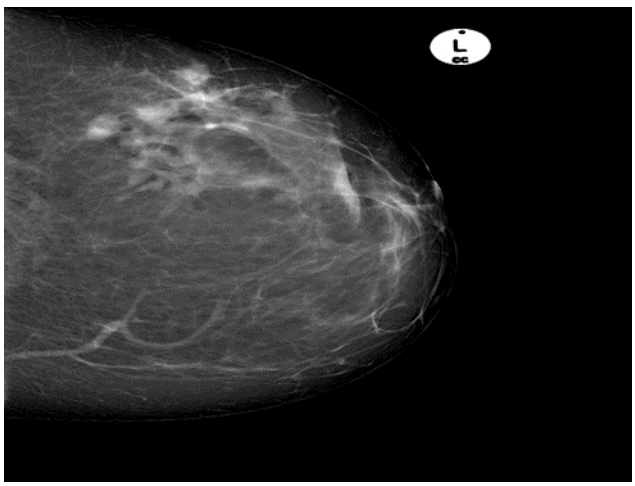




Figure 1 Figure 2 Figure 3

Case 2: Figure 1 CXR and Figure 2 CT chest, both showing spiculated left upper lobe mass. Figure 3 is a post-op CXR following excision of the mass.



Case 3: The mammogram reflects increased architectural distortion and density in the upper outer quadrant. Sonography confirmed a sinus with a linear fluid collection measuring 11-12cm in length, 5mm wide, with reactive lymph node. No masses noted.

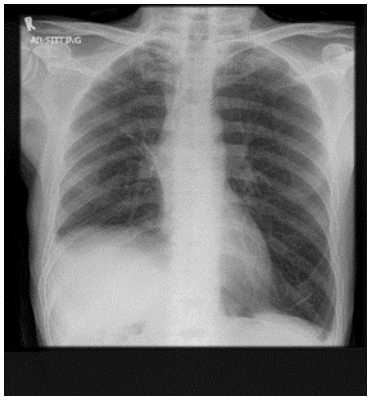
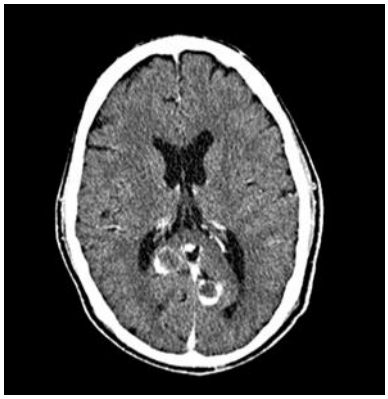
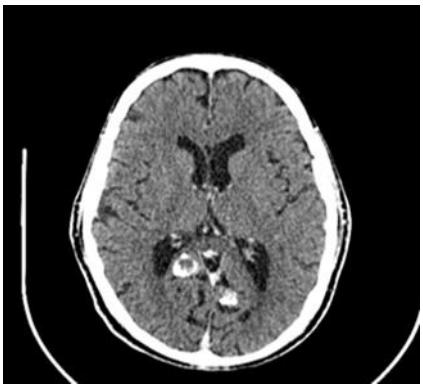




Figure 1 Figure 2 Figure 3

Case 5: Figure 1 CXR done in 2012 following right partial pneumonectomy, Figure 2 CT brain in 2012 showing cerebral parenchymal lesions with peripheral enhancement, and figure 3 CT brain in 2018 showing essentially unchanged cerebral parenchymal lesions with peripheral enhancement, and calcification noted.

Appendix 3: Basic laboratory tests at diagnosis of IFD

Table 8: Basic laboratory tests done during diagnosis of IFD

Case			**1**	**2**	**3**	**4**	**5**
FBC	WCC(*X10^9/L)	4.0-10	6.61	8.5	3.00	7.10	9.46
	HB(g/dL)	13.5-18	17.4	9.6	10.4	14.3	15.8
	MCV(fL)	79-96	80.3	80.1	82.8	85.7	90.2
	PLTS(*X10^9/L)	138-454	285	489	279	253	234
DIFF	Neutro(*X10^9/L)	2.0-7.6	3.76	5.73	1.4	6.8	4.31
	Lymph(*X10^9/L)	1.0-4.2	2.27	1.96	0.9	0.62	4.19
	Mono(*X10^9/L)	0.1-0.8	0.50	0.59	0.28	0.70	0.60
	Eosino(*X10^9/L)	0-0.44	0.02	0.17	0.00	0.16	0.27
	Basophil(*X10^9/L)	0-0.10	0.02	0.06	0.00	0.04	0.06
INFLAMM	ESR (mm/hr)	0-15	40	-	-	-	15
	CRP(mg/L)	0-5	9	-	-	-	37
RENAL	Na^+(mmol/L)	135-145	136	140	137	141	139
	K^+(mmol/L)	3.5-5.1	4.9	5.2	4.4	4.3	4.7
	Urea(mmol/L)	2.6-7.0	4.9	5.2	4.9	4.5	4.4
	Creat(µmol/L)	60-104	95	96	66	89	79
	eGFR(ml/min/1.73m^2)	>60	>60	>60	>60	>60	>60
LIVER	Bili total(µmol/L)	0-21	12	7	3	9	5
	Bili conj(µmol/L)	0-6	4	3	<1	3	2
	Tot Prot(g/L)	60-83	73	88	64	80	
	Albumin(g/L)	35-54	53	46	41	49	49
	ALP(U/L)	42-124	91	103	98	92	69
	GGT(U/L)	0-62	77	24	94	40	37
	ALT(U/L)	5-39	26	16	26	28	6
	AST(U/L)	5-39	23	28	25	24	22
THYROID	TSH(mIU/L)	0.26-4.3	2.3	3.1	0.36	1.21	2.27
GLUCOSE	Fasting(mmol/L)	4-6.9	5.3	6.4	5.8	6.8	6.2
	HBA1C%	<5.7		5.4		4.8	
AUTOIMM	ANA titer		Neg	Neg	Neg	Neg	Neg

(FBC=full blood count, Diff=differential count, Inflamm=inflammatory markers, Autoimm=autoimmune disease screen, WCC=total white cell count, HB=hemoglobin, MCV=mean corpuscular volume, PLTS=platelets, Neutro=neutrophils, Lymph=lymphocytes, Mono=monocytes, Eosino=eosinophils, ESR=erythrocyte sedimentation rate, CRP=C-reactive protein, Na+=serum sodium, K+=serum potassium, Creat=creatinine, eGFR=estimated glomerular filtration rate, ANA=antinuclear antibody)

Appendix 4a: Baseline Laboratory and Immunology Results

Table 9: Basic immunology results

Case			**1**	**2**	**3**	**4**	**5**
WCC (*x10^9/L)		4.0-10	6.61	8.5	***3.00***	7.10	9.46
Differential Count (*x10^9/L)	Neutro	2.0-7.6	3.76	5.73	***1.4***	6.8	4.31
	Lymph	1.0-4.2	2.27	1.96	***0.9***	***0.62***	4.19
Hemoglobin (12-18 g/dl)			17.4	***9.6***	***10.4***	14.3	15.8
Platelets (138-454 X10^9)			285	489	279	253	234
Total IgG (7.0-16 g/L)			16.43	16.9	16.44	16.24	12.91
Total IgA (0.7-4.0 g/L)			3.25	3.77	3.77	1.78	2.53
Total IgM (0.4-2.3 g/L)			1.67	1.67	0.64	1.27	0.81
CD3 cells (527-2846 cells/µl)			1253	1134	668	1325	2429
CD4 cells (332-1642 cells/µl)			795	726	400	***218***	1606
CD8 cells (170-811 cellsµ/l)			458	418	232	1034	886
B cells (78-899 cells/µl)			265	256	119	281	1021
NK cells (67-1134 cells/µl)			892	257	217	367	877
Total complement levels			810	790	690	717	852
Serum CLAT			-	Negative	***Positive***	***Positive***	***Positive***

(NK=Natural killer cells; CLAT=cryptococcal latex agglutination test)

Figure 1: Line chart representation of the total leucocyte count, as well as absolute neutrophil and lymphocyte counts. Units is cells/L.

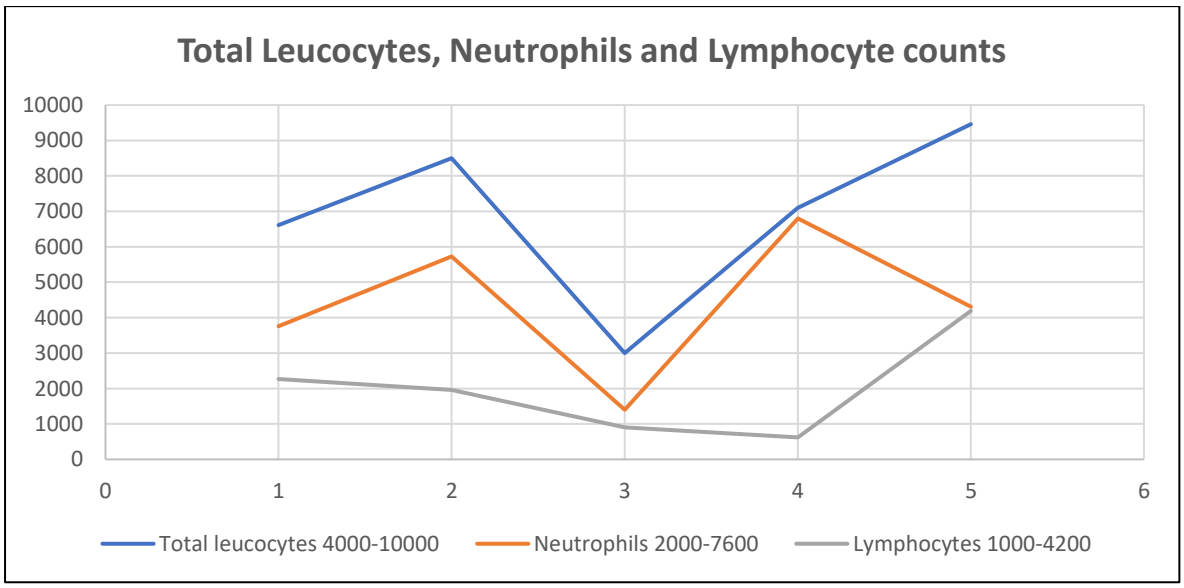
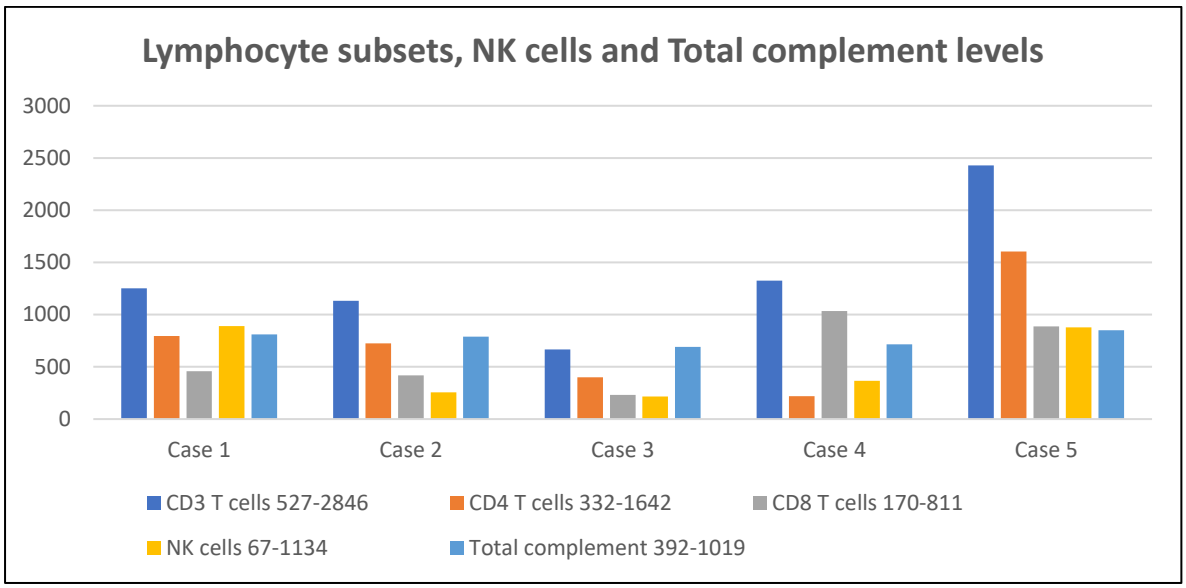


Figure 2: Bar graph representation of Lymphocyte subsets, natural killer (NK) cells, and Total complement levels. Units is cells/µl.



Appendix 4b: Recall titers to vaccines, Neutrophil function and Vitamin D levels

Table 10: Antibody recall titers, neutrophil function, and Vitamin D levels

Case	**1**	**2**	**3**	**4**	**5**
Current antibody levels					
C. diphtheriae IgG	0.19	0.10	0.06	0.46	0.06
C. tetani IgG	0.02	0.01	0.31	1.50	0.56
H. influenza B IgG	19.93	0.23	0.68	0.09	>9.00
S. pneumonia IgG	47.3	283	50.70	75.20	123.75
Oxidative burst for Neutrophils					
Wash	0-10%	3.7	3.9	16.9	2.4
fMLP	1-20%	8.5	14.1	45.6	9.7
PMA	99-100%	99.6	100	100	100
E. coli	95-100%	99.6	100	99.9	100
Serum Vitamin D Level in nmol/L (<50 =Deficient, 50-72.5=Insufficient, >72.5=Sufficient)					
	52	**47.2**	53.1	**45.1**	60.2

Table 11: Reference ranges of the vaccine recall titers

Addendum to **"Current antibody levels".** This is a measure of the current antibody recall titers to various vaccines; based on National Health Laboratory Services guidelines.

Corynebacterium diphtheriae IgG (IU/mL)

Protective Ranges:

<0.01 IU/mL	No protection.
0.01 - 0.09 IU/mL	Minimal protection.
0.10 - 0.99 IU/mL	Safe protection. Booster recommended.
1.00 - 1.49 IU/mL	Booster after 5 years.
1.50 - 1.99 IU/mL	Booster after 7 years.
>2.00 IU/mL	Booster after 10 years.

Clostridium tetani IgG (IU/mL)

Protective Ranges:

<0.01 IU/mL	No protection.
0.01 - 0.10 IU/mL	No safe protection. Booster recommended.
0.11 - 0.50 IU/mL	Sufficient protection. Booster improves protection.
0.51 - 1.00 IU/mL	Sufficient protection. Booster not necessary.
1.01 - 5.00 IU/mL	Long term protection. Booster after 5-10 years.
>5.00 IU/mL	Long term protection. Booster after 10 years.

Haemophilus influenza B IgG (mg/L)

Protective Ranges:

At 0-1 years	>0.84 mg/L
At 1-2 years	>1.00 mg/L
At >2 years	>1.50 mg/L

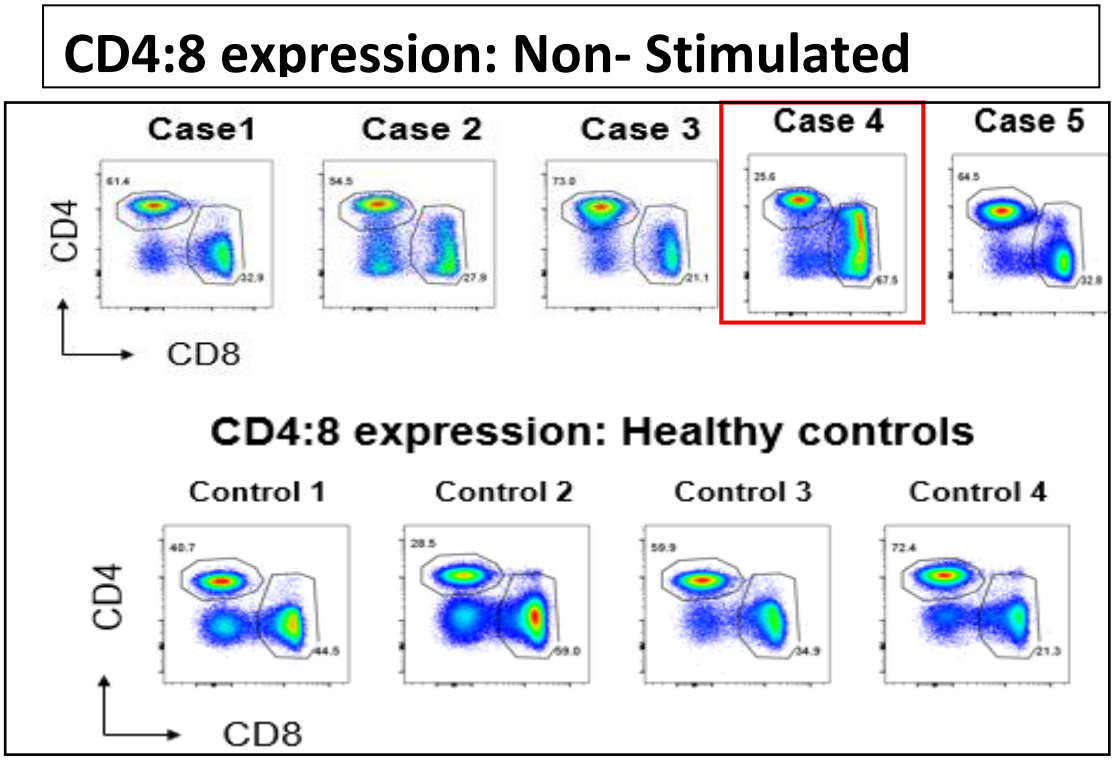
Streptococcus pneumoniae IgG (mg/L)

Protective Ranges:

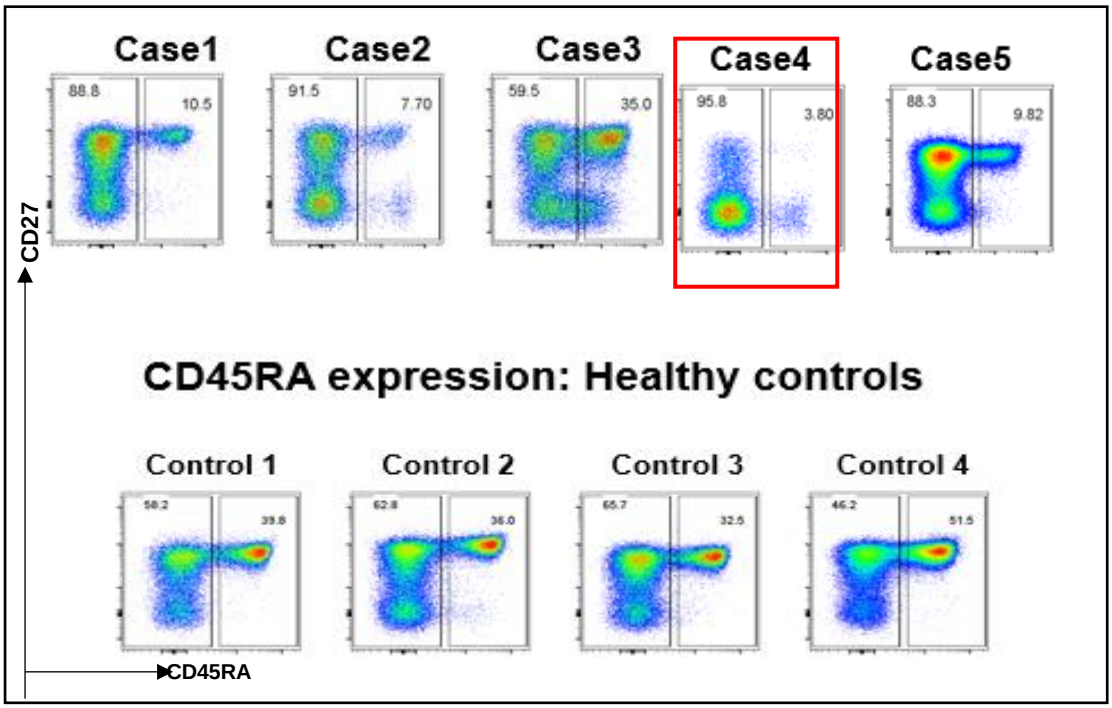
At 0-1 years	> 9.20 mg/L
At 1-2 years	> 4.60 mg/L
At 2-3 years	>12.30 mg/L
At 3-4 years	>14.60 mg/L
At >4 years	>35.00 mg/L

Appendix 4c: Flow cytometry plots

Figure 3: Flow cytometry series

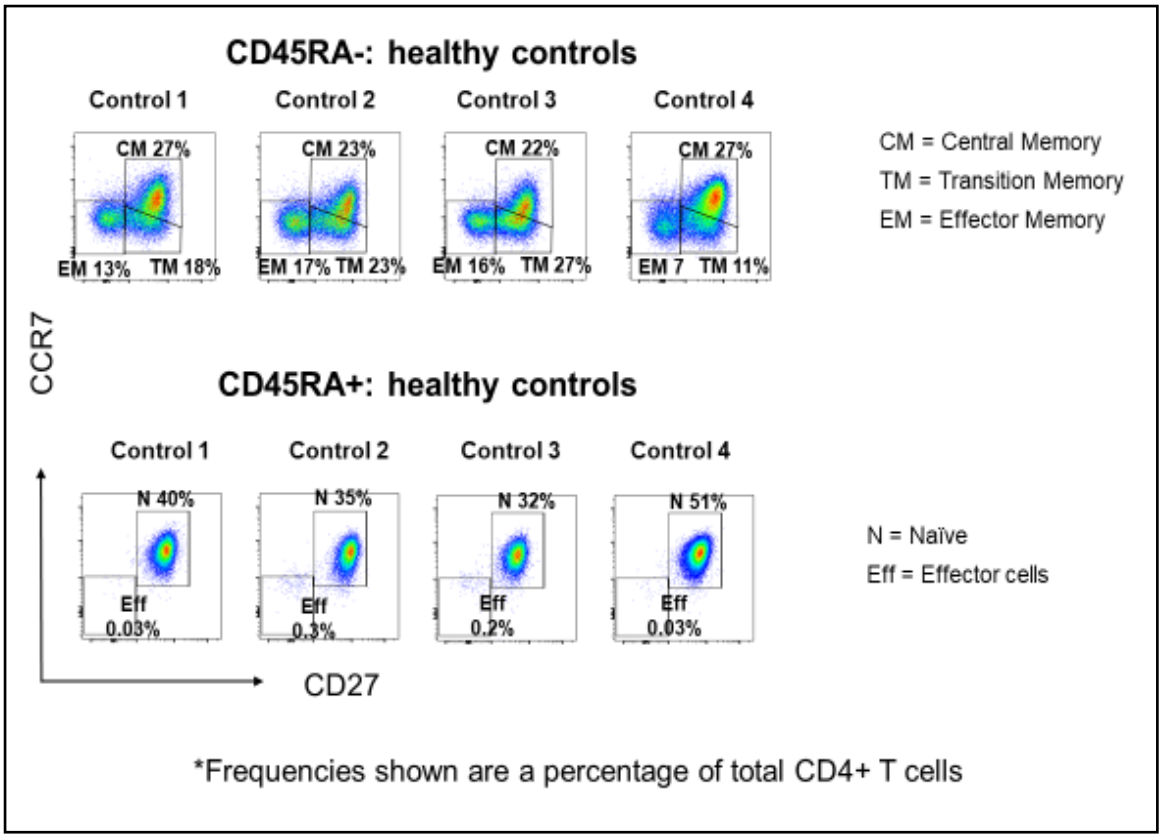


Notice that the CD4:CD8 ratio is inverted for Case 4

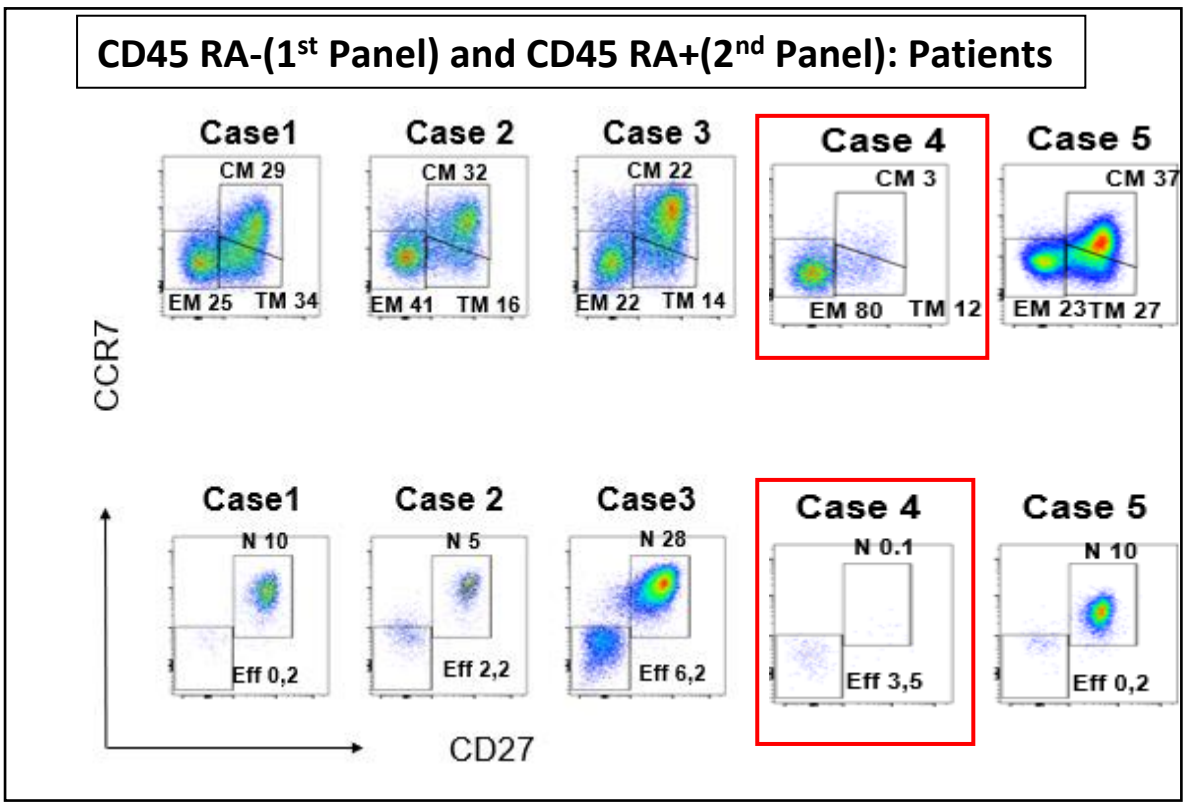


Low CD45RA expression observed for all patients; especially Case 4.

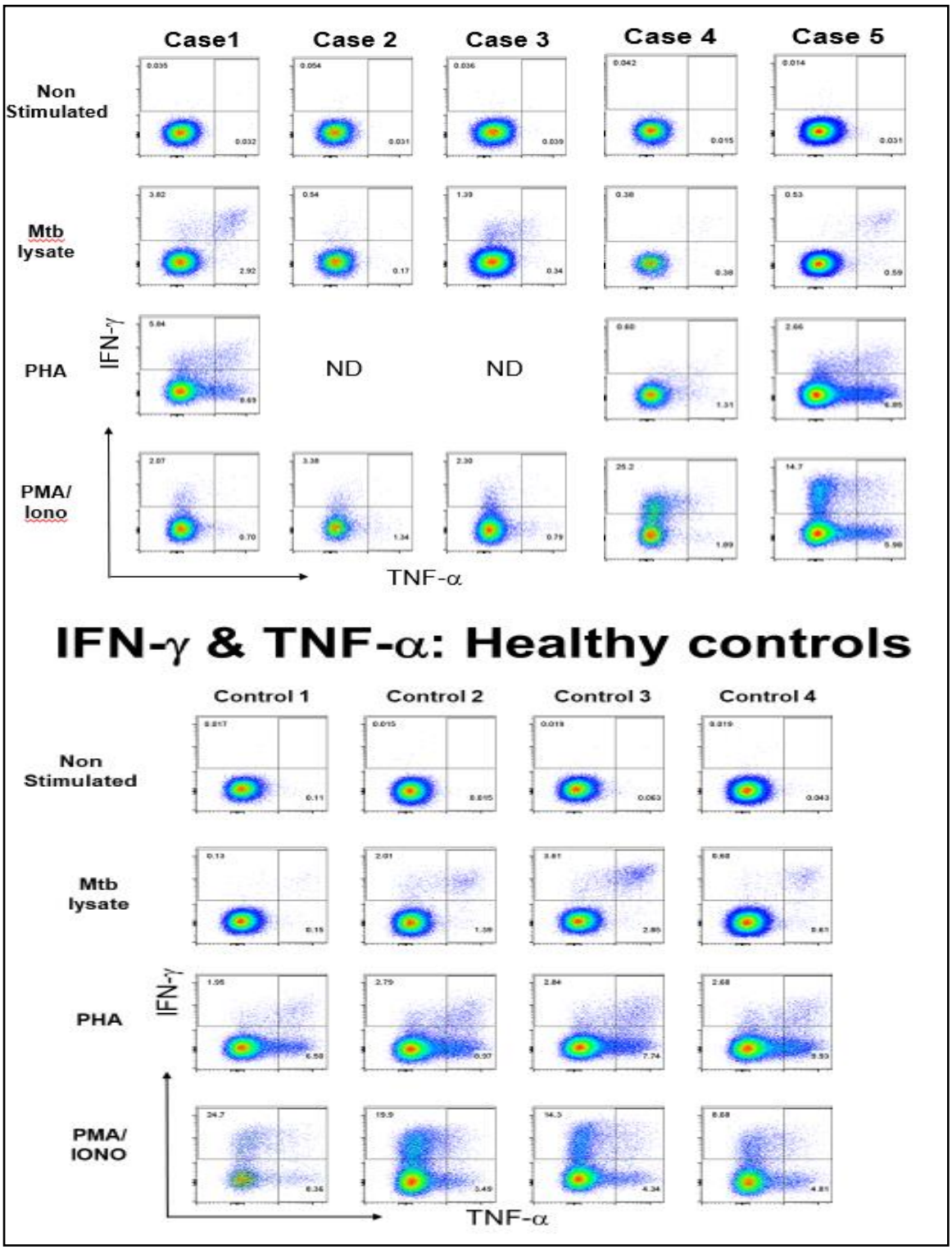
CD4+ CCR7/CD27 on CD45RA-/+



CD4+ CCR7/CD27 on CD45RA-/+

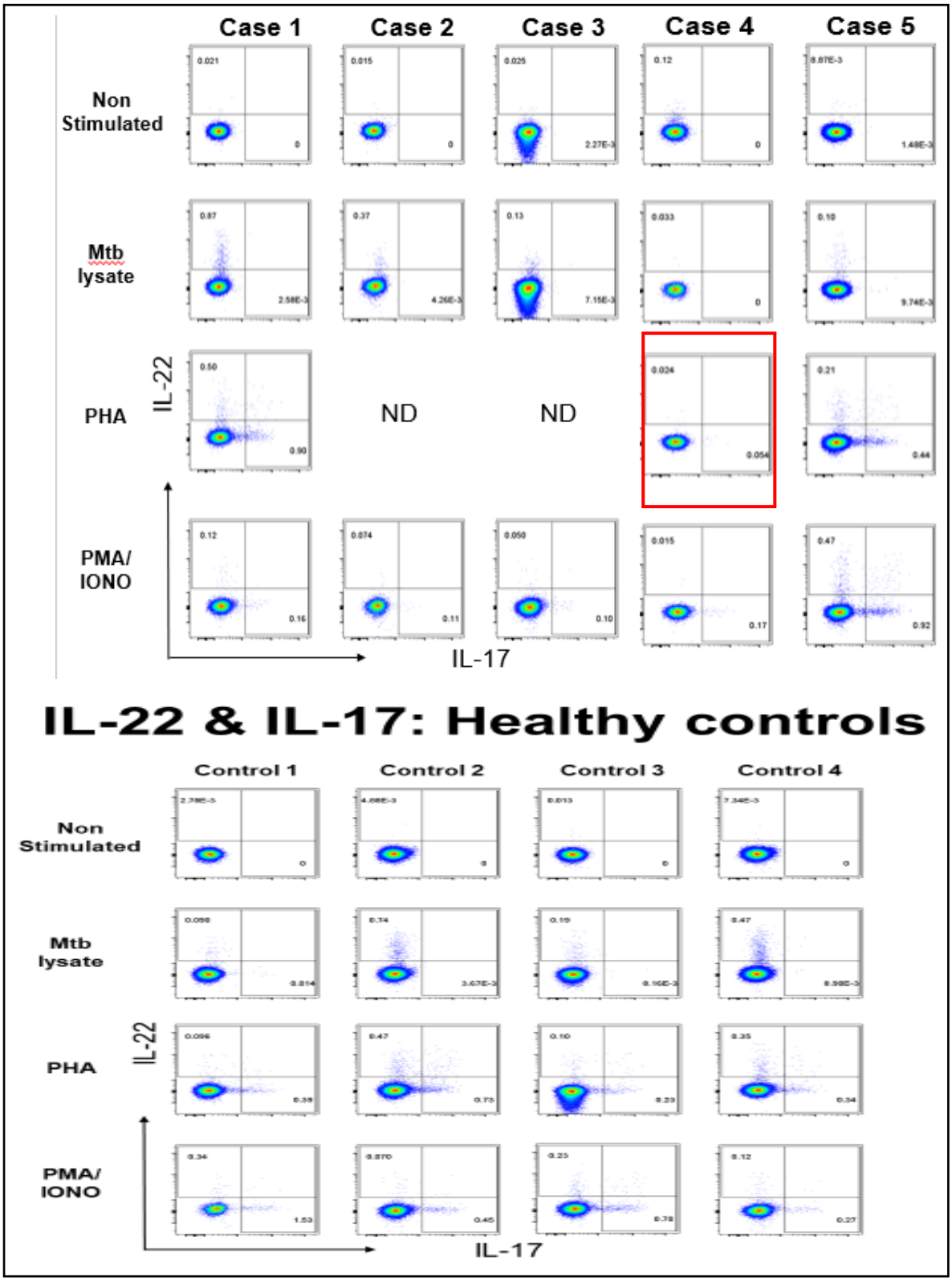


CD4+ cytokine expression: IL-22 & IL-17



Note that Case 4 had absent CD27 and CCR7 expression; therefore, showing absence of naïve and memory T cell populations.

CD4+ cytokine expression: IL-22 & IL-17



Compared to healthy controls, all patients had normal stimulated and unstimulated CD4+ cytokine expression for IFN-γ and TNF-α.

CHAPTER 3: APPENDICES

RESEARCH PROTOCOL.

Immunological evaluation of HIV-negative invasive fungal disease at Groote Schuur Hospital; Cape Town, South Africa.

Principal Investigator
A/Prof. Jonathan Peter; FCP (SA) PhD (UCT)
Head of Division of Allergology and Clinical Immunology,
Department of Medicine
University of Cape Town/Groote Schuur Hospital
Observatory, Cape Town, 7925
Telephone; +27832317004
Email: Jonny.Peter@uct.ac.za

Master's candidate
Dr. Vonwicks Czelestakov Onyango
Medical Registrar
Department of Medicine
University of Cape Town/Groote Schuur Hospital
Observatory, Cape Town, 7925
Telephone:-+27846615395
Email: - drvonczelo@gmail.com

Co-supervisor
A/Prof Sipho Dlamini, FCP(SA) Cert. Infectious Diseases(SA)
Department of Medicine
Division of Infectious Diseases and HIV
University of Cape Town/Groote Schuur Hospital
Observatory, Cape Town, 7925
Telephone:-+27837219504
Email: - sk.dlamini@uct.ac.za

Collaborators/Co-investigators

A/Prof. Wendy Burgers
Department of Pathology
Division of Medical Virology
Institute of Infectious Disease and Molecular Medicine/University of Cape Town
Observatory, Cape Town, 7925
Telephone:-+27825109071
Email: wendy.burgers@uct.ac.za

Mr. Mohau Steven Makatsa
PhD student
Department of Pathology
Division of Medical Virology
Institute of Infectious Disease and Molecular Medicine/University of Cape Town
Observatory, Cape Town, 7925
Telephone:-+27837731573
Email: MKTMOH003@myuct.ac.za

Dr. Claire Hoving
Institute of Infectious Disease and Molecular Medicine/University of Cape Town
Observatory, Cape Town, 7925
Telephone:-+ 27825109071
Email: Claire.Hoving@uct.ac.za

Dr. Anne Puel
Génétique Humaine des Maladies Infectieuses
INSERM UMR 1163
Université Paris Descartes-Sorbonne Paris Cité
Institut Imagine
Pièce 421-B1
24 boulevard du Montparnasse
75015 PARIS
FRANCE
Telephone: +33(0)142754200
Email: anne.puel@inserm.fr

LIST OF ABBREVIATIONS

ABBREVIATION	
HIV	Human immune-deficiency virus
AIDS	Acquired immune deficiency syndrome
CD e.g. CD4	Cluster of differentiation
IL e.g. IL17	Interleukin
TH/Th	T-helper cells
CARD9	Caspase recruitment domain-containing protein 9
CNS	Central nervous system
IFN	Interferon
GM-CSF	Granulocyte-monocyte colony stimulating factor
TNF	Tumor necrosis factor
PID	Primary immune deficiency
FBC	Full blood count
CRP	C-reactive protein
ESR	Erythrocyte sedimentation rate
CT scan	Computerized tomography scan
MRI	Magnetic resonance imaging
NHLS	National health laboratory services
CCR	C-chemokine receptor
HREC	Human research and ethics committee
UCT	University of Cape Town
CRF	Case report form
APACHE	Acute physiology and chronic health evaluation
CHC	Community health clinic
MMed	Master of medicine degree
GIT system	Gastro-intestinal system
HIb	*Hemophilus influenze* type B
PHA mitogen	Phytohemagglutinin
PMA mitogen	Phorbol myristate acetate
VZV	Varicella zoster virus

FIGURES, TABLES AND APPENDIX

ABSTRACT

Whereas fungi are ubiquitous, only a small number cause infections in humans. Innate and adaptive immune responses are usually effective in preventing disease or restricting to non-invasive infections. Invasive and severe fungal disease is usually restricted to an immunocompromised host. Secondary immunodeficiencies, especially HIV/AIDS; account for the majority of patients presenting with invasive and/or severe fungal disease, but there is a small group of patients with no evidence of a secondary cause; yet with an increased susceptibility to disease. A working hypothesis is that these patients may either possess an already described yet undiagnosed primary immunodeficiency; or carry novel genetic mutations resulting in an increased susceptibility to disease.

No African studies have conducted detailed immunological or genetic analysis of HIV-negative patients presenting with invasive fungal disease This study will be a cross-sectional study to describe the clinical and immunological characteristics of a series of such patients, seen at Groote Schuur Hospital in Cape Town; South Africa. Future studies will then examine the genetics of susceptibility in this cohort, looking for novel mutations. Future studies will then examine the genetics of susceptibility in this cohort, looking for novel mutations.

INTRODUCTION

Fungi are a heterogeneous group of eukaryotes consisting of different morphological forms, and mainly identified by their lack of chlorophyll and presence of a rigid cell wall [1]. The main forms include yeasts and yeast-like fungi (reproduce by budding) e.g. *Candida* and *Cryptococcus*; moulds (grow by branching and extension of hyphae) e.g. *Aspergillus*, and dimorphic fungi (which behave as yeasts in the host but as moulds in vitro) e.g. *Histoplasma* [1, 2]. While thousands of species exist, only around 300 species cause disease in humans. The forms of infections include superficial infections of the skin e.g. ring worms, mucocutaneous infections e.g. vulvovaginal candidiasis, oral thrush, and local non-invasive infections e.g. Aspergillomas (found in old tuberculosis cavities). Invasive fungal infections occur when fungi penetrate deep tissues and/or systemic circulation from where they can be disseminated to various body systems. They are usually seen in immunocompromised patients, where they contribute a significant burden of opportunistic infections, and are often associated with high morbidities and mortalities [2, 3].

Given the ubiquitous nature of fungi, the immune responses to fungal infections involve both innate and adaptive mechanisms (see Figure 1) [4]. The first-line innate mechanism is the presence of physical barriers in the form of skin and mucous membranes, which is complemented by cell membranes, cellular receptors and humoral factors [4, 5]. Cells of the innate system e.g. Natural killer cells, neutrophils, dendritic cells and macrophages, bind components of fungal cell walls using pattern recognition receptors and initiate killing by means of phagocytosis. C-type lectins such as Dectin-1 and Toll-like receptor 2 are particularly important for anti-fungal immunity. These cells also process and present fungal antigens to CD4+ T cells, which differentiate toward the protective Th1 and Th17 pathways [6]. Key cytokines necessary include IFN-γ, IL-17, and IL-12. TH1 and TH2 cells are the most important components of the adaptive anti-fungal immune response [6, 7]. Antibodies that kill fungal elements via antibody-mediated cytotoxicity and activation of complement are adjunctive [8]. In majority of instances, fungal immunity is effective. Invasive fungal infections are thus restricted to patients with primary or secondary immunodeficiency.

Table 1 lists known secondary immunodeficiency states that increase susceptibility to invasive fungal diseases. In Africa, the commonest risk factor for invasive fungal disease is advanced HIV. In fact, mucosal candidiasis and cryptococcal CNS infections are AIDS-defining illnesses [9]. Hematological malignancies, e.g. leukemia and lymphomas; plus their immunosuppressive chemotherapies, are other immunodeficiencies predisposing to invasive fungal infections [10, 11].

In our infectious disease clinics and medical wards, we have found a small group of patients with invasive fungal infections but no known secondary immune deficiency. This project is focused on the clinical and laboratory characterization of these patients. Do these patients have an undiagnosed secondary immune deficiency, an already described primary immunodeficiency, or do they carry novel genetic mutations with an increased susceptibility to invasive fungal disease? In studies of similar patients in other countries, genetic disorders of immune functions have been found. Table 2 lists the known genetic defects that have been associated with invasive fungal infections. For instance, homozygous mutations in *CARD9* is associated with deep dermatophytosis of CNS and GIT [12, 13, 14]. Other described susceptibilities include isolated CD4 lymphopenia, severe congenital neutropenia, leucocyte adhesion deficiency, and in-born errors of the phagocytic respiratory burst activity, necessary

for fungal killing. Endemic mycoses have also been linked to congenital errors of IFN-γ/IL-12 signaling pathway and acquired auto-antibodies to both IFN-γ and GM-CSF [15].

To our knowledge, there are no African studies that have conducted clinical and immunological assessments with or without genetic investigation in HIV-negative cohorts of invasive fungal disease without an obvious underlying secondary immunodeficiency. Characterizing these patients would be useful not only to diagnose and care for individual patients, but also to potentially identify novel genes involved in anti-fungal immunity unique to African populations.

PURPOSE OF THE STUDY

The aim of this project is to establish the clinical and immunological characteristics of HIV-negative patients with invasive fungal diseases, treated at the Groote Schuur Hospital; Cape Town, South Africa.

Hypothesis

We hypothesize that HIV-negative patients with invasive fungal diseases will not be found to have any discernible abnormalities in their baseline immunological work-up; yet may be found to have as yet uncharacterized genetic abnormalities affecting immune defense against fungal disease.

OBJECTIVES

1. To determine the clinical and baseline immunology characteristics of HIV-negative patients treated for invasive fungal disease at Groote Schuur Hospital.
2. Perform cytokine stimulation assays to examine functional pathways involved in susceptibility to fungal infections, including but not limited to TNF-α, IFN-γ, IL1β, IL-6, IL17A, and IL-22.
3. Conduct whole exome or genome sequencing of patients treated for invasive fungal disease, but with no secondary immunodeficiency and normal baseline immunological investigation.

Please note that the scope of this MMed project is restricted to objective 1. The details of investigations to be undertaken in Objective 2 and 3 are dependent on the results of objective 1 and will be performed in collaborations with international collaborators.

In addition, we also hope that the data generated from this study will provide useful information to develop a reasonable set of immune diagnostics to identifiable treatable immune problems in a developing country setting.

METHODS

Study Design

This study is a descriptive, cross-sectional, case series.

Study Site

The study will be conducted at the Infectious Diseases and Clinical Immunology clinics of Groote Schuur Hospital, Cape Town, South Africa.

Study Population and Sampling

The study will include all patients who have been treated for invasive fungal infections at Groote Schuur Hospital. The following categories of patients will NOT be included in the study:

- Patients who were/are HIV-positive at the time of infection
- Patients who have a documented secondary cause for immunosuppression other than HIV, e.g. cancer, autoimmune diseases, hematological malignancy, and organ transplant
- Patients who are below 18 years of age.
- Patients who are unable/refuse to give consent

The reason for excluding these patients is that the study is based on the hypothesis that the subjects to be studied may have an underlying PID. Therefore, we will exclude patients with known secondary cause(s) of fungal immune deficiency.

The patients involved in the study will have in-patient medical records from doctors, nurses, and any other allied health workers who managed them, reviewed and relevant data extracted (see appendix 2 for case report form). Routine investigations done will be captured from the National Health Laboratory Sciences, as well as Radiology and Imaging databases; and the relevant data from this will be included in the study. Patients will then be reviewed in the Immunology clinic and bloods taken for an in-depth immune work-up (detailed below) to exclude known PIDs associated with increased fungal susceptibility.

MEASUREMENTS

The data to be collected will include:

Demographic data for the patients

- Age
- Sex
- Self-reported ethnicity
- Gender

Clinical Characteristics

Family history of fungal infections, detailed genogram

Admission history of invasive fungal infections

1. Body system(s) involved

- CNS
- Respiratory
- Gastrointestinal
- Other

2. Diagnostic investigations done during admission

- Hematological tests e.g. FBC with differential counts
- Renal Function tests
- Liver Function tests
- Markers of inflammation e.g. CRP, ESR
- Microbiological tests e.g. Blood Cultures, Urine analysis, CSF studies
- Any baseline immunological testing e.g. Lymphocyte subsets (T-, B- and NK-cell numbers)
- Imaging reports e.g. Chest X-ray, Ct scans, MRI studies
- Tissue studies e.g. from biopsy, Endoscopy studies

3. Definitive etiological diagnosis i.e. the identity of the fungus (Genus and species), and the method used to arrive at the identity

4. Management given, including the medication used, in what route of administration, what duration, and the nature of secondary prophylaxis given

5. The current status of the subject e.g. the nature of follow up appointment given, and their current status of health.

A case report form will be constructed and validated against international samples. It will then be used to collect data from each subject of interest (See Appendix 2)

IMMUNOLOGICAL WORK-UP

All patients enrolled will undergo a baseline immunological work-up. They will undergo this work-up irrespective of immunological testing done at the time of admission, provided that they are culture negative at the time of their study visit. They should be considered as treated by the infectious disease team.

Those tests to be performed/available through the NHLS lab would include:

1. Immunoglobulin levels and measurement of vaccine responses to tetanus, HIb and total pneumococcal
2. FBC with differential count
3. Total Haemolytic complement
4. DHR (dihydrorhodamine) testing for neutrophil respiratory burst function.
5. Lymphocyte subsets and basic T-cell proliferation in response to mitogens PHA and PMA (performed at Tygerberg hospital NHLS)
6. Vitamin D levels

Those tests to be performed by co-investigators in the Department of Pathology: -

1. Naïve and memory CD4 and CD8 subsets as well as TH1 and TH17 numbers (measured via CXCR3, CCR6+ and CCR4+)
2. Innate and T cell cytokine production to a range of mitogens and PRRs (e.g. TLR,

lectins) and recall antigens, such as candida (and other available fungal antigens) and *Mycobacterium tuberculosis*.

Exploratory assays to be attempted depending on agreement of overseas laboratory to perform without cost: -
1. Autoantibody measurements to IFN-γ, IL17A and GM CSF

SAMPLE STORAGE AND GENETIC TESTING

In addition to the baseline testing as outlined above, we will store serum and peripheral blood mononuclear cells (PBMCs) for future functional and cytokine work depending on the results of objective 1. We may need to ship some samples to overseas labs of collaborating researchers for such, and for more advanced testing, including genetic tests. We will inform the HREC of planned further analyses; and patients will be required to consent to sample storage for 15 years, as well as sample shipment overseas.

STATISTICAL ANALYSIS

Coded data will be entered into Red-cap secure data-base for analysis.

Descriptive statistics will be used to characterize the targeted sample in terms of demographics, clinical history and response to management. Continuous variables will be described using means (standard deviations) or medians (inter-quartile ranges), while categorical variables will be expressed as frequencies and percentages. Depending on the nature and distribution of the variable, group comparisons will be conducted using either parametric tests (two-sample t-test) or non-parametric tests (Mann-Whitney U test) for continuous variables, while Chi-square or Fisher's Exact tests will be used to test for associations between categorical variables. Data will be presented in form of tables, graphs and charts.

A $p<0.05$ will be considered statistically significant where appropriate.

Statistical analysis will be performed using the statistical software, STATA.

TIMELINES

July 2016-April 2017	Complete project design, HREC approvals, collect data from medical records
April-Sept 2017	Data collection from patients
Sept 2017	Complete data collection
Sept-Oct 2017	Data processing
Oct-Dec 2017	Data analysis, Report writing and Preparation for journal articles

BENEFITS OF THE STUDY

The clinical review and testing performed may diagnose previously unidentified disorder in individual patients; this may offer individual patient-level benefit. Analysis of the data may be useful in setting up a diagnostic algorithm for HIV-negative patients suspected to have an underlying susceptibility to invasive fungal diseases. If indicated, the data will be used to motivate for further genetic testing. Identification of novel genes and pathways may have future

benefit to individual patients, patients with similar clinical phenotypes, may influence treatment strategies, and may provide fundamental insights on fungal immunity in humans.

ETHICAL CONSIDERATIONS

Permission to conduct this study will be sought from the UCT Human Research Ethics Committee (HREC). The study is considered to carry very minimal risk to patients.

Subject Identification and Confidentiality

All the information obtained during the study will be handled, stored and confidential. All the data collected will be entered in a password-protected UCT-hosted database. All the Study documents, materials, and patient Case Report Forms (CRFs) will be regarded as strictly confidential and will be stored in a secure place that is only accessible to the study staff. All subjects will be de-identified in the study with the use of a study number not linked to any other personal identifiers.

Informed Consent Procedure

Study participants will be identified from their medical records available in the Infectious Diseases Unit of the Groote Schuur Hospital. They will each be introduced by the Supervisors to the Lead investigator, who will explain the nature of the study. The lead investigator will make sure that each patient understands that participation is voluntary, and that his/her subsequent standard of care will not be affected if he/she decides not to participate. The lead investigator will then provide the patient with a copy of the informed consent (Appendix 1); and answer all the preliminary questions that the patient might have about the study. For any illiterate patient, the informed consent will be read to the patient in the presence of a witness to ensure that the patient understands the full terms as contained in the informed consent. The signature of the said witness shall be captured in the enrolment of such patients.

Patient's Risks, Benefits and Re-imbursements

Patients who are enrolled in this study will benefit from a review of their clinical status by participating in a preliminary search for a possible underlying immune dysfunction. The results of all testing will be conveyed back to both the patient and treating clinician. There will be minimal risk of venepuncture, which includes mild discomfort, and bruising. We will not take more than 50mls of blood on one, maximum two occasions (4 weeks apart); the second sample will only be collected if we are unable to isolate more than 20 million PBMCs from the first sample. Patients will be reimbursed R150 to cover cost of transport and time-off-work.

BUDGET

A sum of R5000 is allocated to all MMed projects. Additional compassionate support for testing will be sought from Ampath private immunodiagnostics laboratory as well as the Cambridge Immunology Laboratory performing autoantibody testing. Immunological tests performed by co-investigators will be covered by their budgets. Additional funding will be sought to cover any costs related to further genetic and functional immunology to be performed in this patient cohort.

***Figure 1:* - Innate and Adaptive Responses to Fungal infections: -Adapted from Romani et al. *Nature Review of Immunology*; 2004**

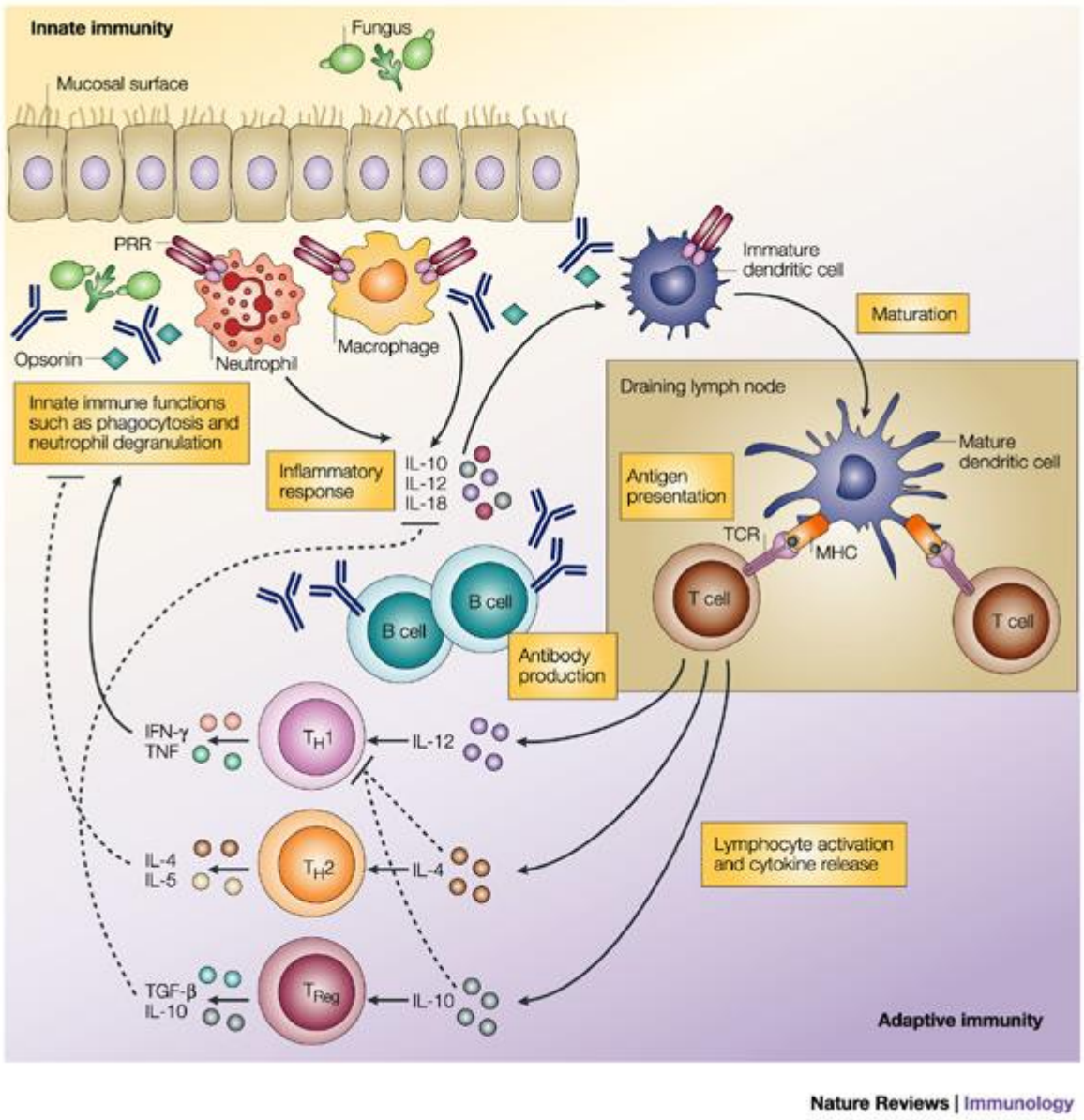


***Table 1:* - Causes of secondary immunodeficiency leading to increased susceptibility to invasive fungal infections**

SYSTEM/CATEGORY	EXAMPLES/ASSOCIATED STATES
Vascular	Central venous catheterization
Autoimmune diseases	Systemic lupus erythromatosus, rheumatoid arthritis
Trauma	Major surgery (especially abdominal)
Immunosuppressants	Chemotherapy, Steroids, and other immunosuppressant medications
Infections	HIV/AIDS, Tuberculosis (especially post tuberculous fibrosis of lungs) Severe sepsis/septic shock Prolonged use of broad-spectrum antibiotics
Metabolic	Diabetes, Hypothyroidism
Nutritional	Severe malnutrition Parenteral nutrition
Neoplasms	Hematological malignancies e.g. leukemia, lymphoma Solid organ malignancy
Organ failure	Renal failure Hemodialysis Liver failure Secondary bone marrow failure Solid organ transplant Hematopoietic stem cell transplant
Critical Illness	Prolonged ICU stay Acute pancreatitis Fungal colonization APACHE II score >20

Table 2: **-Primary immunodeficiencies associated with invasive fungal diseases *(*Adapted from Lanternier et al *'Primary immunodefiencies underlying fungal infections'*** Curr Opin Pediatr 2013, 25:736–747

Fungal susceptibility	Disease	Associated infections	Immunological phenotype	Gene, transmission
Deep dermatophytosis	CARD9 deficiency	CMC	Deficit of IL-17-producing T cells	CARD9, autosomal recessive
Aspergillosis	STAT3 deficiency	Staphylococcus aureus	STAT3 deficiency, pithelial lung dysfunction	STAT3, autosomal dominant
	Leukocyte adhesion deficiency, type 1	Bacteria	Impaired neutrophil adhesion	CD18, autosomal recessive
	Severe congenital neutropenia	Bacteria invasive candidiasis	Neutropenia	ELA2, autosomal dominant, HAX1, autosomal recessive
Invasive candidiasis	Severe congenital neutropenia	Bacteria, invasive candidiasis	Neutropenia	ELA2, autosomal dominant, HAX1, autosomal recessive
	Leukocyte adhesion deficiency type 1	Bacteria	Impaired neutrophil adhesion	CD 18 autosomal recessive
Candida CNS infection	Chronic granulomatous disease	Bacteria, Scedosporium	NADPH oxidase complex defect in phagocytes	CYBB, X-linked or autosomal, CYBA, NCF1, NCF2, NCF4, autosomal recessive [54,55&,56&,85,86, 89–94]
	CARD9 deficiency	Dermatophytes, CMC	Impaired Candida killing by PMN	CARD 9, autosomal recessive
Cryptococcosis	Autoantibodies against GM-CSF	No	Neutralizing anti-GM-CSF autoantibodies	Unknown
	Autoantibodies against IFN-g	Mycobacteria	Neutralizing anti-IFN-g autoantibodies	
	CD40 ligand deficiency	Pneumocystis, bacteria	Hypo IgG, IgA, high IgM levels, T/B cell cooperation defect	CD40L, X-linked [167,168]
	MonoMAC syndrome	Environmental mycobacteria, viruses, fungi	Monocytopenia, deficit of dendritic cells, B and NK cell lymphopaenia	GATA2, autosomal dominant [96]
Coccidioidomycosis	Interleukin-12Rb1 deficiency	Mycobacteria, Salmonella		IL12RB1, autosomal recessive [154]
	STAT1 GOF mutation	CMC, viruses		STAT1, autosomal dominant
Histoplasmosis	IFN-gR1 deficiency	Mycobacteria, Salmonella		IFNGR1, autosomal dominant
	Idiopathic CD4 lymphopenia	Pneumocystis, Cryptococcus,	CD4 T cells <300 cells/mm^3	UNC119, autosomal dominant [76], MAGT1, X-linked

				[77], RAG1, autosomal recessive
	DOCK8 deficiency	Viruses and bacteria	T-cell defect	DOCK8, autosomal recessive [21,22,25]
	X-linked CD40L deficiency	Pneumocystis, bacteria	Hypo IgG, IgA, high IgM levels, T/B cell copperation defect	CD40L, X-linked
Pneumocystosis	SCID	Bacteria, viruses, fungi, mycobacteria	No T cells, with or without B and NK cell lymphopenia	>30 genes JAK3, autosomal recessive RAG1, autosomal recessive RAG2, autosomal recessive, ARTEMIS, autosomal recessive ADA autosomal recessive
	X-linked CD40 ligand deficiency	Pneumocystis, bacteria	Hypo IgG, IgA, high IgM levels, T/B cell cooperation defect	CD40L, X-linked [167,168]
	MHC class II deficiency	Bacteria, viruses, fungi	T-cell defect	CIITA, RFXANK, RFXC, RFXAP, autosomal recessive
	NEMO deficiency	Pyogenic bacteria, mycobacteria, viruses	T-cell defect	NEMO, X-linked [19]
	DOCK8 deficiency	Viruses, bacteria	T-cell defect	DOCK8, autosomal recessive
	X-linked recessive Wiskott–Aldrich syndrome	Bacteria, viruses	T-cell defect	WASP, X-linked recessive
	CARD11 deficiency		T-cell defect, increased transitional B cells, hypogammaglobulinemia	CARD11, autosomal recessive

Consent form

FACULTY OF HEALTH SCIENCES

Department of Medicine

J-Floor, Old Groote Schuur Hospital, Observatory, 7925 Cape Town; South Africa Phone: +27 21 4066200; Fax: +27 21 4486815

Study Title: - Immunological evaluation of HIV-negative invasive fungal disease at Groote Schuur Hospital; Cape Town, South Africa.

"The Fungal Disease Project''

*[**Prof. Jonathan Peter**, (Principal Investigator), **Prof. Sipho Dlamini** (Co-supervisor), **Dr. Vonwicks Onyango** (MMed Student)]*

Dear Participant,

Thank you for your interest in this study. This consent form will tell you why we want to do this study. You must be 18 years and above to participate in this clinical study.

Who are the Investigators?

This study will be carried out by investigators from University of Cape Town. These are specialists in immunology and infectious diseases, working at the Groote Schuur Hospital. We are working with other investigators in Pretoria, as well as in Netherlands and France, to study infections caused by fungi in people; especially those who do not have HIV infection.

Purpose and Scope of the Research

Fungi are tiny organisms that are found in the environment (in soil, air, clothes etc.). We easily recognize them when they cause our food to go bad, for example, the moulds or yeasts that grow on bread, cooked potatoes or vegetables, once these foods have been stored for some time. Some of these fungi also cause infections in humans. In most people, fungi do not cause severe disease beyond the skin or mucous membranes (e.g. mouth and genitals), for example, ring worms seen on the head and body, or thrush, often seen as whitish sticky material in the mouth, or as vaginal discharge, especially among pregnant women.

Occasionally, some people do suffer from invasive fungal infections. This means that the infection goes beyond the skin and mucous membranes, into the blood stream. Once in the

blood, the fungal infections can spread to any major organs of the body and cause severe disease; e.g. meningitis, pneumonia, or severe diarrhea.

It is unusual for a person to suffer from invasive fungal infections in the absence of an underlying immune system problem. Secondary causes of immune system problems such as HIV remain the most common explanation.

You are being approached to participate in this study because you suffered such a severe infection with a fungal organism. You do not have any obvious problem with your immune system.

However, in the absence of these, such as in your situation, it may be explained by another as yet unknown problem with your immune system. This may involve your 'genes' or genetic material. Everyone has 'genes', and these are responsible for why people in a family look like each other, but different from others. For example, some families have tall members, others short, while others may have a particular shape of eyes, nose and feet. These genes are inherited from the mother and father; and are passed on to the children from generation to generation. In some cases, problems with these genes may be responsible for various types of infections and diseases. We want to look closely to try to identify the possible problem with your immune system so as to potentially help both you and other similar patients.

In future, the information and samples that you will provide for this study may also help us to better understand how the human immune system fights fungal infections. Other investigators elsewhere may also use them to study other diseases. You will not get any direct benefits from this study. But we hope that the information we get may benefit others who have similar and related diseases.

Please take as much time as you need to talk about the study with your family, friends, study staff and other health care providers as you would like. We are very ready to clarify anything that you may not understand; and to answer any questions about anything related to the study.

What will the Study Involve?

If you agree to participate in this study, we will review your hospital folder with you and ask any additional questions pertaining to your history with fungal infections. This interview should last approximately 1 hour. We will then ask you for a blood sample of about 50mls (about 3-4 table-spoons full). We may also ask you to return four weeks after your first visit for a second blood sample, if we are unable to store sufficient blood cells from your first blood sample.

What will happen to the Samples?

Once we have collected the blood sample from you, we will test some of it in our laboratory here at Groote Schuur Hospital, and send some of it to a more advanced laboratory in Pretoria for further tests. We may need to send some of the samples, including your genetic material to overseas laboratories of our scientific collaborators for even more specialized testing which is not available locally. We will store the rest of your samples in our special laboratory here, together with other samples given by the other people participating in this study. The samples will not be sold. We will store them for a maximum of 15 (fifteen) years for future scientific research.

What will happen to the data?

To protect your privacy, we will assign you a study number. This will replace your name in all the samples and data related to you. We will do our best to keep this information confidential. We will do this by creating a private computer data-base with the study numbers, which will be protected by a password, and will only be accessible to the study staff. In order to promote greater understanding of your condition, we may need to share your data with our scientific collaborators and other investigators across the world in future. When we share this information, people will not know your name. Other investigators who want to use your data (without your name) will have to first seek permission from our central repository. They will commit to using this data for scientific research only.

Voluntary nature of participation and right to withdrawal from the Study

It is your personal decision to participate in this study. In other words, it is by your free will that you can say 'Yes' and join the study; or 'No' you don't want to join. If you participate in the study, you can change your mind later and decide that you don't want to continue participating anymore, and that you don't want your blood sample to be used in the study. Please, let us know and we will destroy the sample. If your sample has already been tested and data obtained from it at the time that you change your mind, we shall use such information in our data analysis. Such data may also have been shared already with other investigators. In that case we will not be able to destroy this data. However, should you request it; we can remove your data from the central repository. That means no additional researchers can get your data.

Whether you decide to join this study or not, the way we look after you in this clinic will be the same. It is your decision whether to be in this study or not.

Potential risks associated with the Research

We must tell you that there are some risks with this study. For instance, things might not go very well when taking your blood sample. Most of the time, when we take blood, it is safe. But sometimes, when we take blood, some people feel a bit faint, and may have bruising, swelling or infection at the site. If this happens, let us know and you will be treated.

How participants' privacy will be protected

We will store your blood samples, together with those of other participants in the study, in a locked freezer in our laboratory. Also, whenever we send them for processing, they will be under lock and key. The samples, as well as all other information and data obtained from you will be assigned your study number in order to protect your personal identity. All this information will be stored in a computer data-base which will be protected by a password. It is always possible that someone may find out that you participated in this study. However, it is very unlikely that this will happen. We will take all measures to ensure that this does not happen.

Potential benefits of participating in the Study

This study will not help you to immediately get better. However, by conducting additional tests in combination with the information obtained from your clinical review, we hope to get a better understanding of your condition. This may also help others with the same condition as you, in the future. What we are trying to do is difficult and may take a long time. Whether you decide to join this study or not will not affect your treatment at our clinic. Your decision to join or not is up to you.

Participant compensation

We would like to pay you back for the time and money that you'll spend to participate in this study. We will give you R150 (one hundred and fifty rands) to cover the cost of your time away from work and your travel to get to the clinic.

Return of Results

When the study is finished, we will share the study research's general findings with you. We will not put these results in your medical records. We will inform you of anything which is particularly important to your health. In such a case, we will contact you by phone and put you in touch with doctors and other health care workers who can help you. We hope to get preliminary results in about twelve months.

Participant consent

By signing below, I confirm that the reasons for the study, what will be done, the risks and benefits of participating in the study, and what will happen to my samples have been explained to me in a language that I understand.

Your initials here:……..

I confirm that I have been given the opportunity to ask questions about the study by the investigator.

Your initials here:……..

I understand that I may withdraw from the study or refuse future procedures without affecting any future medical treatment.

Your initials here:……..

I agree to have my samples stored as outlined in the informed consent document. I give permission for my specimens to be stored for future research.

Your initials here:……..

I agree to have my samples sent to overseas laboratory as outlined in the informed consent document. I understand that my specimen will not be used for future research without Ethics Committee permission.

Your initials here:……..

I understand that genetic testing will be performed on samples collected from me. I give my permission for genetic testing to be performed.

Your initials here:……..

Name of participant……..

Your initials here:……..

Signature of participant.....

Date.....

Name of person taking consent/investigator……

Your initials here:……..

Signature of investigator…….

Date.....

Name of witness: …….

Your initials here:……..

Signature of witness.....

Date.....

Case report form

Participant details					
Date	dd / mm / yyyy			**Consent**	Y N
Study ID		**Folder ID**		**Subject initials**	
Contact number	☎			**Home address**	
Emergency contact	☎				
Email	@				
Preferred method of contact				**Clinic/CHC**	

Demographics					
DOB	dd / mm / yyyy				
Gender	M	F			
What is your self-identified Ethnicity?			***Were your parents related?***	*Y*	*N*

Medical history						
Family Hx	Y	N	**If yes**		Chronic	Recurrent
Body system	CNS		GIT	Resp		Other
Personal Hx						
Age at Dx			**Date of initial admission and dx**		dd / mm / yyyy	
Body system	CNS		GIT	Resp		Other
Length of stay in hospital		days		wks	**Number of admissions**	
Last admission to hospital	dd / mm / yyyy				**Discharge date**	dd / mm / yyyy
Body system	CNS		GIT	Resp		Other

Body system	Diagnosed condition	Clinical diagnosis	Current problem
CNS			
GIT			
Resp			
Other			

Additional notes								
Medical Hx not obtained	Y	N						
Final Dx								
Management								
Body system 1	**Genus**		**Species**					
	Dx confirmed	Microscopy	Culture		Histology	Other		
Notes	**Anti-fungal Treatment**	Fluconazole	Route	Dur	Voriconazole	Route	Dur	
		Itraconazole	Route	Dur	Posaconazole	Route	Dur	
		Amphotericin B	Route	Dur	Caspofungin	Route	Dur	
		Other	Route	Dur				
	2° prophylaxis given	Y	N					
	If yes	Drug	Route	Dur				
Body system 2	**Genus**		**Species**					
	Dx confirmed	Microscopy	Culture		Histology	Other		
Notes	**Anti-fungal Treatment**	Fluconazole	Route	Dur	Voriconazole	Route	Dur	
		Itraconazole	Route	Dur	Posaconazole	Route	Dur	
		Amphotericin B	Route	Dur	Caspofungin	Route	Dur	
		Other	Route	Dur				
	2° prophylaxis given	Y	N					
	If yes	Drug	Route	Dur				
Body system 3	**Genus**		**Species**					
	Dx confirmed	Microscopy	Culture		Histology	Other		
Notes	**Anti-fungal Treatment**	Fluconazole	Route	Dur	Voriconazole	Route	Dur	
		Itraconazole	Route	Dur	Posaconazole	Route	Dur	
		Amphotericin B	Route	Dur	Caspofungin	Route	Dur	
		Other	Route	Dur				
	2° prophylaxis given	Y	N					
	If yes	Drug	Route	Dur				

Follow up	Y	N				
Where	GSH MOPD		GSH Immunology clinic	GSH ID clinic	CHC _____	DAY HOSPITAL _____

Laboratory results						
		Dates				
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	**Notes**
FBC	**WCC**					
	HB					
	MCV					
	Platelets					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Diff count	**Neutrophils**					
	Monocytes					
	Lymphocytes					
	Eosinophils					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
U & E	**Urea**					
	Creatinine					
	eGFR					
	K^+					
	Na^+					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Liver	**Bili total**					
	Bili conjugated					
	Total protein					
	Albumin					
	ALP					
	ALT					
	AST					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Inflammation	**CRP**					
	ESR					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Immunology	**T cells**					
	B cells					
	NK cells					
	Serum lgG					
	Serum IgA					
	Serum IgM					
	Complement levels					

Microbiology						
		Dates				
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	Notes
CSF	**Polys**					
	Lymph					
	RBC					
	Protein					
	Glucose					
	Bacteria					
	CLAT					
	Culture					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Blood culture						
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Aspirate	**Glucose**					
	Protein					
	LDH					
	ADA					
	Gram stain					
	Bacteria					
	Culture					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Tissue	specify					
	specify					
	specify					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Xray	**Head & neck**					
	Chest					
	Abdomen					
	Other					
		dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	dd / mm / yyyy	
Other	specify					
	specify					
	specify					

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Ethics approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

25 April 2017

HREC REF: 010/2017

A/Prof J Peter
Allergology and Clinical Immunology
UCT Lung Institute

Dear A/Prof Peter

PROJECT TITLE: IMMUNOLOGICAL EVALUATION OF HIV-NEGATIVE INVASIVE FUNGAL DISEASE AT GROOTE SCHUUR HOSPITAL, CAPE TOWN, SOUTH AFRICA (Masters candidate- Dr VC Onyango)

Thank you for submitting your response letter to the Faculty of Health Sciences Human Research Ethics Committee dated 25 April 2017.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30 April 2018.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

The HREC acknowledge that the student, Dr Vonwicks Czelestakov Onyango will also be involved in this study.

Yours sincerely

signature removed to avoid exposure online

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI),and Declaration of Helsinki (2013) guidelines.
The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Approval by

Groote Schuur Hospital

Western Cape Government
Health

GROOTE SCHUUR HOSPITAL
Enquiries: Dr Bernadette Eick
E-mail : *Bernadette.Eick@westerncape.gov.za*

Professor J. Peter
Allergology & Clinical Immunology

E-mail: Jonny.Peter@uct.ac.za

Dear Professor Peter

RESEARCH PROJECT: Immunological Evaluation of HIV-Negative Invasive Fungal Disease at Groote Schuur Hospital, Cape Town, South Africa (Masters Candidate Dr V.C. Onyango)
Your recent letter to the hospital refers.
You are granted permission to proceed with your research, which is valid until **30 April**.
Please note the following:

a) Your research may not interfere with normal patient care.
b) Hospital staff may not be asked to assist with the research.
c) No additional costs to the hospital should be incurred i.e. Lab, consumables or stationary.
d) **No patient folders may be removed from the premises or be inaccessible.**
e) Please provide the research assistant/field worker with a copy of this letter as verification of approval.
f) Confidentiality must be maintained at all times.
g) Should you at any time require photographs of your subjects, please obtain the necessary indemnity forms from our Public Relations Office (E45 OMB or ext. 2187/2188).
h) Should you require additional research time beyond the stipulated expiry date, please apply for an extension.
i) Please discuss the study with the HOD before commencing.
j) Please introduce yourself to the person in charge of an area before commencing.
k) On completion of your research, please forward any recommendations/findings that can be beneficial to use to take further action that may inform redevelopment of future policy / review guidelines.
l) Kindly submit a copy of the publication or report to this office on completion of the research.

I would like to wish you every success with the project.

Yours sincerely
signature removed to avoid exposure online
DR BERNADETTE EICK
CHIEF OPERATIONAL OFFICER
Date: 11 September 2017

C.C. Mr L. Naidoo
Dr H. Aziz
Professor N. Ntusi

G46 Management Suite, Old Main Building
Observatory 7925
Tel: +27 21 404 6288 fax: +27 21 404 6125

Private Bag X,
Observatory, 7935
www.capegateway.go.v.za

Instructions for Authors (South Africa Medical Journal: Author Guidelines)-abbreviated

South Africa Medical Journal: Author Guidelines

For full instructions, please click on the following URL:
http://www.samj.org.za/index.php/samj/about/submissions#Authorship

General article format/layout

Accepted manuscripts that are not in the correct format specified in these guidelines will be returned to the author(s) for correction, which will delay publication.

General:

- Manuscripts must be written in UK English.
- The manuscript must be in Microsoft Word format. Text must be single-spaced, in 12-point Times New Roman font, and contain no unnecessary formatting (such as text in boxes).
- Please make your article concise, even if it is below the word limit.
- Qualifications, full affiliation (department, school/faculty, institution, city, country) and contact details of ALL authors must be provided in the manuscript and in the online submission process.
- Abbreviations should be spelt out when first used and thereafter used consistently, e.g. 'intravenous (IV)' or 'Department of Health (DoH)'.
- Include sections on Acknowledgements, Conflict of Interest, Author Contributions and Funding sources. If none is applicable, please state 'none'.
- Scientific measurements must be expressed in SI units except: blood pressure (mmHg) and haemoglobin (g/dL).
- Litres is denoted with an uppercase L e.g. 'mL' for millilitres).
- Units should be preceded by a space (except for % and ºC), e.g. '40 kg' and '20 cm' but '50%' and '19ºC'.
- Please be sure to insert proper symbols e.g. µ not u for micro, a not a for alpha, b not B for beta, etc.
- Numbers should be written as grouped per thousand-units, i.e. 4 000, 22 160.
- Quotes should be placed in single quotation marks: i.e. The respondent stated: '...'
- Round brackets (parentheses) should be used, as opposed to square brackets, which are reserved for denoting concentrations or insertions in direct quotes.
- If you wish material to be in a box, simply indicate this in the text. You may use the table format –this is the only exception. Please DO NOT use fill, format lines and so on.

Research

Guideline word limit: 4 000 words

Research articles describe the background, methods, results and conclusions of an original research study. The article should contain the following sections: introduction, methods, results, discussion and conclusion, and should include a structured abstract (see below).

The introduction should be concise – no more than three paragraphs – on the background to the research question and must include references to other relevant published studies that clearly lay out the rationale for conducting the study. Some common reasons for conducting a study are: to fill a gap in the literature, a logical extension of previous work, or to answer an important clinical question. If other papers related to the same study have been published previously, please make sure to refer to them specifically.

Describe the study methods in as much detail as possible so that others would be able to replicate the study should they need to. Results should describe the study sample as well as the findings from the study itself, but all interpretation of findings must be kept in the discussion section, which should consider primary outcomes first before any secondary or tertiary findings or post-hoc analyses. The conclusion should briefly summarise the main message of the paper and provide recommendations for further study.

Select figures and tables for your paper carefully and sparingly. Use only those figures that provided added value to the paper, over and above what is written in the text.

Do not replicate data in tables and in text.

Structured abstract

This should be 250-400 words, with the following recommended headings:
Background: why the study is being done and how it relates to other published work.
Objectives: what the study intends to find out
Methods: must include study design, number of participants, description of the intervention, primary and secondary outcomes, any specific analyses that were done on the data.
Results: first sentence must be brief population and sample description; outline the results according to the methods described. Primary outcomes must be described first, even if they are not the most significant findings of the study.
Conclusion: must be supported by the data, include recommendations for further study/actions.
Please ensure that the structured abstract is complete, accurate and clear and has been approved by all authors.
Do not include any references in the abstracts.

Main article

All articles are to include the following main sections: Introduction/Background, Methods, Results, Discussion, Conclusions.
The following are additional heading or section options that may appear within these:
Objectives (within Introduction/Background): a clear statement of the main aim of the study and the major hypothesis tested or research question posed
Design (within Methods): including factors such as prospective, randomisation, blinding, placebo control, case control, crossover, criterion standards for diagnostic tests, etc.
Setting (within Methods): level of care, e.g. primary, secondary, number of participating centres.
Participants (instead of patients or subjects; within Methods): numbers entering and completing the study, sex, age and any other biological, behavioural, social or cultural factors (e.g. smoking status, socioeconomic group, educational attainment, co-existing disease indicators, etc)that may have an impact on the study results. Clearly define how participants were enrolled, and describe selection and exclusion criteria.
Interventions (within Methods): what, how, when and for how long. Typically for randomised controlled trials, crossover trials, and before and after studies.
Main outcome measures (within Methods): those as planned in the protocol, and those ultimately measured. Explain differences, if any.

Results

Start with description of the population and sample. Include key characteristics of comparison groups.
Main results with (for quantitative studies) 95% confidence intervals and, where appropriate, the exact level of statistical significance and the number need to treat/harm. Whenever possible, state absolute rather than relative risks.
Do not replicate data in tables and in text.
If presenting mean and standard deviations, specify this clearly. Our house style is to present this as follows:
E.g.: The mean (SD) birth weight was 2 500 (1 210) g. Do not use the ± symbol for mean (SD).
Leave interpretation to the Discussion section. The Results section should just report the findings as per the Methods section.

Discussion

Please ensure that the discussion is concise and follows this overall structure – sub-headings are not needed:
Statement of principal findings
Strengths and weaknesses of the study
Contribution to the body of knowledge
Strengths and weaknesses in relation to other studies
The meaning of the study – e.g. what this study means to clinicians and policymakers
Unanswered questions and recommendations for future research

Conclusions

This may be the only section readers look at, therefore write it carefully. Include primary conclusions and their implications, suggesting areas for further research if appropriate. Do not go beyond the data in the article.

Illustrations/photos/scans

- If illustrations submitted have been published elsewhere, the author(s) should provide consent to republication obtained from the copyright holder.
- Figures must be numbered in Arabic numerals and referred to in the text e.g. '(Fig. 1)'.
- Each figure must have a caption/legend: Fig. 1. Description (any abbreviations in full).
- All images must be of high enough resolution/quality for print.
- All illustrations (graphs, diagrams, charts, etc.) must be in PDF or jpeg form.
- Ensure all graph axes are labelled appropriately, with a heading/description and units (as necessary) indicated. Do not include decimal places if not necessary e.g. 0; 1.0; 2.0; 3.0; 4.0 etc.
- Scans/photos showing a specific feature e.g. Intermediate magnification micrograph of a low malignant potential (LMP) mucinous ovarian tumour. (H&E stain). –include an arrow to show the tumour.
- Each image must be attached individually as a 'supplementary file' upon submission (not solely embedded in the accompanying manuscript) and named Fig. 1, Fig. 2, etc.

Tables

- Tables should be constructed carefully and simply for intelligible data representation. Unnecessarily complicated tables are strongly discouraged.
- Large tables will generally not be accepted for publication in their entirety. Please consider shortening and using the text to highlight specific important sections, or offer a large table as an addendum to the publication, but available in full on request from the author
- Embed/include each table in the manuscript Word file - do not provide separately as supplementary files.
- Number each table in Arabic numerals (Table 1, Table 2, etc.) and refer to consecutively in the text.
- Tables must be cell-based (i.e. not constructed with text boxes or tabs) and editable.
- Ensure each table has a concise title and column headings, and include units where necessary.
- Footnotes must be indicated with consecutive use of the following symbols: * † ‡ § ¶ || then ** †† ‡‡ etc.

Do not: Use [Enter] within a row to make ‘new rows’:
Rather: Each row of data must have its own proper row:
Do not: use separate columns for n and %:
Rather: Combine into one column, n (%):
Do not: have overlapping categories, e.g.:
Rather: Use <> symbols or numbers that don’t overlap:

References

NB: Only complete, correctly formatted reference lists in Vancouver style will be accepted. Reference lists must be generated manually and not with the use of reference manager software. Endnotes must not be used.

- Authors must verify references from original sources.
- Citations should be inserted in the text as superscript numbers between square brackets, e.g. These regulations are endorsed by the World Health Organization, [2] and others.[3,4-6]
- All references should be listed at the end of the article in numerical order of appearance in the Vancouver style (not alphabetical order).
- Approved abbreviations of journal titles must be used; see the List of Journals in Index Medicus.
- Names and initials of all authors should be given; if there are more than six authors, the first three names should be given followed by et al.
- Volume and issue numbers should be given.
- First and last page, in full, should be given e.g.: 1215-1217 not 1215-17.
- Wherever possible, references must be accompanied by a digital object identifier (DOI) link). Authors are encouraged to use the DOI lookup service offered by CrossRef:
- On the Crossref homepage, paste the article title into the ‘Metadata search’ box.
- Look for the correct, matching article in the list of results.
- Click Actions > Cite
- Alongside 'url =' copy the URL between { }.
- Provide as follows, e.g.: https://doi.org/10.7196/07294.937.98x