Host Susceptibility to Blastomycosis: A Scoping Review and Case-Control Association Study

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Abstract

Blastomycosis is a pulmonary disease caused by *Blastomyces dermatitidis*, a dimorphic fungus endemic to Manitoba and northwestern Ontario. Immunosuppression is a major risk factor affecting disease susceptibility, yet host immunity is not well understood. Genetic immunodeficiencies can also influence disease, with variants in *IL6*, *GATA2*, and *VDBP* shown to influence susceptibility. However, additional genetic factors in disease susceptibility and severity remain undetected. Our study seeks to establish what is known about susceptibility to blastomycosis and explore genetic risk factors in a case-control cohort. We conducted a scoping review to establish current knowledge on blastomycosis immunity and a literature review to identify candidate genes influencing susceptibility to fungal and mycobacterial infections. Exomes from 18 blastomycosis cases and 9 controls were sequenced, variants were identified, and filtered according to best practices. We performed candidate gene prioritization and variant aggregation to identify genetic associations and explored the full exome dataset. The scoping review included 58 articles on susceptibility to blastomycosis. TNF-α, GM-CSF, $CD4^+$ deficiency, and the IL-12-IFN-γ pathway had the most evidence as susceptibility factors. The literature review identified 86 candidate genes relevant to fungal and mycobacterial infections. One hundred and three genetic variants in 42 candidate genes were identified in the exome dataset. No variants associated with susceptibility were identified in a single-variant analysis although two non-synonymous variants in *TYK2* were enriched among cases suggesting a possible role in susceptibility. Gene-based association analysis found *TLR1* and *GATA2* enriched in controls ($p = 0.024$ and 0.051, respectively) suggesting a possible protective effect, although *GATA2* has previously been associated with blastomycosis susceptibility. Gene cluster analysis identified genetic variants in genes of chromatin remodeling, proteasome, and intraflagellar transport significantly enriched in cases (false discovery rates <14%). Case enrichment of intraflagellar transport genes is interesting as there are previous case reports of blastomycosis and defects in mucociliary function. The findings in this thesis show novel associations with blastomycosis susceptibility. A better understanding of host immunity and genetic predisposition to blastomycosis can help to inform clinical practice for improved outcomes.

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1000G	1000 Genomes
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OHD)	25-hydroxyvitamin D
AD	Autosomal dominant
AIDS	Acquired immunodeficiency syndrome
AP-1	Activating protein 1
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
AR	Autosomal recessive
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BAD1	*Blastomyces* adhesion-1
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guérin
BCL10	BCL10 immune signaling adaptor
BI-Eng2	*Blastomyces* endoglucanase-2
BTK	Bruton's tyrosine kinase
BWA-MEM	Burrow-Wheeler Aligner maximal exact matches
C3	Complement protein 3
C5	Complement protein 5
CADD	Combined Annotation Dependent Depletion
CARD	Caspase activation and recruitment domain
CARD9	Caspase activation and recruitment domain family member 9
CD	Cluster of differentiation
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CGD	Chronic granulomatous disease
CI	Confidence interval
CLR	C-type lectin receptor
CMC	Chronic mucocutaneous candidiasis
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CR3	Complement receptor 3
CXCL	CXC motif ligand
CXCR	CXC motif chemokine receptor
dbSNP	Single Nucleotide Polymorphism Database
DC	Dendritic cell
DOCK8	Dedicator of cytokinesis 8
DP	Depth of coverage
DppIVA	Dipeptidyl peptidase IVA
DRK1	Dimorphism-regulating histidine kinase
EBV	Epstein-Barr virus

EIA	Enzyme immunoassay
eQTL	Expression quantitative trait locus
FcRγ	Fc receptor γ chain
FDR	False discovery rate
FERM	Protein 4.1, Ezrin, Radixin, Moesin
FS	Fisher strand
GATA2	GATA binding protein 2
GATK	Genome Analysis ToolKit
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gnomAD	Genome Aggregation Database
GTEx	Genotype-Tissue Expression
GQ	Genotype quality
GVCF	Genomic variant calling file
GWAS	Genome wide association study
HIES	Hyper-immunoglobulin E syndrome
HIV	Human immunodeficiency virus
HSC	Health Sciences Centre
HSV	Herpes simplex virus
ICAM-1	Intracellular adhesion molecule 1
ICL	Idiopathic $CD4^+$ lymphocytopenia
IEI	Inborn errors of immunity
IFNAR1	Interferon alpha and beta receptor subunit 1
IFN-γ	Interferon gamma
IFN-γR1	Interferon-γ receptor subunit 1
IFT	Intraflagellar transport
IL	Interleukin
IL12Rβ1	Interleukin-12 receptor subunit 1
IL23R	Interleukin-23 receptor
Indels	Insertions and deletions
JAK	Janus kinase
JBI	Joanna Briggs Institute
JCW	JC Wilt Infectious Diseases Research Centre
LFA-1	Lymphocyte function-associated antigen
LOF	Loss of function
MAb	Monoclonal antibody
MAF	Minor allele frequency
MALT1	Mucosa-associated lymphoid tissue 1
MHC	Major histocompatibility complex
MonoMAC	Monocytopenia and mycobacterial infection
MPO	Myeloperoxidase
MR	Mannose receptor
MRSA	Methicillin resistant *Staphylococcus aureus*
MQ	Root mean square mapping quality
MQRankSum	Mapping quality rank sum test

MyD88	Myeloid differentiation primary-response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κB
NHC	Network-based Heterogeneity Clustering
NK	Natural killer
OR	Odds ratio
PAP	Pulmonary alveolar proteinosis
PC	Principal component
PCA	Principal component analysis
PCD	Primary ciliary dyskinesia
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pLOF	Putative loss of function
PolyPhen-2	Polymorphism Phenotyping v2
PRISMA-ScR	Preferred Reporting Items for Systematic review and Meta-Analyses extension for Scoping Reviews
PRR	Pathogen recognition receptor
QD	Quality by depth
QUAL	Quality
ReadPosRankSum	Read position rank sum test
ROBO	Roundabout guidance receptor
RORγt	Retinoic acid receptor-related orphan receptor gamma
ROS	Reactive oxygen species
RUNX1	Runt-related transcription factor 1
SIFT	Sorting intolerant from tolerant
SKAT	Sequence kernel association test
SKAT-O	Optimized sequence kernel association test
SNP	Single nucleotide polymorphism
SOR	Strand odds ratio
STAT	Signal transducer and activator of transcription
SWI/SNF	Switch/sucrose non-fermentable
SYK	Spleen tyrosine kinase
TGF-β	Transforming growth factor beta
Th	$CD4^+$ T helper
Th1	T helper cell type 1
Th2	T helper cell type 2
Th17	T helper cell type 17
Ti/Tv	Transition/transversion
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TSP-1	Thrombospondin 1
TYK2	Tyrosine kinase 2
UCSC	University of California Santa Cruz

VCF	Variant calling file
VDBP	Vitamin D binding protein
VDR	Vitamin D receptor
VEP	Variant effect predictor
WHO	World Health Organization

Chapter 1: Introduction

1.1 The Burden of Fungal Infections and The Influence of Host Immunity

Fungi are eukaryotic microorganisms found ubiquitously in the environment occupying niches in decomposition of organic matter, symbiotic relationships with plants, and colonization in humans. The vast majority of the estimated 2.2 million fungal species are non-pathogenic with few species capable of skin surface infections, opportunistic, or primary invasive infections (1,2). We are constantly interacting with fungi in our environment with minimal risk of infection and disease. Our immune systems, composed of physical barriers and mechanisms (e.g., epithelial linings, mucus and ciliary clearance, and endothermy), innate, and adaptive immune responses, are highly effective at protecting us from most fungi (3). Yet, it is estimated that 150-300 million people have fungal infections and over 1.6 million die per year from invasive fungal infections (4–6).

In recent decades, there has been increasing incidence of fungal infections coinciding with medical advancement of immunosuppressive therapies, emergence of human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS), and increasing life expectancy and aging of the population (3,5,7). These factors highlight the requirement of effective immune function to combat against fungal infections. Prior to the emergence of immunosuppressive therapies and HIV, invasive fungal infections were less common but present at a baseline dependent on host and pathogen factors (3). Pathogen factors consist of the fungus species, strain, and initial fungal load (e.g., number of spores inhaled or inoculated). Each pathogenic or opportunistic fungus has virulence factors that alter the fungal load required to establish infection and subsequently develop disease in the host. The host's environment and genetics adjusts the baseline fungal load required to establish productive infection. Common risk factors for acquiring fungal infections include healthcare contact, medical indwelling devices (8), immunosuppression (3), older age (≥65) (9), male sex (10), and proximity to the fungus for endemic mycoses (11). Host genetic variation is a major contributor to the heterogeneity of infectious disease phenotypes. Each person has a unique set of genetic variants that alters the functioning of the immune system from minute expression changes to

complete loss of protein function. The combination of environmental risk factors and unique genetic architecture of each individual also contributes to vast phenotypic heterogeneity in clinical manifestations of infectious disease (12). This clinical heterogeneity is not well understood and current efforts have focused on extreme phenotypes with underlying rare and deleterious mutations as well as large-scale genome-wide association studies (GWAS) for common phenotype-altering variation (13).

The introduction of broadly immunosuppressive (e.g., corticosteroids, tacrolimus, cyclosporine, sirolimus, mycophenolic acid, and methotrexate) and targeted immunomodulatory therapies (e.g., monoclonal antibodies and small kinase inhibitors) greatly improved the treatment of autoimmune conditions, chronic inflammatory diseases, infectious disease cytokine storms, and facilitated progress in liquid and solid organ transplantations. However, the resulting immunosuppression lowers the barrier of immunity, increasing rates of fungal infection. HIV similarly induces cell-mediated immunodeficiency with the depletion of $CD4^+$ T helper (Th) cells. Advanced HIV or $CD4^+$ cell counts below 200 cells/µL are associated with AIDS-defining invasive fungal diseases such as pneumocystis pneumonia (*Pneumocystis jirovecii*), cryptococcosis (*Cryptococcus neoformans*), and histoplasmosis (*Histoplasma capsulatum*) (5). Blastomycosis is not an AIDS-defining infection but from existing case reports, $CD4^+$ deficiency appears to increase the risk of widespread atypical dissemination and central nervous system (CNS) involvement (14,15). The increased incidence and severity of fungal infections due to targeted immunomodulatory therapies and HIV reveal important immune factors required to protect against these infections.

The most common invasive fungal pathogens are nosocomial *Candida* and environmentally ubiquitous *Aspergillus* both belonging to the Ascomycota phylum. *Candida* species, such as *C. albicans*, *C. auris*, *C. glabrata*, and *C. parapsilosis*, are commonly found as commensals in humans on mucosal and epithelial tissues of the oral cavity, gastrointestinal, and genitourinary tracts (16). Infection typically occurs in the setting of host immunosuppression enabling mucocutaneous surface infections and invasion into tissues and the bloodstream (16). *Aspergillus* species are saprophytic fungi that exist in a filamentous hyphal form in the environment. Inhalation of *Aspergillus* spores can exacerbate asthma (allergic aspergillosis) and

in the setting of immunosuppression, infection can develop into chronic pulmonary or invasive aspergillosis (16). Another group of pathogenic fungi are the *Onygenales* family of thermally dimorphic fungi in the Ascomycota phylum characterized by their conversion from environmental hyphae to yeast at higher temperatures (30-37°C) when inhaled by a host. Clinically relevant members include *Blastomyces*, *Histoplasma*, *Coccidioides*, *Paracoccidioides*, and *Emergomyces*. Additional clinically relevant thermally dimorphic fungi include *Talaromyces* and *Cryptococcus* although the latter is unique, belonging to the Basidiomycota. *Histoplasma capsulatum* and *Coccidioides* species *C. immitis* and *C. posadasii* are found in North America and regions of South America and are the causative agents of histoplasmosis and coccidioidomycosis, respectively (11). Both are characterized by high rates of latent asymptomatic or acute infections with the potential for reactivation in the setting of immunosuppression (16). Paracoccidioidomycosis is caused by *Paracoccidioides brasiliensis* and *P. lutzii* and limited to Brazil and surrounding countries (11). Emergomycosis, caused by *Emergomyces* spp., is diagnosed sporadically with few diagnostic tests available but has been reported in Africa, North America, Europe, and Asia (18). Phylogenetically, *Blastomyces*, *Histoplasma*, *Emergomyces*, and *Paracoccidioides* are closely related within the *Ajellomycetaceae* family (17). Penicilliosis, caused by *Talaromyces marneffei*, is limited to Southeast Asia and often associated with advanced HIV and immunosuppression (11,19). Overall, thermally dimorphic fungi represent a large portion of the pathogenic fungal species owing to their ability to survive and thrive at high internal temperatures of mammalian hosts.

1.2 *Blastomyces* spp. Epidemiology, Infection, and Disease

1.2.1 Molecular Characteristics of Blastomyces

Blastomyces spp. are thermally dimorphic fungi and etiological agents of blastomycosis in humans. *Blastomyces* belongs to the Ascomycota phylum and *Ajellomycetaceae* family and characterized by an environmental mold phase as septate hyphae and yeast phase at temperatures around 33-37°C (17,21). The life cycle of *Blastomyces* is consistent with the Ascomycota and other *Ajellomycetaceae* with both sexual and asexual cycles. The sexual life cycle is heterothallic with two mating-type loci "α" alpha domain (MAT1-1) and the "a" HMG

domain genes (MAT1-2) (22). Heterothallic sexual reproduction requires two haploid hyphal structures, the ascogonium and antheridium from opposite mating types to fuse and form the diploid zygote and cleistothecium fruiting body (22,23). The zygote undergoes meiosis followed by mitosis to form eight ascospores (22,24). The asexual life cycle of *Blastomyces* hyphae involves the formation of genetically identical haploid conidiospores. Ascospores and conidiospores are then released into the environment. Inhalation of the aerosolized spores is the most common route of entry for *Blastomyces* infection and conidiospores appear to be the more likely cause than the sexual ascospores. McDonough and Lewis reported that out of 76 clinical isolates of *Blastomyces*, only one formed a cleistothecium when paired with itself noting potential contamination of heterokaryotic conidia rather than ascospore infection (23). The spores convert to single broad-based budding yeast at internal host temperatures through the activation of the dimorphism-regulating histidine kinase (DRK1) for dimorphic phase transition (25). During this conversion, the fungal cell surface changes from predominantly 1,3-β-glucan to 1,3-α-glucan. In the yeast phase, the cell surface contains chitin, 1,3-α-glucan, low amounts of 1,3-β-glucan, mannans, and other glycoproteins such as the *Blastomyces* adhesion 1 (BAD1) protein bound to chitin. The single broad-based budding of *Blastomyces* is unique and differs from other dimorphic fungi such as *Histoplasma* and *Emergomyces* which have narrow-based budding and *Paracoccidioides* with multi-budding from the parent yeast (17,18).

1.2.2 Epidemiology and Ecological Niche

Blastomyces is endemic to North American and Africa with sporadic cases outside these regions. The *Blastomyces* genus is composed of the following species: *B. dermatitidis*, *B. gilchristii*, *B. helicus*, *B. percursus*, *B. parvus,* and *B. emzantsi* (26). *B. dermatitidis* is the predominant causative agent of blastomycosis in North America followed by *B. gilchristii*, *B. helicus*, and *B. parvus*. The endemic regions of *Blastomyces* in Canada are spread across southern Saskatchewan (27), Manitoba (28), Ontario (29), and Québec along the St. Lawrence River (30). *B. helicus* has been reported in western Canada (Alberta and Saskatchewan) (31). A sporadic case of cutaneous blastomycosis was also reported in New Brunswick (32). Blastomycosis in Canada is most prevalent in northwestern Ontario with incidence rates

between 1.87 and 7.11 cases per 100,000 population reported by Crampton et al. (28) and 0.87-10.94 cases per 100,000 population reported by Brown et al. (29). Hyperendemic regions in northwestern Ontario includes the Kenora catchment area with an incidence rate of 117.2 (95% confidence interval (CI): 90.3-152.4 per 100,000 population) and surrounding communities with incidence rates upwards of 404.9 cases per 100,000 population (33). The incidence rates of blastomycosis in Manitoba range from 0.17 to 7.11 cases per 100,000 population (28). The city of Winnipeg has the highest incidence of blastomycosis at 1.87 to 7.11 cases per 100,000 population. The frequent travel of inhabitants to Kenora and northwestern Ontario during the spring and summer months likely contributes to the incidence of blastomycosis, however, cases have also been reported among persons who have not left the city. Blastomycosis in the United States is found in most midwestern states along the Mississippi River, the Great Lakes, and states along the east coast (11). Cases of blastomycosis are reported to public health in Wisconsin, Minnesota, Michigan, Louisiana, and Arkansas (34) although blastomycosis has been reported in most midwestern and east coast states (11). Wisconsin has the highest incidence rate of blastomycosis with hyperendemic regions and several outbreaks reported (35,36). Benedict et al. report annual incidence rates near 3 cases per 100,000 population in Wisconsin with rates upwards of 10-42 cases per 100,000 population in the Menominee, Lincoln, and Vilas counties (34). Outbreaks of blastomycosis have been reported in Wisconsin including 48 cases in Vilas county (1984) and 55 cases in Marathon county (2013) (35,36). Blastomycosis cases have also been reported across Africa with *B. dermatitidis* as the causative agent (11). Maphanga et al. performed whole genome sequencing on 20 *Blastomyces* clinical isolates from South Africa between 1964 and 2014 revealing isolates previously assigned to *B. dermatitidis* were *B. percursus* and *B. emzantsi* (37). Schwartz et al. further confirmed *B. percursus* and *B. emzantsi* as the major *Blastomyces* species in Africa and the Middle East with few cases of blastomycosis caused by *B. dermatitidis* and *B. gilchristii* (38).

Blastomyces has been isolated and traced back to areas with moist organic-rich soil and decaying matter near freshwater ecosystems (39,40). Patient exposures include beaver dams (35,41), a water-exposed petroleum filtering shed (42), railroad work (43), forestry (44), paper mills (45), road construction (46), and canoeing and camping (47). These cases were located in

endemic regions with a combination of sporadic cases (41–43) and outbreaks (35,45–47). However, *Blastomyces* was only isolated in two of these studies (35,42). Attempts to isolate and determine the exact niche of *Blastomyces* remains a challenge with inconsistent isolation likely due to its periodic appearance highly dependent on environmental conditions (48). Successful methods include the direct inoculation of mice with environmental sample to propagate for culturing and microscopic analysis (35,42) as well as polymerase chain reaction (PCR)-based sequencing of environmental samples (48). *In vitro* studies of *Blastomyces* corroborate the requirement for moisture and proximity to waterways to aid in releasing asexual conidia from the hyphae that may ultimately be inhaled resulting in infection (49).

1.2.3 Blastomyces infection and Blastomycosis

Environmental *Blastomyces* in the hyphal phase can release and aerosolize spores when disrupted which are subsequently inhaled by a mammalian host (50). A phase transition of the spores into the yeast phase occurs at the internal host temperature around 37°C. *Blastomyces* establishes infection within the lungs utilizing yeast-specific virulence factors such as BAD1 for adhesion to innate phagocytic cells (neutrophils, macrophages, and dendritic cells (DC)) and the epithelial barriers. Innate cells phagocytose spores and yeast but can fail to kill *Blastomyces* resulting in intracellular replication and implying a dual extracellular and intracellular role in infection (51). The incubation period for the development of blastomycosis after successful infection is 2 to 6 weeks (21). Blastomycosis is most often limited to the lungs with clinical manifestations ranging from asymptomatic infection, mild acute bacterial pneumonia-like disease with uncomplicated resolution, to life-threatening acute respiratory distress syndrome (ARDS) or chronic granulomatous lung disease (21). Patients with mild blastomycosis are often not diagnosed in cases of sporadic infection. Roughly 50% of individuals in blastomycosis outbreaks have been found to be asymptomatic with self-limiting infections (21,35,39). The remaining 50% of individuals present with fever, coughing, night sweats, chills, chest pain, weight loss, and shortness of breath often requiring antifungal therapy (21,35). Acute manifestations are similar to bacterial or viral pneumonia and in more severe cases, can progress to respiratory failure and ARDS. Chronic manifestations of blastomycosis includes a

granulomatous presentation similar to tuberculosis and lung malignancies with radiological findings of lung consolidations and masses (21,39). Hematogenous dissemination and extrapulmonary blastomycosis occurs in around 25-40% of patients most commonly to the skin, bones, CNS, and genitourinary tract (21). Infection can also be acquired through direct inoculation of cutaneous wounds but this is rare (21). Immunocompromised patients are at a higher risk of dissemination and more severe manifestations contributing to a higher mortality rate from blastomycosis (52). Persons living with HIV and idiopathic $CD4^+$ lymphocytopenia (ICL) with low $CD4^+$ cell counts have been observed to have atypical dissemination to the liver, kidneys, spleen, pancreas, adrenal gland, lymph nodes, and heart (53–55). The uncommon and endemic nature of blastomycosis and similarities to more common diseases can often lead to delays in diagnosis and treatment with empirical antibiotics, antimycobacterials, and, in certain cases, immunosuppressive agents (56,57). These delays are associated with a higher risk of mortality from blastomycosis and highlight the requirement of accurate diagnostic tools for expedited antifungal therapy (9).

1.2.4 Diagnosis and Treatment

The diagnosis of blastomycosis is complicated by the need for diagnostic tools not routinely used. A high degree of suspicion for fungal etiology is required and the inclusion of blastomycosis in the differential diagnosis can differ by geographical location (presence in endemic region) and awareness of *Blastomyces*. Current diagnostic methods for blastomycosis include culturing, microscopy, enzyme immunoassay (EIA), complement fixation, immunodiffusion antibody detection, and PCR (58). Culture and microscopy are the gold standards for diagnosing blastomycosis (59). Staining of respiratory secretions (sputum or bronchoalveolar lavage (BAL)) for microscopy and identification of broad-based budding yeast is the most common method for diagnosing blastomycosis in endemic areas. Culture takes approximately 1-4 weeks to grow, limiting use for prompt diagnosis, but establishes a definitive diagnosis (21). However, both methods often require samples that may be difficult to obtain such as sputum and BAL fluid or require invasive methods such as lung biopsy (60). The MVista® *Blastomyces* EIA test is an antigen test for diagnosis of blastomycosis from urine, cerebrospinal

fluid, serum, and BAL fluid (59–61). Cross reactivity of the EIA test with *Histoplasma* reduces the ability of the test to definitively diagnose blastomycosis with the overlap of endemicity of both pathogens (11). No commercial PCR diagnostics are available for *Blastomyces* detection but real-time PCR has been used to detect *Blastomyces* DNA in clinical specimens with high specificity and sensitivity (62). 1,3-β-glucan detection assay, commercially available as Fungitell®, does not reliably detect *Blastomyces* likely due to surface antigen switching from 60% 1,3-β-glucan and 40% 1,3-α-glucan in the hyphal phase to 95% 1,3-α-glucan and 5% 1,3-β-glucan in the pathogenic yeast phase (63,64). Overall, the diagnosis of blastomycosis relies primarily on culture identification, microscopy, and the EIA tests. Each of these tests is limited by their diagnostic rate with culture and microscopy often requiring difficult to obtain samples with the potential for nondiagnostic outcome and EIA having lower sensitivity. Therefore, the available diagnostics do not rule out blastomycosis. However, successful diagnosis allows physicians to immediately start antifungal therapy and prevent further disease worsening.

The treatment of blastomycosis relies primarily on antifungal therapy to eliminate the fungal infection. Amphotericin B and itraconazole are the recommended antifungal agents depending on the severity of cases. Amphotericin B is a polyene class antifungal agent binding ergosterol, an essential fungal component for cell membrane integrity similar to human cholesterol, with a broad antifungal spectrum and no known resistance in dimorphic fungi (65). The drawback of this antifungal is the requirement for intravenous administration and host toxicity due to host cholesterol binding. The development of the liposomal formulations of amphotericin B has improved host tolerance and has been recommended for treatment of severe and disseminated cases of blastomycosis (65,66). After an induction phase with improvement of severe or disseminated cases on amphotericin B, patients are often switched to itraconazole, a triazole antifungal agent, for 6-12 months to ensure fungal clearance and reduce the risk of blastomycosis relapse (66). Itraconazole is also the recommended antifungal agent for mild and moderate pulmonary blastomycosis (66). The triazole family of antifungals inhibit the synthesis of ergosterol (67). Other triazole antifungals used to treat blastomycosis includes fluconazole, ketoconazole, posaconazole, and voriconazole (66). *Blastomyces* is

intrinsically resistant to echinocandins (caspofungin, micafungin, and anidulafungin) which target surface glucans, reducing their effectiveness for treatment of blastomycosis (66).

1.3 Blastomycosis Risk Factors and Outcomes

1.3.1 Risk Factors for the Acquisition of Blastomycosis

The acquisition of blastomycosis requires exposure to *Blastomyces* with insufficient immunity to clear the infection. The risk factors for *Blastomyces* exposure revolve around the proximity to endemic regions and higher risk activities that disrupt the ground. Choptiany et al. showed that persons with an outdoor occupation in Manitoba or northwestern Ontario had higher risk of acquisition (odds ratio (OR) 3.2 95% CI: 1.2-8.4) compared to those with indoor occupations (68). Outbreaks of blastomycosis corroborate this risk with blastomycosis outbreaks associated with outdoor occupations such as forestry, construction/excavation, and paper mills (44–46). Outdoor activities including hunting, camping, canoeing, fishing, and exploring beaver ponds have documented outbreaks implicating these activities as risk factors for acquiring infection (69). Males have been found to be at a higher risk of acquiring blastomycosis, although this finding may be due to higher proportions of outdoor occupations rather than higher biological susceptibility (10,68,69).

Both immunocompetent and immunosuppressed persons can acquire blastomycosis. However, immunosuppression for any reason has been independently associated with an increased risk of acquiring blastomycosis (68). A history of pneumonia as a marker for decreased immunity was also independently associated with blastomycosis risk (70). A possible explanation may be immune status-dependent variation in infectious fungal dose required to develop blastomycosis (3). Another risk factor for blastomycosis acquisition are immune-mediated diseases (e.g., lupus erythematosus and rheumatoid arthritis) independent of corticosteroid and methotrexate immunosuppression (68). Age above 65 has been associated with an increased risk of blastomycosis although this does not account for higher rates of immunosuppression and comorbidities such as diabetes mellitus (9,71). Individuals of Hmong ancestry have also been reported to be at a higher risk of blastomycosis compared to the

general population due to genetic variation in interleukin 6 gene (*IL6*) and reduced T helper type 17 (Th17) response (36,72).

1.3.2 Risk Factors for Blastomycosis Severity and Mortality

The clinical presentation of blastomycosis is heterogenous ranging from subclinical to severe and life-threatening disease. The risk factors that may predispose individuals to more severe outcomes relate to immune status and the diagnosis and treatment of fungal disease. Immunosuppressive therapy, primary immunodeficiency, or acquired immunodeficiency can impact severity of blastomycosis. McBride et al. and Villacorta Cari et al. conducted retrospective cohort studies that demonstrated that those who were immunosuppressed were at a greater risk of severe pulmonary disease and mortality from blastomycosis (71,73). A systematic review and meta-analysis of blastomycosis mortality by Carignan et al. reported a general mortality rate of 6.6% increasing to 37% for those who are immunocompromised (52). The risk of severe disease and mortality may also be higher for those who are immunosuppressed from solid organ transplantation compared to those immunosuppressed for any other reason (73). There is also an association between age ($\geq$65) and blastomycosis mortality (9), although this again, may be due to a higher rate of immunosuppression amongst older patients (71). Other risk factors include diabetes mellitus and delay in blastomycosis diagnosis. Diabetes mellitus was associated with severe blastomycosis (71) and blastomycosis-related intensive care unit admission (74). Delay in diagnosis may also contribute to disease progression and mortality from blastomycosis (9). Acquired cell-mediated immunodeficiency due to advanced HIV and ICL have been shown to increase the risk of CNS and atypical dissemination to the visceral organs (53). Studies on the contribution of genetic immunodeficiency are limited to a study of genetic variation in *IL6* and a case report of GATA2 deficiency that predisposed individuals to blastomycosis (72,75).

1.4 Antifungal Immunity and Genetic Susceptibility to Invasive Fungal Infections

1.4.1 Physiological and Innate Immune Barriers

Invasive fungal infections are caused by fungal pathogens that evade host barriers and innate immunity to establish infection within a host. Skin and mucosal infections colonize without breaching host barriers. Invasive fungal infections are uncommon often requiring the host to be in an immunocompromised state (3). The routes of entry for most fungi are inhalation and cutaneous inoculation (76). The genitourinary and gastrointestinal tracts are also routes of entry limited to *Candida* and the mycobiome and will not be discussed here (1). Fungal elements are inhaled into the respiratory tract which is lined with an epithelial layer of ciliated epithelial cells and a mucous layer. The mucous layer functions to entrap particulate and microbes, prevent the adherence of pathogens, and utilize antimicrobial peptides such as defensins and surfactants (77). The ciliated epithelial cells push the mucus and collected debris out of the respiratory tract through mucociliary clearance (77). Dysfunction of the mucous membranes or defective clearance mechanisms such as primary ciliary dyskinesia (PCD), an autosomal recessive (AR) genetic disorder caused by genetic variants in genes involved in ciliary structure and function, enable fungal invasion of the respiratory tract (78,79). Similarly, cystic fibrosis (CF) leads to higher risk of acquiring *Aspergillus* infections due to highly viscous mucus preventing mucociliary clearance of *Aspergillus* spores (80). The skin is another physiological barrier composed of stratified squamous epithelial tissue that is connected by tight junctions and continuously shed, preventing fungal entry. Breach of the epithelial tissue is required for cutaneous inoculation of fungal elements (3). Once inside the host, invasive fungal infections must also adapt to mammalian endothermy, the homeostatic core temperature of humans and mammals around 37°C, that prevents the growth of most known fungi (3,81).

1.4.2 Fungal Pathogen Recognition

The most critical step in antifungal immunity is the host recognition of invading fungal pathogens. Innate immune cells, macrophages, neutrophils, DCs, and epithelial cells, express pathogen recognition receptors (PRRs) that recognize common fungal antigens to initiate the innate immune response. Two families of PRRs have been shown to be important in antifungal

immunity: c-type lectin receptors (CLRs) and toll-like receptors (TLRs) (82). Dectin-1, dectin-2, mincle, and mannose receptor are CLRs that recognize fungal β-glucans, α-mannans, α-mannose, and mannosylated polysaccharides, respectively (83,84). Antigen recognition by CLRs initiates signaling cascades through spleen tyrosine kinase (SYK) and the caspase activation and recruitment domain family member 9 (CARD9)-BCL10 immune signaling adaptor (BCL10)-mucosa-associated lymphoid tissue 1 (MALT1) signalosome complex to activate nuclear factor κB (NF-κB) signaling (83,85,86). Mincle signals through the Fc receptor γ chain (FcRγ) and SYK to the CARD9-BCL10-MALT1 signalosome to activate NF-κB signaling (87). Genetic deficiencies in the above-mentioned genes can predispose individuals to fungal infections. For example, AR loss of function (LOF) variants in *CARD9* increase susceptibility to chronic mucocutaneous candidiasis (CMC), invasive candidiasis, and aspergillosis due to reduced tumour necrosis factor alpha (TNF-α) expression and Th17 cell differentiation (88,89). TLRs are a family of PRRs recognizing a variety of fungal, bacterial, and viral antigens. The extracellular TLR1, TLR2, TLR4, and TLR6 as well as endosomal TLR9 have been shown to recognize fungal antigens (90–93). The general mechanism for TLRs is antigen recognition and binding followed by homodimerization or heterodimerization to initiate downstream signaling through myeloid differentiation primary-response gene 88 (MyD88) (94). TLR2 recognizes phospholipomannan and zymosan (1,3-β-glucan), homo- or hetero-dimerizes with TLR1 or TLR6 to initiate signaling, and activates NF-κB and activating protein 1 (AP-1) for the expression of proinflammatory cytokines (76,84,94). TLR4 is well known for its binding to bacterial lipopolysaccharide but it also binds fungal surface O-linked mannans (83). TLR9 is found within endosomes of innate immune cells and recognizes unmethylated single stranded DNA CpG islands from phagocytized pathogens (94). Genetic deficiencies in the TLRs increase susceptibility to fungal infections. Genetic variants impairing the expression or function of TLR1, TLR2, TLR4, TLR6, and TLR9 are associated with increased risk of candidiasis and aspergillosis (90,92,93). Knockout of *MyD88* in mice prevented effective vaccination against *B. dermatitidis*, *H. capsulatum*, and *C. posadasii* with impaired interleukin (IL) 17 and interferon gamma (IFN-γ) production (95).

1.4.3 Innate Immunity

Alveolar macrophages and epithelial cells are the first innate immune cells to recognize fungi in the lungs. Lung epithelial cells detect fungal pathogens including *B. dermatitidis*, leading to increased production of IL-17 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (96). Alveolar macrophages are tissue-resident macrophages activated with GM-CSF (97). Upon the recognition of fungal antigens with PRRs, alveolar macrophages phagocytose and entrap fungi within intracellular phagosomes. These are low pH intracellular compartments containing antimicrobial compounds to lyse fungal cells (97). Antigens from the lysed fungal cells can be processed and presented on major histocompatibility complex (MHC) molecules for the activation of Th effector cells (97). Signaling cascades from PRRs on innate immune cells upregulate the production of proinflammatory cytokines including TNF-α, IL-12, IL-6, IL-1β, IL-23, and GM-CSF, as well as chemokines such as CXC chemokine ligand (CXCL) 2 and CXCL8 (or IL-8) for the recruitment of immune cells to the site of infection (77,98). Hematopoietic-derived innate immune cells, polymorphonuclear neutrophils, monocyte-derived macrophages, and DCs, are recruited to the site of infection to enhance the innate inflammatory response. Neutrophils are the most abundant innate immune cell and the first to reach the site of infection. They are short-lived immune cells that utilize three main mechanisms for controlling infections: phagocytosis, degranulation, and neutrophil extracellular traps (NET). Neutrophils recognize fungi with PRRs to initiate their antifungal mechanisms. Phagocytosis is similar to macrophages with the addition of myeloperoxidase (MPO) to form HOCl from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase generated H_2O_2 and chlorides to kill fungal cells (99). Degranulation is the extracellular release of granules containing antimicrobial compounds to lyse fungal cells (100). NET is an infection control mechanism where neutrophils are lysed with their genomic DNA chromatin structures unraveled and combined with antimicrobial peptides. This keeps invading pathogens entangled to reduce spread and kills fungal cells (83).

Innate immune cells rely on these antimicrobial mechanisms to kill invading fungi. The impairment of these mechanisms can prevent innate cells from controlling infection and potentially worsening fungal disease. Genetic deficiency of CXC motif chemokine receptor

(CXCR) 1 reduces neutrophilic degranulation and *Candida* killing, increasing susceptibility to *Candida* infection in humans (101). Chronic granulomatous disease (CGD) is a genetic disorder that reduces the effectiveness of the NADPH oxidase system (102). Loss of NADPH oxidase function prevents reactive oxygen species (ROS) generation for fungal killing by oxidative burst. CGD presents as a phagocytic cell defect with characteristic susceptibility to filamentous fungi such as *Aspergillus* (103). Patients with CGD are also at a higher risk of tuberculous mycobacterial infection (102). Oxidative burst in neutrophils is supplemented with MPO (99). Defects in MPO can increase susceptibility to invasive *Candida* infection but MPO is ineffective against *B. dermatitidis* (16,104).

1.4.4 Adaptive immunity

The cell-mediated immune response is critical for antifungal immunity. The communication between innate immune cells and Th cells dictates the course of fungal infection. DCs are phagocytic innate immune cells that play a major role in fungal antigen recognition, processing, and presentation to naïve Th cells. They are found in both lymphoid and nonlymphoid tissues as tissue resident and migratory DCs. DCs can be found adhered to the epithelial layer of the respiratory tract sampling antigens using a broad arsenal of PRRs (105). The detection of invading microbes can result in phagocytosis and antigen processing for MHC presentation. DCs then migrate to the lymph nodes where they present the antigen on MHC molecules to lymph node resident DCs and naïve $CD4^+$ Th cells (105). The cytokine profiles produced from PRR signaling cascades dictate subset differentiation and priming of naïve $CD4^+$ Th cells (98). For effective antifungal immunity, cytokine profiles will generally be of the Th type 1 (Th1) and Th17 responses.

The production of IL-12 by DCs stimulates $CD4^+$ Th cells to differentiate into Th1 cells producing IFN-γ (106). IFN-γ stimulates antimicrobial functions in innate immune cells including the acidification and fusion of phagosomes and lysosomes and the increased production of ROS and reactive nitrogen species (107). The IL-12 and IFN-γ axis is indispensable for dimorphic fungal immunity. Genetic variants in the IL-12 receptor subunit 1 (IL12Rβ1) and IFN-γ receptor subunit 1 (IFN-γR1) have both been implicated in dimorphic fungal infections. AR LOF genetic

variants in *IL12RB1* are reported to contribute to disseminated paracoccidioidomycosis and coccidioidomycosis (108,109). Disseminated histoplasmosis and coccidioidomycosis have been reported in patients with autosomal dominant (AD) LOF genetic variants in IFN-γR1 (110,111). Genetic immunodeficiency in signal transducer and activator of transcription (STAT) 4 involved in the signaling cascade from the IL-12 receptor as a transcription factor to upregulate IFN-γ expression has also been observed in paracoccidioidomycosis (112). The requirement of the Th1 immune response against dimorphic fungal pathogens is similar to antimycobacterial immunity (113). Both groups of pathogens share similar pathogenesis and clinical presentations, often contributing to misdiagnosis (21). These similarities point towards dimorphic fungal immunity and antimycobacterial immunity sharing immunological pathways for infection control.

The differentiation of naïve Th cells into Th17 subsets requires IL-6, transforming growth factor beta (TGF-β), and IL-10 (114). The upregulation of these cytokines can be through the IL-23 pathway. Th17 cells produce IL-17A, IL-17F, IL-22, as well as IFN-γ, GM-CSF, and TNF-α (115). These cytokines are essential for immunity against *Candida* and *B. dermatitidis* (72,116,117). Th17 cells also recruit neutrophils to the site of infection through the upregulation of CXC chemokines (e.g., CXCL1, CXCL2, and CXCL8) (115). Genetic variation in the Th17 pathway has also been implicated in genetic susceptibility to fungal pathogens including dimorphic fungi. Genetic variation in *IL6* has been shown to increase the risk of blastomycosis in persons of Hmong ancestry due to an impaired Th17 response (72). *Candida* infections have also been shown in patients with immunodeficiencies in the Th17 response including LOF variants in IL-23 receptor (*IL23R*), *STAT3*, *IL17RA*, and *IL17F* as well as autoantibodies against the IL-17A, IL-17F, and IL-22 cytokines (113,116,118,119). Hyper-immunoglobulin E syndrome (HIES) is a genetic disorder characterized by elevated IgE and heightened risk of fungal and bacterial infections due to impaired Th17 differentiation (113). Genetic variation in *STAT3*, *DOCK8*, and *ZNF341* can cause AR or AD forms of the disorder and have been implicated in *Candida*, *Aspergillus*, and *Cryptococcus* susceptibility (120–122).

The Th type 2 (Th2) response is typically reserved for allergic and humoral immunity and not generally considered effective for antifungal immunity except for *Pneumocystis* infection

(76). IL-4 is produced by innate immune cells to drive a Th2 response, inhibiting both Th1 and Th17 differentiation (76). Th2 skewed antifungal immune response may also contribute to chronic fungal infection (98). Cytotoxic $CD8^+$ T cells are not generally considered to be involved in the antifungal immune response. However, $CD8^+$ T cells can compensate for $CD4^+$ T cell deficiency in vaccine-induced immunity to *B. dermatitidis* and *H. capsulatum* as well as mucosal *Candida* infections (89,123). Humoral immunodeficiencies are not associated with increased risk of fungal infections. However, passive transfer of antibodies can ameliorate fungal disease outcomes (98).

1.5 Rationale, Hypothesis, and Objectives

1.5.1 Rationale

Blastomycosis is a rare pulmonary disease with vast clinical heterogeneity. Nearly 50% of infected individuals develop blastomycosis requiring medical intervention and antifungal therapy (21). Blastomycosis can progress to severe pulmonary and disseminated forms requiring intensive care measures. The risk factors for the acquisition and severity of blastomycosis largely revolve around the immune status of patients. Immunosuppression for any reason predisposes individuals to severe blastomycosis and mortality from the disease (52,68,73). Genetic immunodeficiencies have not been explored extensively in blastomycosis unlike other dimorphic fungi. Currently, genetic variation in *IL6*, *GATA2*, and *VDBP* are the only genetic markers known to influence susceptibility to blastomycosis (72,75,124). Studies of genetic immunodeficiency in dimorphic fungi have demonstrated similar genetic requirements for immunity as those necessary for mycobacterial disease (109,110,112,125–127). From experimental studies, host immunity to blastomycosis requires the Th1 and Th17 immune responses to control infection (128–131). These similarities suggest that blastomycosis host immunity may follow the trends discovered for general antifungal and antimycobacterial immunity. In this thesis, we have conducted a scoping review to systematically identify the currently known host susceptibility factors for naturally acquired blastomycosis. We have also identified genetic variants in exome sequences from a blastomycosis case-control cohort that may influence susceptibility to blastomycosis and could further support that similar immune

mechanisms for blastomycosis are shared with antifungal and antimycobacterial immunity. The findings of these studies will be beneficial for improving our understanding of host immunity in blastomycosis and potentially inform clinical practice and future therapeutic development.

1.5.2 Hypothesis

We hypothesize that genetic risk factors conferring increased susceptibility to fungal and mycobacterial diseases contribute to an increased risk of developing symptomatic blastomycosis.

1.5.3 Objectives

The objectives for the scoping review on host susceptibility to blastomycosis are as follows:

1. Conduct a systematic scoping review in accordance with the Joanna Briggs Institute (JBI) and Preferred Reporting Items for Systematic review and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR).
2. Establish the current state of knowledge about host susceptibility to blastomycosis.
    a. Identify host susceptibility factors directly required for blastomycosis immunity.
    b. Identify reports of single target immunomodulatory therapies and blastomycosis.
    c. Characterize the contribution of $CD4^+$ immunodeficiency on blastomycosis.
    d. Identify host targets of *B. dermatitidis* virulence factors.
3. Determine the immunological components and pathways known to be involved in blastomycosis immunity.

The objectives for the blastomycosis exome sequencing case-control study are as follows:

1. Conduct a literature review to generate a candidate gene list of genetic risk factors in fungal and mycobacterial diseases.
2. Perform exome sequencing and bioinformatic analysis to identify genetic variants in a blastomycosis case-control cohort.
3. Assess the association of genetic variants with blastomycosis susceptibility.

Chapter 2: Materials and Methods

2.1 Literature Review to Identify Candidate Gene Set

The literature review was conducted to generate a list of genes and genetic variants known to increase susceptibility and severity of fungal or mycobacterial diseases. The candidate list was used to test the hypothesis that blastomycosis susceptibility shares host genetic risk factors with fungal and mycobacterial infections. Primary and secondary peer-reviewed literature was considered for this review. The inclusion of genes required evidence of genetic susceptibility to the relevant infectious diseases. Observational studies (case-control and cohort studies, case reports, and case series) describing genetic associations with infectious disease susceptibility and experimental studies demonstrating molecular dysfunction in response to the infectious agent were included in the literature review (12). Literature describing important genes involved in the function of candidate genes were also included regardless of fungal or mycobacterial susceptibility association. For example, AR LOF IL-12p40 (IL12B) variants are associated with mycobacterial disease due to a loss of IL-12 and IL-23 response (126). The IL-12p35 subunit (IL12A) heterodimerizes with IL12B to form the IL-12 signaling cytokine (126). Therefore, IL12A was also included as a candidate gene. Receptor-ligand partners, quaternary protein structure subunits, signaling cascade adaptor proteins, and co-receptors were also considered for inclusion. This review was not performed systematically and rather through the identification of peer-reviewed primary and secondary literature. As a result, some susceptibility genes in fungal or mycobacterial diseases may have been missed. The candidate gene list can be found in Appendix A.

The included genes were screened to provide contextual information about the infectious agents, the molecular dysfunction contributing to disease susceptibility, and the source of evidence. The resulting primary immunodeficiency was also documented if available. The candidate gene list generated from this literature review was analyzed with STRING (v. 12.0) to identify pathways and biological processes relevant to their function (132). The STRING analysis was performed with the full STRING network, high confidence (0.7) interaction score, and a false discovery rate (FDR) of 5%. Clusters were generated with a k-means clustering setting of 11 and annotated according to STRING provided databases with the most relevant and enriched

pathways. The gene clusters were annotated with Inkscape (v. 1.2) (133). The candidate gene list generated from this literature review was used for downstream analysis. Genetic variants contained within candidate genes were retained to prioritize testing the hypothesis and condense the exome data to facilitate deleterious genetic variant discovery. The list also provides support for putative functional effects of any candidate genetic variants that are supported by *in silico* and statistical analyses.

2.2 Scoping Review Protocol

2.2.1 Study Design and Registration

This scoping review protocol was designed with guidance from the Joanna Briggs Institute (JBI) and Peters et al. (134) and in accordance with the Preferred Reporting Items for Systematic review and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) (135). The protocol was preregistered in the Open Science Framework (https://osf.io/) database under DOI https://doi.org/10.17605/OSF.IO/HZYBU.

2.2.2 Eligibility Criteria

Population

The study population consists of any human or animal diagnosed with blastomycosis, animal model infected with *Blastomyces*, and any *in vitro* cell models (macrophages, neutrophils, DCs, epithelial cells, T cells, B cells, and natural killer (NK) cells). Studies using animal models were included for the purpose of comparing immunity requirements across species where sufficient data exists.

Concept

The main concept of this scoping review was to review the essential immune mechanisms in *Blastomyces* infection and blastomycosis. Invasive fungal diseases are rare and often a result of acquired or cryptic primary immunodeficiencies in otherwise healthy individuals (3). The identification of genetic and immunological causes for susceptibility can inform relevant immune mechanisms and guide the development or repurposing of therapeutic agents for

managing infections (136). This includes clinical studies reporting primary (e.g., inborn errors of immunity (IEI)) and secondary immunodeficiencies (e.g., advanced HIV), autoimmune disorders (e.g., autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)) (137), and immunomodulating medications (e.g., TNF-α and IL-6 blockers) (138). Genetic and immunological studies using *in vitro*, *in vivo*, *in situ*, and *in silico* methods in cell lines, animal models, and bioinformatic analyses of any of the above were considered to characterize host susceptibility to blastomycosis. Experimental studies exploring *Blastomyces* virulence factors that target host factors were also considered to characterize pathogen requirements for infection. The summarized inclusion criteria can be found in Table 1. Environmental and behavioural risk factors were considered although their utility as markers for specific host immune mechanisms may be limited and rather serve as potential confounders.

Table 1. Summary of study eligibility criteria.

Inclusion criteria	Studies examining host susceptibility in the context of *Blastomyces* spp. infection and blastomycosis
	Studies reporting clinical, genetic, or immunological deficiencies (e.g., primary or acquired immunodeficiencies, autoimmune disorders, immunomodulatory medications, inborn errors of immunity, etc.)
Exclusion criteria	Secondary or tertiary literature
	No mention of blastomycosis
	No mention of host immunity in clinical, genetic, or immunological contexts
	Not written in English, French, or Spanish

Context

This scoping review considered any clinical study or basic research reporting host-specific mechanisms impacting susceptibility to *Blastomyces* spp. infection and development of blastomycosis. Case reports and case series describing relevant contributing factors such as immunomodulatory medications were included. Studies using tissues, cell models, animal models, and computational analyses can reveal insights into the host immunity and susceptibility that may not present clinically. Therefore, these studies were included to build the network of host susceptibility in addition to the clinically present genetic and immunological findings. Studies broadly examining immunity or with limited interpretation of

the specific underlying immune mechanisms were excluded. Serological studies were excluded as they are often for the purpose of developing diagnostics and their context of host susceptibility is limited. Clinical studies and case reports describing only corticosteroids or broadly immune dampening medications may not allow for deriving specific immune mechanisms. Therefore, these studies were also excluded. The focus of this scoping review was on naturally acquired immunity rather than vaccine-induced immunity. This is due to blastomycosis vaccination being conducted only in mouse models with the use of adjuvants and intradermal administration. These conditions may not be directly comparable to natural immunity.

2.2.3 Information Sources

The PubMed, MEDLINE, EMBASE, Scopus, and Cochrane bibliographical databases were used to search for primary literature published in or before February 2023. Secondary literature containing relevant sections on host susceptibility to blastomycosis was scanned to extract references. The reference section and articles citing each included paper were also assessed to include literature not captured in the database search.

2.2.4 Search Strategy

Peer-reviewed primary literature and non-reviewed grey literature (conference abstracts) published in English, French, and Spanish were considered for eligibility in the scoping review. The terms “blastomycosis” and “*Blastomyces*” were included in all searches to capture any literature primarily or partially studying blastomycosis. To refine the search to host immunity studies, the following terms including variations were used: “predisposition”, “immunodeficiency”, “immunosuppression”, “immunocompromise”, “idiopathic”, “autoimmune”, “inflammatory”, “autoantibody”, “monoclonal”, “inhibitor”, “phagocyte”, “lymphocyte”, “monocyte”, “cytokine”, “chemokine”, “innate”, “cell-mediated”, “humoral”, “pattern recognition receptor”, “PRR”, “lectin”, “TLR”, “toll-like”, “mannose”, “complement”, “opsonize”, “inborn error of immunity”, “IEI”, “PID”, “PIDD”, “heterozygous”, “homozygous”, “loss of function”, “gain of function”, and “haploinsufficiency”. These terms attempt to capture

clinical, observational, genetic, immunological, and molecular studies of host susceptibility to blastomycosis. The list of search strategies used for each database can be found in Table 2. The papers were downloaded and exported to Rayyan (www.rayyan.ai) to remove duplicates and assess abstracts for eligibility. The eligible papers were then exported into Mendeley for full-text assessment for inclusion and data extraction.

Table 2. Bibliographical database search strategy.

Database	Search strategy
PubMed	((Blastomycosis) OR (blastomyces)) AND (((immun*) OR (predispose*) OR (cytokine) OR (idiopath*) OR (immunomodulat*) OR (immunodeficien*) OR (immunosuppress*) OR (immunocompromis*) OR (idiopath*) OR (autoimmun*) OR (inflammatory) OR (autoantibod*) OR (monoclonal) OR (inhibitor) OR (phagocyt*) OR (lymphocyte) OR (monocyte) OR (humoral) OR (cell-mediated) OR ("inborn errors of immunity") OR (IEI) OR (PID) OR (PIDD) OR (heterozygous) OR (homozygous) OR ("loss of function") OR ("gain of function") OR ("haploinsufficiency") OR (PRR) OR ("pattern recognition receptor") OR (innate) OR (chemokine) OR (lectin) OR (TLR) OR (toll-like) OR (mannose) OR (mannose-binding) OR (complement) OR (opson*)))
EMBASE & MEDLINE	((Blastomycosis or Blastomyces) and (immun* or predisposition or cytokine or idiopath* or immunomodulat* or immunodeficient* or immunodeficien* or immunosuppress* or immunocompromise* or autoimmun* or inflammatory or autoantibod* or monoclonal or inhibitor or phagocyt* or lymphocyte or monocyte or humoral or cell-mediated or inborn errors of immunity or IEI or PID or PIDD or heterozygous or homozygous or loss of function or gain of function or haploinsufficiency or PRR or pattern-recognition or innate or chemokine or lectin or TLR or toll-like or complement or opson* or mannose or mannose-binding)).ti,ab.
Scopus	((TITLE-ABS (blastomycosis) OR TITLE-ABS (blastomyces)) AND (TITLE-ABS-KEY (immun*) OR TITLE-ABS-KEY (predisposition) OR TITLE-ABS-KEY (cytokine) OR TITLE-ABS-KEY (idiopath*) OR TITLE-ABS-KEY (immunomodulat*) OR TITLE-ABS-KEY (immunodeficien*) OR TITLE-ABS-KEY (immunosuppress*) OR TITLE-ABS-KEY (immunocompromis*) OR TITLE-ABS-KEY (autoimmun*) OR TITLE-ABS-KEY (inflammatory) OR TITLE-ABS-KEY (autoantibod*) OR TITLE-ABS-KEY (monoclonal) OR TITLE-ABS-KEY (inhibitor) OR TITLE-ABS-KEY (phagocyt*) OR TITLE-ABS-KEY (lymphocyte) OR TITLE-ABS-KEY (monocyte) OR TITLE-ABS-KEY (humoral) OR TITLE-ABS-KEY (cell-mediated) OR TITLE-ABS-KEY ("inborn errors of immunity") OR TITLE-ABS-KEY (iei) OR TITLE-ABS-KEY (pid) OR TITLE-ABS-KEY (pidd) OR TITLE-ABS-KEY (heterozygous) OR TITLE-ABS-KEY (homozygous) OR TITLE-ABS-KEY ("loss of function") OR TITLE-ABS-KEY ("gain of function") OR TITLE-ABS-KEY (haploinsufficiency) OR TITLE-ABS-KEY (PRR) OR TITLE-ABS-KEY ("pattern recognition receptor") OR TITLE-ABS-KEY (innate) OR TITLE-ABS-KEY (chemokine) OR TITLE-ABS-KEY (lectin) OR TITLE-ABS-KEY (TLR) OR TITLE-ABS-KEY (toll-like) OR TITLE-ABS-KEY (complement) OR TITLE-ABS-KEY (opson*) OR TITLE-ABS-KEY (mannose*) OR TITLE-ABS-KEY (mannose-binding*)))
Cochrane	"Blastomycosis" OR "Blastomyces"

2.2.5 Selection Strategy

One reviewer screened the titles and abstracts of all journal articles identified across database searches. Uncertainty in eligibility was resolved through discussion with the other authors. The same reviewer also assessed full-text articles for inclusion and performed data extraction. Resolution of uncertainty again occurred through discussion with the other authors.

2.2.6 Data Extraction

Data from the included literature was extracted using the data extraction template adapted from the JBI data extraction template (Table 3) (139). The template is composed of four categories: study characteristics, participant characteristics, study methodology, and principal study findings. Study characteristics captured basic information such as the author(s), the year of publication, the type of study and the general design, as well as the objectives set by the author(s). Participant characteristics is broad and captured information relevant to the type of study. Any population(s) of interest, mammalian species, cell types, and tissues used were extracted. Eligibility criteria, sample size, age, sex, comorbidities, medications, and any relevant risk factors were also extracted if available. The study methodology consisted of extracting the general study design and the techniques used to derive the study findings. Study findings sought to extract information regarding host susceptibility and consisted of identified host genes, proteins, pathways, and mechanisms of immunity as well as any reported clinical or immunological deficiencies. The proportion of subject phenotypes and proposed genotype or characteristics was also extracted in addition to any reported environmental, behavioural, or clinical risk factors. Statistical analyses and supporting evidence for the findings were extracted. The process and template for extracting data was an iterative process. A pilot data extraction was conducted to refine the data collection strategy by modifying the template.

Table 3. Data extraction template for the scoping review.

Study Characteristics	
Title	
Year	
Authors	
Journal	
Location	
Type of study	
Study design	
Objective(s)	
Participant Characteristics	
Species	
Cell type(s) (if applicable)	
Population(s) (if applicable)	
Eligibility criteria (if applicable)	
Sample size(s)	
Age	
Sex	
Comorbidities & medications	
Risk factors (if applicable)	
Treatment	
Study Findings	
Methodology	
Experimental host factors	
Primary immunodeficiency	
Secondary immunodeficiency	
Immunomodulatory medications	
Deficiencies	
CD4 Lymphocyte counts	
Etiology/phenotype	
Genotype frequencies	
Statistical evidence	
Evidence	
Confounding evidence	
Supporting papers	
Comments and concerns	

2.2.7 Data Analysis and Presentation

The collected data was used to inform the current state of knowledge in blastomycosis host immunity and susceptibility. A flow chart demonstrating the process for including and presenting the number of included papers was generated using Inkscape (v. 1.2). In addition, the included papers were further categorized by study findings as follows: host susceptibility factors and immunodeficiencies, immunomodulatory agents, $CD4^+$ T cell deficiency, and host targeting *Blastomyces* virulence factors. Each included study's findings were aggregated by category to summarize the contribution to host susceptibility to blastomycosis. Findings of host susceptibility in humans and other susceptible mammals were also compared and discussed.

2.3 Blastomycosis Case-Control Exome Sequencing Study Design

2.3.1 Ethics Statements

The study of genetic risk factors for acquisition of blastomycosis was approved by the University of Manitoba Biomedical Research Ethics Board H2003:006.

2.3.2 Genetic Risk Factors for Acquisition of Blastomycosis

This study, originally proposed in 1999, was designed to explore genetic risk factors for acquiring blastomycosis. The initial proposal was to identify polymorphisms in 15 genes relating to the recognition of microbial surface antigens and innate immune response in a blastomycosis case-control cohort. An epidemiological study was conducted in the same blastomycosis case-control cohort to identify behavioural and environmental risk factors for the acquisition of blastomycosis. The results of this study were published by Choptiany et al. in 2009 (68). Delays in the genetic analysis and advancement of genetic technologies led to the modification of the genetic study to use an exome sequencing approach. This approach has major advantages over the original study as polymorphisms across the entire exome, including the 15 genes, were sequenced, and explored as genetic risk factors for blastomycosis acquisition. The wealth of data generated allowed for extensive analysis but introduced different limitations to the interpretation of the results.

The present study was initially carried out in 2011 with the recruitment of participants from the original epidemiological study and the inclusion of participants that had recently been diagnosed and treated for blastomycosis. The original participants were contacted by Dr. John Embil at the Health Sciences Centre (HSC) in Winnipeg, Manitoba. Additional participants were recruited through advertisements posted at the HSC and surrounding clinics as well as in Kenora, Ontario. Cases or individuals diagnosed and successfully treated for blastomycosis were recruited to participate in the exome sequencing study. These participants each selected a control with similar risk factors for acquiring blastomycosis. In total, 30 cases and 20 controls were enrolled between 2011 and 2014 to participate in the genetic study. Informed consent was obtained from each participant at the HSC clinic. A blood sample was collected from all participants. Only basic contact information was collected as this study was intended to be pilot for a larger case-control cohort exome sequencing study.

2.4 Exome Sequencing

The blood sample processing was conducted by Dr. Yoav Keynan's laboratory at the University of Manitoba. Genomic DNA was extracted from each blood sample from the case-control cohort using the Qiagen DNeasy Blood & Tissue Mini Spin Column kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The concentration of DNA was determined using the Qubit 3 fluorometer. The DNA samples were transferred to the JC Wilt Infectious Diseases Research Centre (JCW) for storage and exome sequencing. The exome sequencing was conducted by Dr. Emma Lee in 2016 at the JCW. Paired end 150 bp DNA exome libraries were prepared with the Illumina Nextera Rapid Capture Exome kit and Illumina human exome panel (Illumina, San Diego, United States). Each sample was individually sequenced on the Illumina MiSeq platform to obtain an average of 30 times exome depth. The Illumina enrichment sequencing analysis report (v. 2.5.1.3) was generated for each exome to assess sequencing run quality and report the exome sequencing summary statistics.

2.5 Bioinformatic Processing and Analysis

2.5.1 Bioinformatic Processing

The Illumina sequencing pipeline output paired-end exome sequencing files in FASTQ format. There were 28 samples with 56 FASTQ files saved on the Digital Research Alliance of Canada research network clusters. Quality control with FastQC (v. 0.11.9) was performed on each paired-end sequencing file for independent confirmation of the Illumina enrichment sequencing analysis results. No adapter sequences were detected, and most sequencing files passed the per base sequence quality check. No samples were removed after the quality check. The Genome Analysis ToolKit (GATK) best practices for detecting germline mutations was used as a standardized protocol for exome sequence processing and variant discovery with compatibility for downstream analysis (140). Exome sequencing studies for the discovery of genetic immunodeficiencies and IEI have used the GATK best practices workflow for case-control cohorts (141,142) and single-patients (112,143).The Picard software (v. 2.23.3) and GATK (v. 4.1.9.0) tool suites were used for most pre-processing of the exome sequences (140,144).

The paired FASTQ files were converted to unmapped BAM format with Picard FastqToSam to add read groups which includes the following sequencing information: sequencing platform, library preparation kit, sample name, and unique read group identifier. For all samples, the Illumina MiSeq, Illumina Nextera kit, and unique sample and read group identifiers were used for the read groups. This information is required for the assessment of sequencing biases and differences between sequencing runs. The Picard MarkIlluminaAdaptors tool tagged any adaptor sequences for downstream removal to reduce artifact sequences. Unmapped BAM files were converted to single interleaved FASTQ files containing matched paired-ends of each exome sequence with Picard SamToFastq. The output FASTQ was used for reference-based sequence alignment with the Burrow-Wheeler Aligner maximal exact matches (BWA-MEM) (145). BWA-MEM (v. 0.7.15) was run with default settings with the following inputs and options: enabled multi-threading, input query set to interleaved paired-end FASTQ, and the “M” flag for downstream Picard compatibility with local primary alignments (removal of shorter secondary alignments). The GRCh38 human reference genome was used for BWA-MEM

reference-based sequence alignment as the most up-to-date human genome build with downstream bioinformatic software compatibility. The GRCh38 reference-aligned BAM output from BWA-MEM was merged with the unmapped BAM containing the read group sequencing information with the Picard MergeBamAlignment tool. The merged BAM alignment retained insertions and deletions and clipped overhanging bases to keep only the matching, paired-end sequences. The merged BAM alignment was then run through Picard MarkDuplicates to mark and remove duplicates and artifactual paired end reads that could inflate variant confidence (146). The final pre-processing step was base quality score recalibration with GATK tools BaseRecalibrator and ApplyBQSR. This step improves the accuracy of base calling confidence with an empiric machine learning model that adjusts quality scores to reduce the impact of systematic sequencing errors (147). The GRCh38 Single Nucleotide Polymorphism Database (dbSNP) v.138 and Mills and 1000 Genomes (1000G) gold standard indel databases were used for base recalibration of SNPs and indels, respectively.

2.5.2 Variant Identification and Filtering

Genetic variants were identified from the base quality score recalibrated exome sequencing alignments using the GATK HaplotypeCaller tool. Genomic variant calling file (GVCF) outputs for each individual were generated using the default settings. A database of the per-sample GVCFs was created with GATK GenomicsDBImport for streamlining of joint call genotyping with GATK GenotypeGVCF. Default settings with the optimization option --genomicsdb-shared-posixfs-optimizations was used for both tools. Variant discovery at the joint call genotyping step allows for variant information across all samples to be used to improve the sensitivity and provide more accurate genotypes (140). The output variant calling file (VCF) from GenotypeGVCF was the starting VCF for variant filtering.

The genetic variants were filtered according to GATK best practices (140). Due to the small sample size of 28, below the minimum recommended 30 samples for variant quality score recalibration, we performed GATK hard filtering of genetic variants according to the best practices for single nucleotide polymorphisms (SNPs) and insertions and deletions (indels). The SNPs were filtered as follows: quality by depth (QD) < 2, quality (QUAL) < 30, strand odds ratio

(SOR) > 3.0, Fisher strand (FS) > 60.0, root mean square mapping quality (MQ) < 40.0, -12.5 < mapping quality rank sum test (MQRankSum) < 12.5, and -8.0 < read position rank sum test (ReadPosRankSum) < 8. Indels were filtered as follows: QD < 2.0, QUAL < 30.0, FS > 200.0, and -20.0 < ReadPosRankSum < 20.0. The genetic variants were further filtered to improve variant quality according to Carson et al. with VCFtools v.0.1.16 and SnpSift v.5.0 as follows: per-variant missingness > 12%, genotype quality (GQ) < 20, and depth of coverage (DP) < 8 (148). The VCFtools --bed option with the Nextera exome targeted regions BED file was also run to retain only the variants within targeted exome regions in the Nextera Rapid Capture Exome v. 1.2 library. The Nextera Rapid Capture Exome v. 1.2 targeted regions BED file was lifted over from GRCh37 to GRCh38 with the University of California Santa Cruz (UCSC) LiftOver webtool (https://genome.ucsc.edu/cgi-bin/hgLiftOver) (149). Excess heterozygosity was filtered at a phred score of 30. Variant quality metrics were monitored at each filtering step with GATK CollectVariantCallingMetrics.

2.5.3 Functional Variant Annotation and Filtering

We evaluated three functional variant annotation tools: ANNOVAR, Ensembl variant effect predictor (VEP), and SnpEff (150–152). Each tool was evaluated based on relevance in the literature and suite of available annotations. Based on a search of recent primary peer-reviewed literature using exome sequencing and genetic variant association analyses, ANNOVAR and Ensembl VEP were used more frequently than SnpEff (153–157). Both ANNOVAR and Ensembl VEP offer extensive annotation tools for evaluating human genetic variants. We selected ANNOVAR (v. 2019October24) for functional variant annotation. ANNOVAR *table_annovar.pl* was used with VCF input and the GRCh38 reference human genome to annotate the genetic variants (150). The refGene database was used to incorporate genetic variant function (e.g., exonic, intronic), corresponding gene, variant consequence (e.g., non-synonymous), and variant nomenclature (e.g., TYK2 NM_003331 exon15 c.T2051G p.I684S). The following ANNOVAR filter-based annotations were also included: dbSNP v.150 (158), ClinVar (2021) (159), 1000G population allele frequencies based on the phase 3 genetic variant data from 2,504 individuals (v. 5, October 2014) (160), and dbnsfp v.42a. The dbnsfp42a package

contains the following *in silico* predictors: SIFT, PolyPhen-2 (HumDiv and HumVar), LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, VEST4, MetaSVM, MetaLR, MetaRNN, GERP++, PhyloP, SiPhy, CADD, and DANN.

Functional variant filtering was performed using SnpSift v. 5.0 *filter* (161), and command line parsing by *grep*, *awk*, and *sed* tools on the VCF format. Several datasets were generated for the analysis of genetic variants that will be discussed in the following sections. For sample characterization, SnpSift v. 5.0 *filter* was used to filter out variants with 1000G all population allele frequency below 5%. For rare variant analyses, SnpSift v. 5.0 *filter* was used to filter out variants with 1000G all population allele frequency above 5%. Putative loss of function (pLOF) analyses retained genetic variants with the following consequences: frameshift indels, stop gain, and splicing mutations. Moderate variant analyses retained all pLOF variants as well as non-synonymous, inframe indels, start loss, and stop loss variants. The candidate gene dataset was based on the literature review described in Section 2.1. Variants within the candidate genes were extracted using command-line parsing tools *grep*, *awk*, and *sed*. Additional filtering of the candidate gene dataset was conducted as described above for rare, moderate, and pLOF filtering.

2.6 Sample-level Filtering

2.6.1 Sex Inference and Sample Relatedness

The 28 sample exomes were assessed for sample-level filtering with PLINK v. 1.9 (162). The PLINK v. 1.9 tools for sex inference and relatedness were run on all samples. The sex of each sample was predicted by --check-sex function. This method predicts sex based on the homozygosity rate on the X chromosome with values below 0.3 categorized as male and above 0.7 categorized as female. The results were compared with the available metadata to evaluate concordance. The percentage of males and females in cases is 61% and 39%, respectively (Table 4). In controls the percentage of males and females is 33% and 67%, respectively (Table 4). One control sample was removed with discordant sex prediction.

The relatedness between individuals was assessed by identity-by-descent analysis in PLINK v. 1.9. Relatedness was determined with --genome which evaluates relatedness by

complementary analysis looking at genetic similarities between individuals. Samples with proportions of identity above 0.1 were compared against metadata to evaluate relatedness. Outlier values (>0.6) were considered as potential duplicate sequenced samples. Duplicate samples were removed with VCFtools (v. 0.1.16) (163). Three pairs of participants had predicted relatedness with values between 0.1 and 0.6 and these relationships were confirmed with the available metadata. Two samples had a relatedness value of >0.9 suggesting sample duplication. The control sample with discordant sex was a duplicate of another control leading to removal of this sample from further analyses.

Table 4. Bioinformatic inference of the sex distribution in the case-control cohort with PLINK v. 1.9.

	Case (n=18)	Control (n=9)	Total
Male	11	3	14 (51%)
Female	7	6	13 (49%)

2.6.2 Principal Component Analysis and Covariate Analysis

Principal component analysis (PCA) was performed in PLINK v. 1.9 with --pca on the local case-control cohort to generate principal components for covariate analysis. The case-control cohort was also merged with the 1000G dataset with all populations. Genetic variants with matching dbSNP rsIDs between the case-control cohort and 1000G dataset were used for PCA. This ensures that neither dataset deviates according to missing information. PCA plots were generated using the Tidyverse R package (v. 1.3.1) from the output eigenvector and eigenvalue files (164).

Covariate analysis was performed with univariate and multivariate logistic regression models in R (v. 4.1.1) with the glm function. Inferred sex and the first 10 principal components from the case-control cohort PCA were compared individually (univariate) or combined (multivariate) against blastomycosis case status.

2.7 Damaging Genetic Variant Analysis

We took two approaches to identify damaging genetic variants. Firstly, we divided the candidate gene dataset to subsets containing only the pLOF variants, or those most likely to have severe functional consequences on the protein. Each pLOF variant was assessed for case or control enrichment and, if available, characterized using the Genome Aggregation Database (gnomAD v. 3.1.2) for variant frequencies, ClinVar for disease associations, Genotype-Tissue Expression (GTEx v. 8) project database for tissue-specific gene expression, and published peer-reviewed literature (159,165,166). The next analysis incorporated the moderate genetic variants (non-synonymous, inframe indels, and start loss) from the candidate gene dataset for *in silico* prediction of deleterious effects on protein expression, structure, or function. The Sorting Intolerant from Tolerant (SIFT) (167), Polymorphism Phenotyping v2 (PolyPhen-2) (168), and Combined Annotation-Dependent Depletion (CADD) (169) *in silico* predictors were used to characterize these deleterious effects. A stringent threshold was set requiring genetic variants to be characterized as deleterious or damaging by all three predictors. The threshold for each predictor is as follows: SIFT score ≤ 0.05 (deleterious), PolyPhen-2 HumVar ≥ 0.453 (Probably or possibly damaging), and CADD ≥ 20. The SIFT predictor utilizes sequence alignment and homology to assess protein structure conservation and the effect of amino acid substitution. Amino acid substitutions and sequence conservation affect the likelihood of deleterious mutation (167). The PolyPhen-2 HumVar-trained model was selected as this model is more stringent for identifying highly damaging or Mendelian mutations. HumVar is trained with UniProt disease-causing variants and non-synonymous variants ranging from benign to mildly damaging. HumDiv compares damaging to non-damaging variants and could attribute a higher damaging PolyPhen-2 score than HumVar (170). The CADD predictor provides a quantitative phred score output where scores of 20 or above indicate deleteriousness in the top 1% according to functional prediction and evolutionary conservation (169). A CADD score ≥ 20 was considered deleterious in accordance with previous studies of rare variant genome and exome sequencing (171,172). Each genetic variant enriched in cases or controls and passing the *in silico* prediction thresholds was assessed using gnomAD, ClinVar, GTex, and published peer-reviewed literature (159,165,166).

2.8 Single-variant Association Analysis

The single-variant association analysis was conducted on the candidate gene and exome-wide datasets. The exome-wide dataset was composed of all genetic variants remaining in the dataset after the filtering described in Section 2.5.2. The candidate gene dataset contained only rare genetic variants in prioritized genes from the literature as described in Section 2.5.3. Fisher exact test and logistic regression analyses under the allelic, dominant, and recessive models were performed using PLINK v. 1.9. Logistic regression is a commonly used association test for genetic association studies of binary phenotypes (173). The Fisher exact test is a 2x2 contingency table test that provides an exact p-value. The Fisher exact test is useful for small sample studies as logistic regression requires large sample size (174). We report only the Fisher exact test p-values for the candidate gene analysis. The exome-wide analysis reports both Fisher exact test and logistic regression p-values. No covariates were included in the models as only sex and principal components were available, but these did not associate with blastomycosis phenotype (Section 3.3.2). The p-value output for the exome-wide analyses were -log10 transformed and visualized by Manhattan plots using the R package ggplot2 (v. 3.4.0). The p-value threshold for exome-wide significance is $5x10^{-6}$ but a more lenient exploratory threshold of $5x10^{-4}$ was set similar to another small sample size exome sequencing study (171). For the candidate gene analysis, an exploratory p-value threshold of 0.05 was applied with no multiple testing correction. These thresholds allow for the evaluation of enriched genetic variants and their relevance to antifungal immunity.

2.9 Gene-based Association Analysis

To perform gene-based association analysis, we selected the binary optimized sequence kernel association test (SKAT-O) (175). SKAT-O is a commonly used omnibus test (142,176,177), combining two gene-based tests: burden test and sequence kernel association test (SKAT) (178). The burden test combines all variants to generate a single association score with the assumption that all variants are causal in one direction. SKAT is a variance component test that calculates per-variant association scores with the assumption that variants can be causal in

both directions (178). The SKAT-O omnibus test utilizes a weighted average of the burden test and SKAT test statistics to select the optimal association test (175).

The annotated genetic variants described in Section 2.5.3 were grouped according to their ANNOVAR gene annotation to perform the SKAT-O test. The rare, pLOF, and moderate genetic variants were used for both the candidate gene and exome-wide analyses, unless mentioned otherwise. This method was modified from Borda et al. who performed a small sample size exome sequencing study exploring rare genetic variation in congenital zika syndrome (171). The filtered VCF dataset was converted to PLINK v.1.9 binary file format and command line parsing tools *awk, sed,* and *grep*, as well as the Tidyverse R package (v. 1.3.1) were used to clean the dataset and generate input files (162,164). VcfR R package (v. 1.12.0) and the command line parsing tools were used to generate the setID file. No covariates were applied as described in Section 2.8. We weighted each variant by their 1000G all population minor allele frequencies (MAF) with the Madsen-Browning method $[(1/((MAF_i)(1\text{-}MAF_i)^{1/2})]$ (178,179). Variants without 1000G population frequencies were assigned the lowest allele frequency within our dataset. Small sample size adjustment was applied to the analyses to reduce the risk of false positives (175). Weighted binary SKAT-O was performed with the SKAT R package (v. 2.0.1) using the default settings and results visualized with the ggplot2 package (v. 3.4.0). A p-value of 0.05 was considered significant for the candidate gene analysis with no multiple testing correction. For the exome-wide association analysis, an exploratory threshold of $5x10^{-3}$ was applied to investigate the top associations. Benjamini-Hochberg FDR for multiple testing correction was used to adjust p-values to assess statistical significance at $p < 0.05$.

2.10 Pathway Analysis

Pathway analyses were conducted with two different approaches. The hypothesis-driven pathway analysis approach used binary weighted SKAT-O with gene clusters generated using STRING v.12.0 (132) as mentioned previously in Section 2.1. There was a total of 10 gene clusters including 6 singletons grouped as “other” and analyzed together. The SKAT-O analysis was conducted according to Section 2.9. Briefly, rare pLOF and moderate genetic variants in

candidate gene clusters weighted by Madsen-Browning MAF were analyzed with weighted binary SKAT-O in the SKAT R package (v. 2.0.1).

The exome-wide pathway analysis approach differed from the hypothesis-driven approach by the usage of a systemic gene clustering and association analysis method developed by Zhang et al. called Network-based Heterogeneity Clustering (NHC) (180). This method uses a preconstructed weighted network of protein-protein interactions from the BioGRID, Reactome, STRING, and IntAct Molecular Interaction Databases. Small gene clusters were generated from a list of genes containing genetic variants in cases and controls. We input the exome-wide set of genetic variants with predicted deleteriousness scores of CADD $\geq$ 20. This threshold was used previously by Richardson et al. and Borda et al. to capture only predicted deleterious variation that may influence pathway function (171,172). Gene clusters with $p < 0.05$ and an FDR below 20% were considered significant.

Chapter 3: Results

3.1 Host Susceptibility to Naturally Acquired Blastomycosis: A Scoping Review

3.1.1 Selection of Sources of Evidence

We searched five databases to extract publications relating to host susceptibility to blastomycosis. 4,034 publications were retrieved for a total of 1,758 abstracts for full-text assessment eligibility after deduplication (Figure 1). Full-text assessment and data extraction was conducted on 80 publications with 52 included into the scoping review. Searching through the references and citations of each included article as well as other relevant publications on blastomycosis host immunity, we identified an additional 6 publications that were included for data extraction and analysis (Figure 1). The included publications demonstrate host factors involved in the response against blastomycosis separated into four categories: host susceptibility factors, immunomodulatory therapy, $CD4^+$ T cell deficiency, and host targets for virulence factors. The host susceptibility factor category includes primary and secondary immunodeficiencies that directly contribute to blastomycosis susceptibility. The immunomodulatory therapy category includes host-directed agents that dysregulate specific immune function with concurrent blastomycosis diagnosis. $CD4^+$ T cell deficiency is a known risk for blastomycosis severity and was analyzed separately according to $CD4^+$ T cell count and clinical manifestations. The host targets for virulence factor category includes host factors targeted by *B. dermatitidis* virulence factors that could contribute to pathogenesis. From the full-text assessment, 28 articles were excluded for reasons including unclear host factor or unknown impact of host factor deficiency on blastomycosis outcome, studies of blastomycosis vaccination and vaccine-induced immunity, and studies not related to blastomycosis (Figure 1).

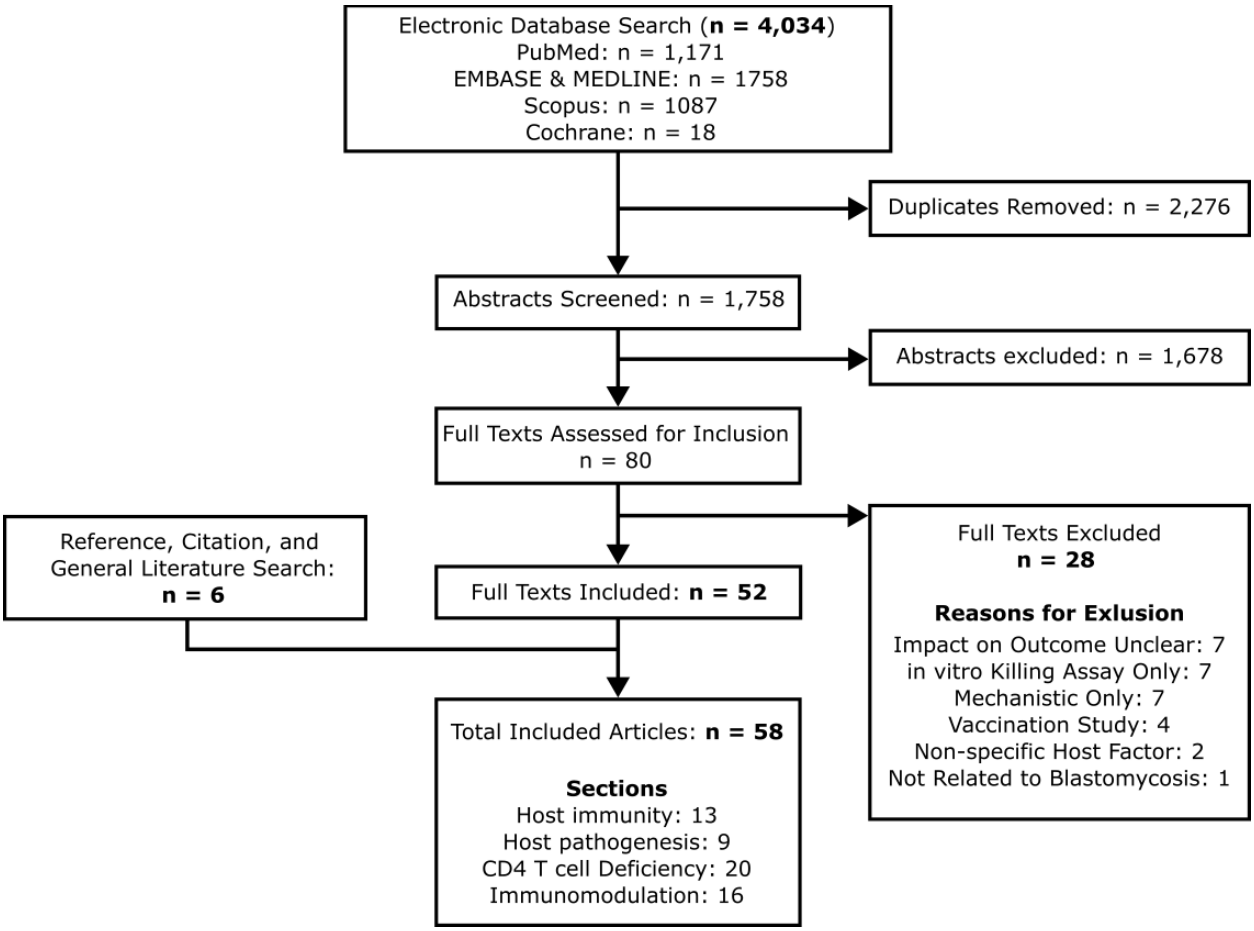

Figure 1. Flow chart for the selection of sources of evidence.

3.1.2 Characteristics of Sources of Evidence

The sources of evidence for the scoping review consisted primarily of case reports and experimental studies as well as case series, retrospective cohort, and case-control studies (Table 5). The study designs varied by category with host susceptibility factors including case reports, case-control, and experimental studies. Immunomodulatory therapies included case reports, retrospective cohort studies, and a case-control study. Publications on $CD4^+$ T cell deficiency consisted of case reports, case series, and retrospective cohort studies. The host-pathogenesis factors included only experimental studies (Table 5). The year of publication ranged from 1978 to 2022. The results of each category will be individually described in the following sections. Only one included article appears in two different categories, host susceptibility factors and host targeting virulence factors, and was categorized in the latter (117).

Table 5. Aggregated count of study designs in included articles per category.

Study Design	Host susceptibility factors	Host-Pathogen Factors	Immunomodulatory Therapy	CD4 Lymphocytopenia	Total Included Studies
Case report	6	-	13	13	32
Experimental study	5	9	-	-	14
Case series	-	-	-	5	5
Retrospective cohort study	-	-	2	2	4
Case-control study	2	-	1	-	3
Total	13	9	16	20	58

3.1.3 Host Susceptibility Factors Involved in Blastomycosis

The host susceptibility factors category was the main focus for the scoping review aimed to capture primary and secondary immunodeficiencies that influence susceptibility and severity of blastomycosis. In total, 10 host susceptibility factors were identified across 14 publications (Table 6). Identified factors include immune factors involved in the adaptive Th1 and Th17 responses, innate macrophage and neutrophil immunity, vitamin D recognition and function, and innate immune barriers. There were three different hosts for host susceptibility factor studies with 5 mouse studies, 1 dog study and 7 human studies. GM-CSF and vitamin D recognition and function factors (vitamin D binding protein (VDBP) and calcifediol) had corroborating evidence in different hosts. IFN-γ had two studies in different mice models demonstrating improved survival in mice through exogenous supplementation. The remaining host susceptibility factors had only a single source of evidence. Genetic susceptibility factors were reported in 3 studies for *IL6*, *GATA2*, and *VDBP* (72,75,124).

Table 6. Host susceptibility factors associated with blastomycosis.

Immune Factors	Evidence	Model	Reference
Pulmonary cilia	Primary ciliary dyskinesia and severe pulmonary blastomycosis.	Human	Guevara et al. 2017 (79)
CFTR	CNS blastomycosis dissemination in patient with CF. No pulmonary evidence and concurrent prednisone treatment.	Human	Gershan et al. (181)
GATA2	Severe pulmonary blastomycosis in setting of monocytopenia and mycobacterial infection (MonoMAC) syndrome due to *GATA2* frameshift insertion (c.871+2_3insT) mutation.	Human	Spinner et al. 2016 (75)
GM-CSF	Blastomycosis ARDS in the setting of GM-CSF autoantibodies and pulmonary alveolar proteinosis.	Human	Deever and Nystrom 2016 (182)
	Blastomycosis ARDS in the setting of pulmonary alveolar proteinosis likely due to GM-CSF deficiency.[±]	Human	Hellman et al. 2017 (183)
	Pulmonary blastomycosis in the setting of pulmonary alveolar proteinosis.[±]	Human	Kellar et al. 1995 (184)
	DppIVA cleavage of GM-CSF improved *Blastomyces* survival. Antibody inhibition of GM-CSF led to >2000-fold increase in fungal burden compared to DppIVA knockout yeast infection alone.	Mouse	Sterkel et al. 2016 (117)
NF-κB & IKK2	Defective NF-κB (Δ*IKK2*) signaling in lung epithelial cells reduced IL-17A and GM-CSF expression and reduced survival with higher fungal burden.	Mouse	Hernández-Santos et al. 2018 (96)
IFN-γ	Exogenous IFN-γ therapy improved survival of BALB/c mice.	Mouse	Brummer et al. 2005 (128)
	Exogenous IFN-γ therapy improved survival of young CD-1 mice to similar levels as adults at lower fungal loads. *	Mouse	Kethineni et al. 2006 (129)
IL-12	Improved BALB/c mice survival with exogenous IL-12 therapy.	Mouse	Brummer et al. 2006 (130)
IL-6	*IL6* and *AS-IL6* variants associated with lower IL-6 expression and higher blastomycosis incidence in persons of Hmong ancestry.	Human	Merkhofer et al. 2019 (72)
VDBP	Significant association of *VDBP* rs4588 in uninfected controls compared to blastomycosis cases.	Human	Sainsbury et al. 2014 (124)
Vitamin D (Calcifediol)	Calcifediol levels were lower in dogs with blastomycosis than in uninfected healthy dogs.	Dog	O'Brien et al. 2018 (185)

* No significant improvement in survival at higher fungal loads. [±] GM-CSF deficiency not clinically confirmed. ARDS, acute respiratory distress syndrome; CF, cystic fibrosis; DppIVA, *Blastomyces* serine protease dipeptidyl peptidase IVA; IFN-γ, interferon gamma; GM-CSF, granulocyte-macrophage colony-stimulating factor; MonoMAC, monocytopenia and mycobacterial infection; VDBP, vitamin D binding protein.

3.1.4 Immunomodulatory Therapies and Blastomycosis

We considered publications reporting immunomodulatory therapy and blastomycosis diagnosis as evidence of opportunistic infection in the setting of host deficiency. A total of 15 publications were included reporting 9 monoclonal antibodies (mAb) and 2 host kinase inhibitors used for therapy with associated blastomycosis diagnoses (Table 7). 11 immunomodulatory agents were described in these studies with 4 involved in TNF-α inhibition (adalimumab, infliximab, etanercept, and certolizumab). The mode of action for each immunomodulatory agent is described in Table 8. 14 patients have been reported to have blastomycosis with concurrent TNF-α mAb therapy across 7 case reports (186–192), 1 case-control (193), and 1 retrospective cohort study (194) (Table 7). Concurrent immunosuppressive agents included methotrexate, corticosteroids, as well as azathioprine and mesalamine. The therapeutic indications for TNF-α inhibition were for the following autoimmune disorders: psoriatic arthritis, psoriasis, ulcerative colitis, juvenile idiopathic arthritis, and Crohn's disease. The manifestations of blastomycosis in TNF-α mAb therapy, where available, were limited to the lungs and skin with the exception of suspected liver dissemination in a single case (Table 7).

The other reported immunomodulatory agents each had only a single patient with a blastomycosis diagnosis. In total, 7 patients, each on unique immunomodulatory agents, were reported across 6 case reports (41,195–199) and 1 retrospective cohort study (200). The mode of action for these agents consisted of inhibiting the following: T and B cell development and activation, Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling, complement system, and T cell immune checkpoints (Table 8). Concurrent immunosuppression was only reported for rituximab (steroid taper) (196) and eculizumab (methylprednisolone, thymoglobulin, and tacrolimus) (195) although the latter provides temporal evidence of eculizumab as the likely agent for blastomycosis reactivation.

Table 7. Reported cases of blastomycosis with concomitant immunomodulatory therapy.

Host Target	Immunomodulatory Agent	No. of Cases	Therapy Indication	Concurrent Immunosuppressive Therapy	Comorbidities	Blastomycosis Manifestations	Reference
TNF-α	Infliximab	1	Psoriatic arthritis	N/A	5-year recurrent MRSA, atherosclerotic heart disease. Simvastatin, metoprolol, and amlodipine. Humoral immunodeficiency (low IgA and IgG4)	Pulmonary	Bessich et al. 2010 (186)
		1	Ulcerative colitis	Previous corticosteroid courses, azathioprine, and mesalamine	Age > 65, diabetes, asthma, and congestive heart failure	Pulmonary	McCann et al. 2013 (187)
		1	Juvenile idiopathic arthritis	Methotrexate	N/A	Cutaneous only	Smith et al. 2015 (188)
	Adalimumab	1	Crohn's disease	N/A	Age > 65, pulmonary hypertension and morbid obesity	Pulmonary and cutaneous	Duke and Egan 2022 (189)
		1	Crohn's disease	N/A	N/A	Pulmonary	Purkey and Shorman 2021 (190)*
		1	Crohn's disease	Methotrexate	N/A	Pulmonary with suspected liver dissemination	Mills et al. 2015 (191)
		1	Psoriasis	Methotrexate	Age > 65	Pulmonary	Otto-meyer et al. 2021 (192)
	Infliximab, adalimumab, etanercept, and certolizumab	6	Autoimmune disorders	Prednisone significantly associated with risk of fungal and mycobacterial infection	N/A	Blastomycosis diagnosis only	Salt et al. 2016 (193)

	Unknown	1	Not reported	Not reported	Not reported	Not reported	Austin et al. 2019 (194)
C5	Eculizumab	1	Single lung transplant for pulmonary fibrosis	Methylprednisolone, thymoglobulin, and tacrolimus	Suspected collagen vascular disease	Rapid onset blastomycosis in transplanted lung after eculizumab initiation	Hirama et al. 2020 (195)
CD20	Rituximab	1	Chronic kidney disease with perinuclear antineutrophil cytoplasmic antibody vasculitis	Steroid taper	Age > 65	Pulmonary (ARDS)	Shingada et al. 2022 (196)
PD-1	Pembrolizumab	1	Metastatic melanoma	N/A	Age > 65	Pulmonary and cutaneous	Ferguson et al. 2019 (197)
CD52	Alemtuzumab	1	Hemophagocytic lymphohistiocytosis	N/A	Heterozygous perforin mutation of unknown significance, polymicrobial sepsis, and EBV reactivation	Pulmonary and disseminated	Gerard et al. 2012 (198)
JAK1/JAK2	Ruxolitinib	1	Polycythemia vera	N/A	Age > 65, COPD, and deep vein thrombosis	Pulmonary and cutaneous	Zeitler et al. 2021 (199)
LFA-1 (CD11a subunit)	Efalizumab	1	Psoriasis treatment	N/A	N/A	Pulmonary and cutaneous	Atanaskova et al. 2009 (41)
BTK	Ibrutinib	1	Hematologic malignancy	Not reported	Not reported	Disseminated	Rogers et al. 2019 (200)

* Blastomycosis diagnosed with urine antigen test only with possibility of histoplasmosis cross-reactivity. ARDS, acute respiratory distress syndrome; BTK, Bruton's tyrosine kinase; C5, complement protein 5; COPD, chronic obstructive pulmonary disease; EBV, Epstein-Barr virus; JAK, Janus kinase; LFA-1, lymphocyte function-associated antigen 1; MRSA, methicillin resistant *Staphylococcus aureus*; PD-1, programmed cell death protein 1; TNF-α, tumour necrosis factor; N/A, not applicable.

Table 8. Immunomodulatory agent mode of action.

Immunomodulator	Mode of action	Reference
Infliximab	MAb binding and inhibiting of TNF-α	(138)
Adalimumab	MAb binding and inhibiting of TNF-α	(138)
Etanercept	MAb binding and inhibiting of TNF-α	(138)
Certolizumab	MAb binding and inhibiting of TNF-α	(138)
Eculizumab	MAb binding and inhibiting C5 cleavage	(201)
Rituximab	MAb binding CD20. Mode of action not well understood but widespread use for treating B-cell non-Hodgkin lymphomas	(202)
Ibrutinib	Irreversible small molecule BTK inhibitor. Inhibition of B cell development	(200)
Pembrolizumab	MAb binding and blocking PD-1 to improve T cell function	(197)
Alemtuzumab	MAb binding CD52 and depleting CD52-expressing immune cells	(203)
Ruxolitinib	Small molecule inhibiting downstream signaling of JAK1 and JAK2	(204)
Efalizumab	MAb targeting CD11a subunit of LFA-1 blocking interaction with ICAM-1 and inhibits T cell recruitment and activation	(205)

BTK, Bruton's tyrosine kinase; C5, complement protein 5; ICAM-1, intracellular adhesion molecule 1; JAK, Janus kinase; LFA-1, lymphocyte function-associated antigen; mAb, monoclonal antibody; PD-1, programmed cell death protein 1; TNF-α, tumour necrosis factor alpha.

3.1.5 Cell-mediated Immunodeficiencies

The deficiency of $CD4^+$ Th cells has been reported in blastomycosis patients to potentially predispose to severe pulmonary disease, CNS, and atypical dissemination (dissemination to visceral organs and lymph nodes) (53,206). We included 20 publications reporting blastomycosis in individuals with low $CD4^+$ cell counts. There were 13 case reports, 5 case series, and 2 retrospective cohort studies for a total of 39 patients with $CD4^+$ deficiency (Table 5). Each included study is listed in Table 9. The underlying reasons for $CD4^+$ deficiency included HIV and AIDS (or advanced HIV), ICL, and malnutrition. The terminology used in Table 9 to describe the cell-mediated immunodeficiencies was preserved from the included reference for consistency.

The $CD4^+$ cell counts ranged from 3-731 cells/μL for 35 patients living with HIV and advanced HIV. Cases were grouped by $CD4^+$ cell counts ≤200 cells/μL and >200 cells/μL. $CD4^+$ cell counts below 200 cells/μL were reported for 26 patients (Table 9). The $CD4^+$ cell count was not known for 6 patients but 5 were included with the ≤200 cells/μL group due to the presence of AIDS-defining illnesses (*P. jirovecii* pneumonia and *Mycobacterium avium* complex) or

characteristics of advanced HIV suggesting low $CD4^+$ cell count. The patient reported by Herd et al. was excluded from analysis due to an uninterpretable $CD4^+$ cell count and absence of AIDS-defining illness. The remaining 3 patients were grouped as having $CD4^+$ cell counts >200 cells/µL with 2 patients reported >200 cells/µL and 1 patient with absolute T cell count ranging from 100-300 cells/µL. Blastomycosis was most commonly reported in patients living with HIV with $CD4^+$ cell counts $\leq$200 cells/µL. The majority of these patients had reported pulmonary blastomycosis with higher rates of CNS and atypical dissemination (45.2% and 38.7%) than cutaneous (35.5%) (Table 10). Patients with $CD4^+$ cell counts >200 cells/µL had a lower rate of CNS and atypical dissemination compared to the $\leq$200 cells/µL group although the sample size is low with only 3 patients.

A total of 4 patients with ICL or severe malnutrition were diagnosed with blastomycosis. The $CD4^+$ cell counts ranged from 78-155 cells/µL for 3 patients with ICL and 1,037 T cells/µL for a single patient with severe malnutrition (Table 9). All patients presented with pulmonary blastomycosis followed by cutaneous in 3 of 4 patients, CNS in 2 of 4 patients, and atypical dissemination in a single patient with $CD4^+$ cell count <100 cells/µL (Table 10). No other illnesses were noted for these patients.

Table 9. $CD4^+$ lymphocytopenia and cell-mediated immunodeficiency in blastomycosis.

Reference	Study Design	Case No.	$CD4^+$ T-cell count	Blastomycosis manifestation	Risk Factors
Battle et al. 2001 (207)	Case report	1	8-200/µL	Pulmonary and cutaneous	HIV/AIDS and concurrent MRSA bacteremia
Cohen et al. 1996 (208)	Case report	1	15/µL	Pulmonary and cutaneous	HIV/AIDS
Dworkin et al. 2005 (9)	Case series	1	38 cells/mm^3	Pulmonary and cutaneous	Advanced HIV
FitzSimons and Ferguson 1978 (209)	Case report	1	T cells: 1037 cells/µL	Pulmonary and cutaneous	Severe malnutrition and anorexia (proper nutrition ameliorated T cell counts)
Fraser et al. 1991 (210)	Case series	1	4 cells/mm^3	Pulmonary, cutaneous, CNS, and systemic dissemination	HIV/AIDS and *P. jirovecii* pneumonia
Guccion et al. 1996 (14)	Case report	1	23 cells/mL	Pulmonary (ARDS), CNS, solid organs, and septicemia	HIV/AIDS, Alcoholic gastritis, pancreatitis, and misdiagnosis with corticosteroid treatment
Harding 1991 (206)	Case series	1	4 cells/µL	Pulmonary, CNS, cutaneous, thyroid, spleen, prostate gland, pancreas, meninges, lymph node, liver, kidney, heart, gallbladder, epididymis, and appendix	HIV/AIDS, *P. jirovecii* pneumonia, and *M avium* intracellulare
		2	T Cells: 0.1-0.3x10^9 cells/L	Pulmonary, CNS, cutaneous, trachea, thyroid gland, testis, pancreas, neurohypophysis, lymph nodes, meninges, liver, kidney, heart, and adrenal glands	HIV
Herd et al. 1990 (211)*	Case report	1	Unknown	Pulmonary	HIV and diabetes mellitus
Jehangir et al. 2015 (212)	Case report	1	4 cells/µL	Pulmonary (ARDS), lymph nodes, liver, and thyroid gland	HIV/AIDS
Kitchen et al. 1989 (213)	Case report	1	<200/mm^3	Pulmonary	HIV/AIDS and tuberculosis
Ludmerer and Kissane 1996 (214)	Case report	1	Unknown	Pulmonary, cutaneous and CNS	HIV and end-stage AIDS, delayed diagnosis, and oral *Candida*
McBride et al. 2021 (73)	Retrospective cohort study	1	79 cells/µL	Pulmonary	HIV/AIDS
		2	78 cells/µL	Pulmonary	ICL
Nelson et al. 1993 (215)	Case report	1	168 cells/mm^3	Pulmonary, cutaneous, and epiglottis	HIV/AIDS and oral *Candida*

Pappas et al. 1992 (53)	Retrospective cohort study	1	28 cells/mm^3	Pulmonary	HIV/AIDS and perianal herpes simplex virus
		2	<50 cells/mm^3	Pulmonary	HIV/AIDS and *Pneumocystis jirovecii* pneumonia
		3	3 cells/mm^3	Pulmonary	HIV/AIDS and CNS cryptococcosis
		4	111 cells/mm^3	Pulmonary	HIV/AIDS
		5	598 cells/mm^3	Pulmonary	HIV
		6	<200 cells/mm^3	Pulmonary	HIV/AIDS and *P. jirovecii* pneumonia
		7	147 cells/mm^3	Pulmonary	HIV/AIDS and Kaposi sarcoma
		8	54 cells/mm^3	Cutaneous	HIV/AIDS and disseminated histoplasmosis
		9	<200 cells/mm^3	CNS	HIV/AIDS and *P. jirovecii* pneumonia
		10	<50 cells/mm^3	Pulmonary, CNS, bone marrow, spleen, adrenal glands	HIV/AIDS and *P. jirovecii* pneumonia
		11	80 cells/mm^3	Pulmonary, bone marrow, psoas, thyroid, lymph nodes	HIV/AIDS and *P. jirovecii* pneumonia
		12	4 cells/mm^3	Pulmonary, CNS, cutaneous, thyroid, kidney, prostate, liver, spleen, heart	HIV/AIDS and *P. jirovecii* pneumonia
		13	Unknown	Pulmonary, CNS, cutaneous, liver, spleen, kidney	HIV/AIDS wasting syndrome
		14	40 cells/mm^3	Pulmonary, CNS, kidney, liver, spleen, lymph nodes	HIV/AIDS wasting syndrome
		15	12 cells/mm^3	Pulmonary and CNS	HIV/AIDS
Siderits et al. 2010 (55)	Case report	1	155 cells/µL	Pulmonary, cutaneous, adrenal gland, vocal cords, and CNS	ICL
Srinath et al. 1994 (216)	Case report	1	43 cells/mm^3	Pulmonary	HIV/AIDS
Tan et al. 1993 (54)	Case series	1	Unknown	Pulmonary, liver, CNS, spleen, pancreas, kidneys, bone marrow, and lymph nodes	HIV/AIDS and *M. avium* complex
		2	Unknown	Pulmonary, CNS, cutaneous, and blood	HIV/AIDS and *M. avium* complex
Tirmizi et al. 2016 (217)	Case report	1	104 cells/µL	Pulmonary and CNS	HIV/AIDS

Witzig et al. 1994 (15)	Case series	1	651-731 cells/mm^3	Pulmonary and cutaneous	HIV and *Mycobacterium avium* complex
		2	17 cells/mm^3	Pulmonary and cutaneous	HIV/AIDS with previous history of oral thrush, syphilis, and tuberculosis
		3	Unknown	CNS only (only *P. jirovecii* identified in lungs)	HIV/AIDS, *P. jirovecii* pneumonia, and rheumatoid arthritis
Zhou et al. 2012 (218)	Case report	1	<100 cells/µL	Pulmonary, cutaneous, and CNS	ICL and misdiagnosis with prednisone treatment

* Excluded from analysis. AIDS, acquired immunodeficiency syndrome; ARDS, acute respiratory distress syndrome; CNS, central nervous system; HIV, human immunodeficiency virus; ICL, idiopathic $CD4^+$ lymphocytopenia; MRSA, methicillin resistant *Staphylococcus aureus*.

Table 10. Clinical manifestations of patients living with HIV, ICL, or experiencing severe malnutrition and blastomycosis by $CD4^+$ cell count.

HIV $CD4^+$ Cell Counts						
≤200 cells/µL (n = 31) Clinical manifestations		No. of Cases		AIDS-defining illnesses	No. of Cases	
	Pulmonary	28	90.3%	*P. jirovecii* pneumonia	9	29.0%
	Cutaneous	11	35.5%	*M. avium* complex	3	9.7%
	CNS	14	45.2%	Cryptococcosis	1	3.2%
	Atypical	12	38.7%	HSV	1	3.2%
>200 cells/µL (n = 3)						
	Pulmonary	3	100%	*P. jirovecii* pneumonia	0	0.0%
	Cutaneous	2	50.0%	*M. avium* complex	1	25.0%
	CNS	1	25.0%	Cryptococcosis	0	0.0%
	Atypical	1	25.0%	HSV	0	0.0%
ICL and malnutrition (n = 4)						
	Pulmonary	4	100%			
	Cutaneous	3	75.0%			
	CNS	2	50.0%			
	Atypical	1	25.0%			

AIDS, acquired immunodeficiency syndrome; CNS, central nervous system; HIV, human immunodeficiency virus; HSV, herpes simplex virus; ICL, idiopathic lymphocytopenia.

3.1.6 Host Factor-targeting Blastomyces Virulence Factors

We identified 9 experimental studies demonstrating interactions between *Blastomyces* virulence factors and host targets. The studies required evidence of the interaction but not necessarily demonstration of pathogen advantage. In total, 11 host targets were identified with 9 interacting with BAD1 and 2 interacting with dipeptidyl peptidase IVA (DppIVA). All studies used mice models to study these interactions with only a single study using human cells *in vitro* (Table 11).

BAD1 is a pleiomorphic virulence factor involved in the adhesion of *Blastomyces* to phagocytic cells (219,220), modulation of the host immune response (220–224), and reducing binding of complement protein 3 (C3) to the yeast surface (225). Modulation of TNF-α expression by BAD1 had the most evidence with three studies demonstrating reduced TNF-α in the presence of BAD1 (220–222). Binding of BAD1 to the complement receptor 3 (CR3) for phagocyte binding and TNF-α modulation was shown across two studies (219,220). The modulation of IFN-γ, IL-12, and IL-10 were supported by a single study (223). Two studies each demonstrating different functions of DppIVA were included (117,226).

Table 11. Host factors targeted in *B. dermatitidis* pathogenesis.

Virulence factor	Host factor	Pathogenesis/Mechanism	Study type	Model system	Reference(s)
BAD1	CD11b/CD18 (CR3)	Surface bound BAD1 binds CR3 for yeast adherence to macrophages and neutrophils for yeast internalization. Soluble BAD1 binds CR3 for receptor mediated BAD1 endocytosis and immune modulation.	*In vitro, in vivo*	Mice	Newman et al. 1995, Brandhorst et al. 2004 (219,220)
	CD14	BAD1-binding receptor involved in TNF-α inhibition. $CD14^{-/-}$ mice have reduced TNF-α suppression by soluble BAD1.	*In vitro, in vivo*	Mice	Newman et al. 1995, Brandhorst et al. 2004 (219,220)
	CD47	Mimics TSP-1 binding heparin and CD47. BAD1-CD47 binding reduces T cell activation.	*In vitro*	Mice	Brandhorst et al. 2013 (224)
	TNF-α	Surface bound BAD1 inhibits TNF-α production through TGF-β-dependent mechanism. Endocytosed soluble BAD1 inhibits TNF-α production through TGF-β-independent mechanism.	*In vitro, in vivo*	Mice	Finkel-Jimenez et al. 2001, Finkel-Jimenez et al. 2002, Brandhorst et al. 2004 (220–222)
	IFN-γ	BAD1 presence decreases host expression of IFN-γ and increases fungal burden.	*In vitro, in vivo*	Mice	Wuthrich et al. 2006 (223)
	IL-12	BAD1 presence decreases host expression of IL-12 and increases fungal burden.	*In vitro, in vivo*	Mice	Wuthrich et al. 2006 (223)
	IL-10	BAD1 presence increases expression of IL-10 and increases fungal burden.	*In vitro, in vivo*	Mice	Wuthrich et al. 2006 (223)
	C3	BAD1 inhibits binding of C3 to *Blastomyces* yeast cell.	*In vitro*	Mice	Zhang et al. 2001 (225)
DppIVA	GM-CSF	Cleavage of GM-CSF into inactive form.	*In vivo*, *in vitro*	Mice	Sterkel et al. 2016 (117)
	CXCL2	Cleavage of CXCL2 induces neutrophil recruitment but inability to kill yeast.	*In vitro*	Human and Mice	Lorenzini et al. 2017 (226)

BAD1, *Blastomyces* adhesion 1; C3, complement protein 3; CXCL, C-X-C motif ligand; DppIVA, *Blastomyces* serine protease dipeptidyl peptidase IVA; GM-CSF, granulocyte-macrophage colony-stimulating factor; TGF-β, transforming growth factor beta; TNF-α, tumour necrosis factor alpha; TSP-1, thrombospondin 1.

3.2 Exome Sequencing to Identify Host Genetic Variants Associated with Blastomycosis Susceptibility

3.2.1 Sequencing Characteristics

Exomes for 18 blastomycosis cases and 10 household controls were sequenced to identify genetics variants that may underlie blastomycosis susceptibility. The exome sequencing characteristics are summarized in Table 12. Briefly, 944.5 million reads from 28 exomes were generated with an average of 33.6 million reads per sample. A read depth >8 was required for variant inclusion. We had an average 10X coverage for 93.44% of the exome. All paired end sequencing files passed FastQC except 3 sample paired end files for unique samples that failed "per base sequence quality" at the end of the read. GATK base quality recalibration and subsequent quality filtering removed the low-quality sequences.

Table 12. Exome sequencing summary.

	Cases (n = 18)	Controls (n = 10)	Total (n = 28)
Total reads	617,072,250	327,506,266	944,578,516
Average reads	34,281,792	32,750,627	33,612,054
Average coverage depth	61.1X	58.8X	59.8X
Average 10X coverage	93.9%	93.42%	93.44%

3.2.2 Variant Identification and Filtering

We identified 6.98 million genetic variants across the 28 sequences using GATK HaplotypeCaller. Variant filtering was performed according to GATK best practices for variant hard filtering (147) and by filtering parameters described by Carson et al. (148). Table 13 summarizes the variant filtering and variant quality improvement for each step performed. The recommended GATK hard filtering removed 11,575 variants. We next removed 6.85 million variants outside of the targeted exome regions in the Illumina Nextera Rapid Capture Exome v. 1.2 library. Variant quality improved after the removal of these variants with the transition/transversion (Ti/Tv) ratio increasing for variants in dbSNP from 2.02 to 2.84 (normal range: 3.0-3.3) and novel (not in dbSNP) from 0.31 to 0.90. The percentage of variants present

in dbSNP in the dataset increased from 55.65% to 88.64%. The 111,628 variants were further filtered by genotypic information according to Carson et al. (148). 4,733 variants with >12% missing genotypes were removed, further improving the novel Ti/Tv ratio from 0.90 to 1.03 and percentage of variants in dbSNP from 88.64% to 90.03%. The filtering of the genotype quality, read depth, and heterozygosity removed 1,149 variants and marginally improved the quality metrics. In total, 105,746 genetic variants remained after filtering for the exome-wide association analyses. The variants were annotated with ANNOVAR v. 2019October24, and the composition of the exome-wide variant consequences are shown in Figure 2. Intronic, non-synonymous, and synonymous consequences made up the majority of genetic variants with other variants making up only 4% (Figure 2A). Of the 4% other variants, frameshift deletions (31.2%) and non-frameshift deletions (31.0%) were the most frequently observed (Figure 2B).

Variant annotation with ANNOVAR v. 2019October24 was used to filter the exome-wide dataset according to variant consequence, 1000 Genomes all population allele frequency, and corresponding gene. We filtered for rare genetic variants (1000G MAF <5%) with moderate to severe variant consequences (non-synonymous, frameshift indels, non-frameshift indels, stop gain, stop loss, start loss, and splicing) in the genes identified in the literature review (Section 2.1). A total of 103 rare and potentially damaging genetic variants were retained after annotation filtering for the rare genetic variant analysis. The quality of the variant dataset decreased with a dbSNP Ti/Tv ratio of 1.76 and novel Ti/Tv ratio of 0.5 and 84.21% representation in dbSNP overall (Table 13). The composition of variant consequences in this dataset are shown in Figure 2C and individually reported in Table 17.

Table 13. Variant filtering summary statistics.

Variant filter	No. of Variants	Ti/Tv dbSNP	Ti/Tv Novel	% dbSNP
GATK HaplotypeCaller variant calling	6,979,946	2.01	0.31	55.54%
GATK hard filtering	6,968,371	2.02	0.31	55.65%
Removing variants outside exome intervals	111,628	2.84	0.90	88.64%
Missingness	106,895	2.87	1.03	90.03%
Genotype and heterozygosity filtering	105,746	2.87	1.03	90.05%
Rare moderate and pLOF candidate variants	103	1.76	0.50	84.21%

dbSNP, Single Nucleotide Polymorphism database; GATK, Genome Analysis ToolKit; pLOF, putative loss of function; Ti/Tv, transition/transversion.

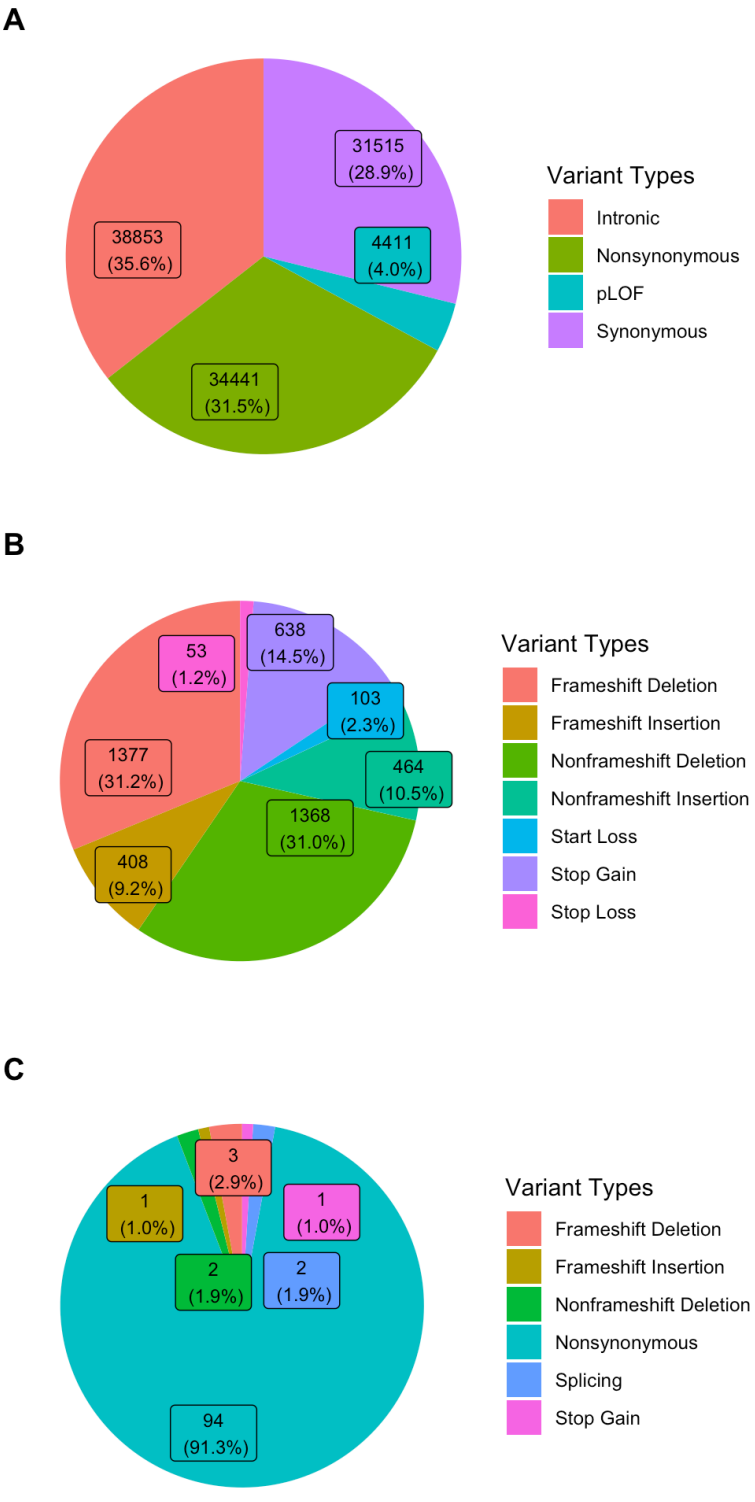

Figure 2. Genetic variant consequences in the exome-wide dataset (A), subdivided by pLOF consequences only (B), and the candidate gene rare variant dataset (C).

3.3 Population and Covariate Analysis

3.3.1 Principal Component Analysis

Principal component analysis (PCA) was conducted to visualize sample clustering to confirm relatedness and monitor for stratification due to genetic ancestry. Principal components (PCs) 1 and 2 explained 8.23% and 6.59% of the total sample variability, respectively and were used for visualization in Figure 3. We observed related individuals clustering together and away from the largest cluster in the bottom right of Figure 3. The top right, leftmost, and bottom right case-control pairs were related according to the relatedness analysis. This further supports the sample relatedness analysis with three pairs of related participants (Section 2.6.1). The remaining samples were all clustered together in Figure 3 except for a single case.

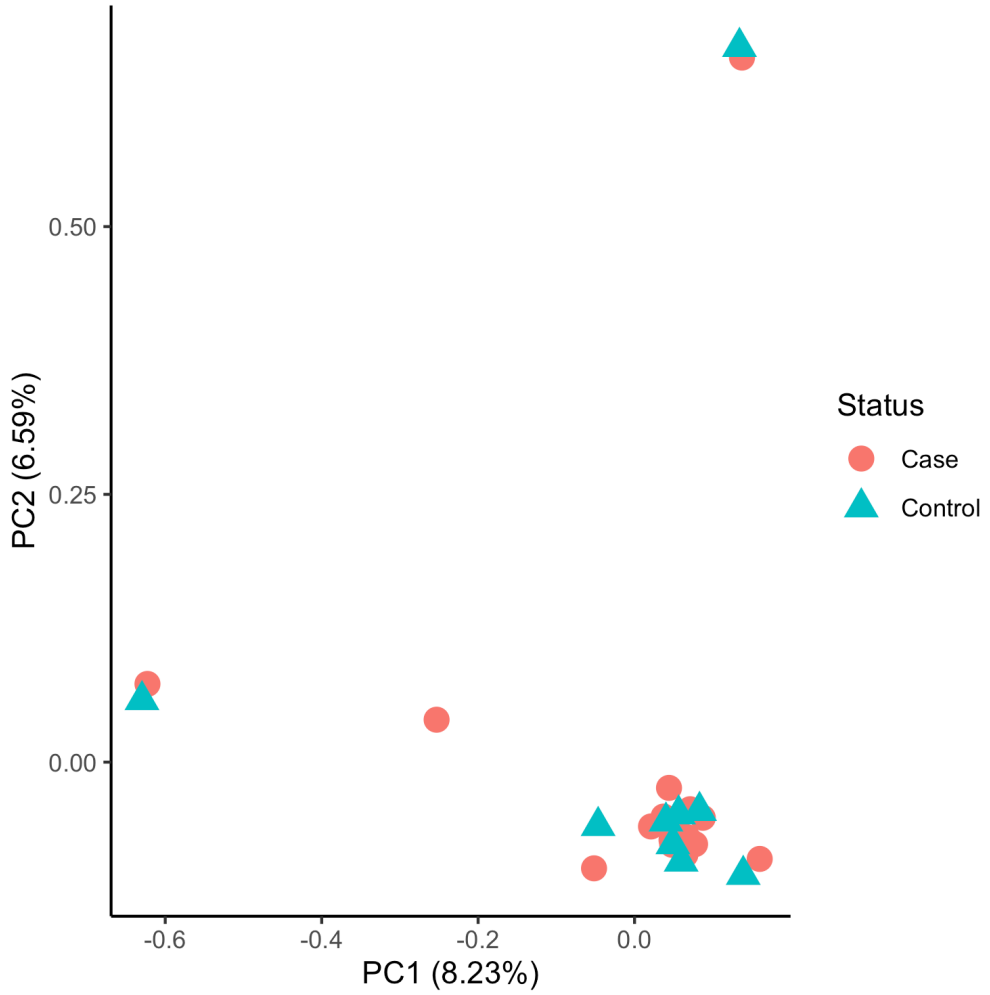

Figure 3. Principal component analysis of the blastomycosis case-control cohort.

To assess the level of stratification due to genetic ancestry differences, the case-control cohort exomes were added to the phase 3 1000G all population GRCh38 dataset (227) (Figure 4). Of the 27 samples, 24 clustered within European populations while 3 samples clustered in South American populations. This finding led to the decision to use allele frequencies for all 1000G populations for downstream analyses instead of only the 1000G European allele frequencies.

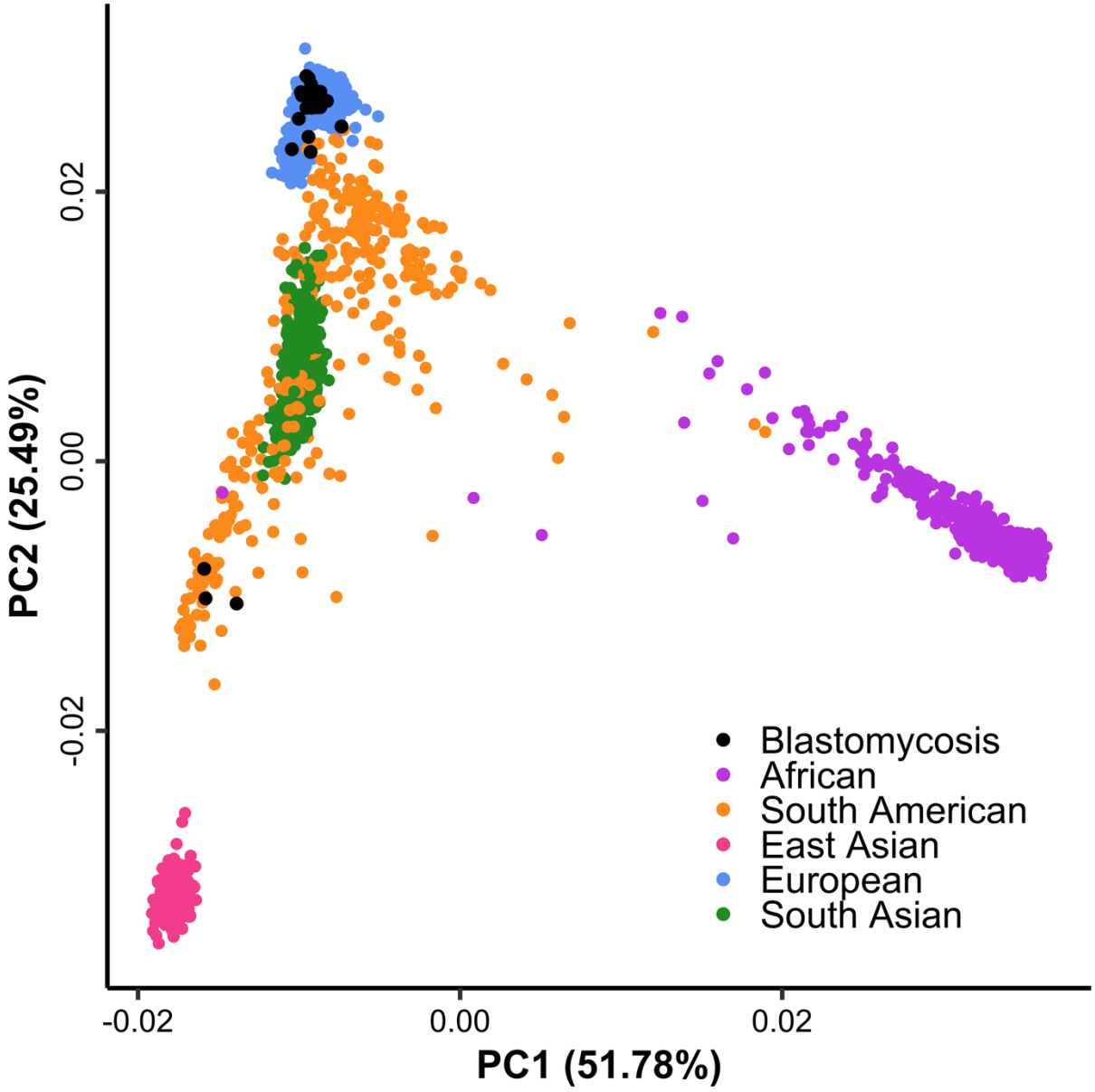

Figure 4. Population distribution of blastomycosis exomes according to 1000 Genome populations using principal component analysis.

3.3.2 Covariate Analysis

We assessed the available metadata for the case-control cohort to evaluate usage as covariates for downstream analyses. Univariate and multivariate logistic regression was performed with the inferred sex and first 10 principal components from the local PCA. No covariate was significantly associated with the blastomycosis phenotype in the univariate analysis ($p > 0.05$) (Table 14). The multivariate analysis did not yield any statistically significant covariates ($p > 0.05$), but sex trended towards significance with a p-value of 0.053 (Table 14). Since no covariates were significantly associated with the phenotype, covariate correction was not performed in the downstream analyses. No adjustment for population stratification was performed as the principal components were not significant in either regression method.

Table 14. Analysis of covariates with logistic regression analysis against blastomycosis case status. Multivariable logistic regression analysis included all reported covariates.

Covariate	Univariate logistic regression p-value	Multivariable logistic regression p-value
Sex	0.181	0.053
PC1	0.804	0.865
PC2	0.611	0.411
PC3	0.516	0.073
PC4	0.288	0.149
PC5	0.845	0.613
PC6	0.805	0.235
PC7	0.627	0.843
PC8	0.443	0.291
PC9	0.332	0.416
PC10	0.883	0.860

3.4 Candidate Gene Association Analysis

3.4.1 Candidate Genes

We conducted a candidate gene approach to prioritize previously associated genes in fungal and mycobacterial disease susceptibility. This approach targets genetic variation in suspected genes that may drive the blastomycosis phenotype enhancing the prior likelihood of association and reducing the multiple testing burden. Studies have used previously associated susceptibility genes for targeted investigations in coronavirus disease 2019 (COVID-19) (228), inflammatory bowel disease (142) and congenital zika syndrome (171). The candidate gene approach used the genes identified from the literature review described in Section 2.1. In total, we searched 45 primary journal articles and 18 reviews to extract 86 candidate genes (see Appendix A). We performed a gene cluster analysis with the candidate genes using STRING v. 12.0 to assess relevant pathways and biological processes. Figure 5 shows nine gene clusters identified and annotated according to STRING provided databases and 6 singletons not associated with the remaining candidate genes grouped as "other". The largest gene cluster, "inflammatory response", contained 19 genes involved in both innate and adaptive response to infectious disease. Two gene clusters contained the fungal recognition receptors. The regulation of immune response pathway contained the CLR signaling pathway and Fc gamma receptors for opsonized pathogen detection (229). The TLR signaling pathway clustered separately containing TLRs and downstream signaling molecules. Innate immune response gene clusters consisted of the chronic granulomatous disease pathway for NADPH oxidase ROS generation, chemokine-mediated signaling pathway for immune cell recruitment and activation, chitin degradation, and vitamin D metabolism. The adaptive immune response gene clusters involved the JAK-STAT signaling pathway for innate and adaptive immune cell communication and the Th17 differentiation pathway.

The following sections explore the genetic variants in the candidate genes by assessing their potential effect on blastomycosis susceptibility utilizing both statistical association analyses and non-statistical bioinformatic approaches.

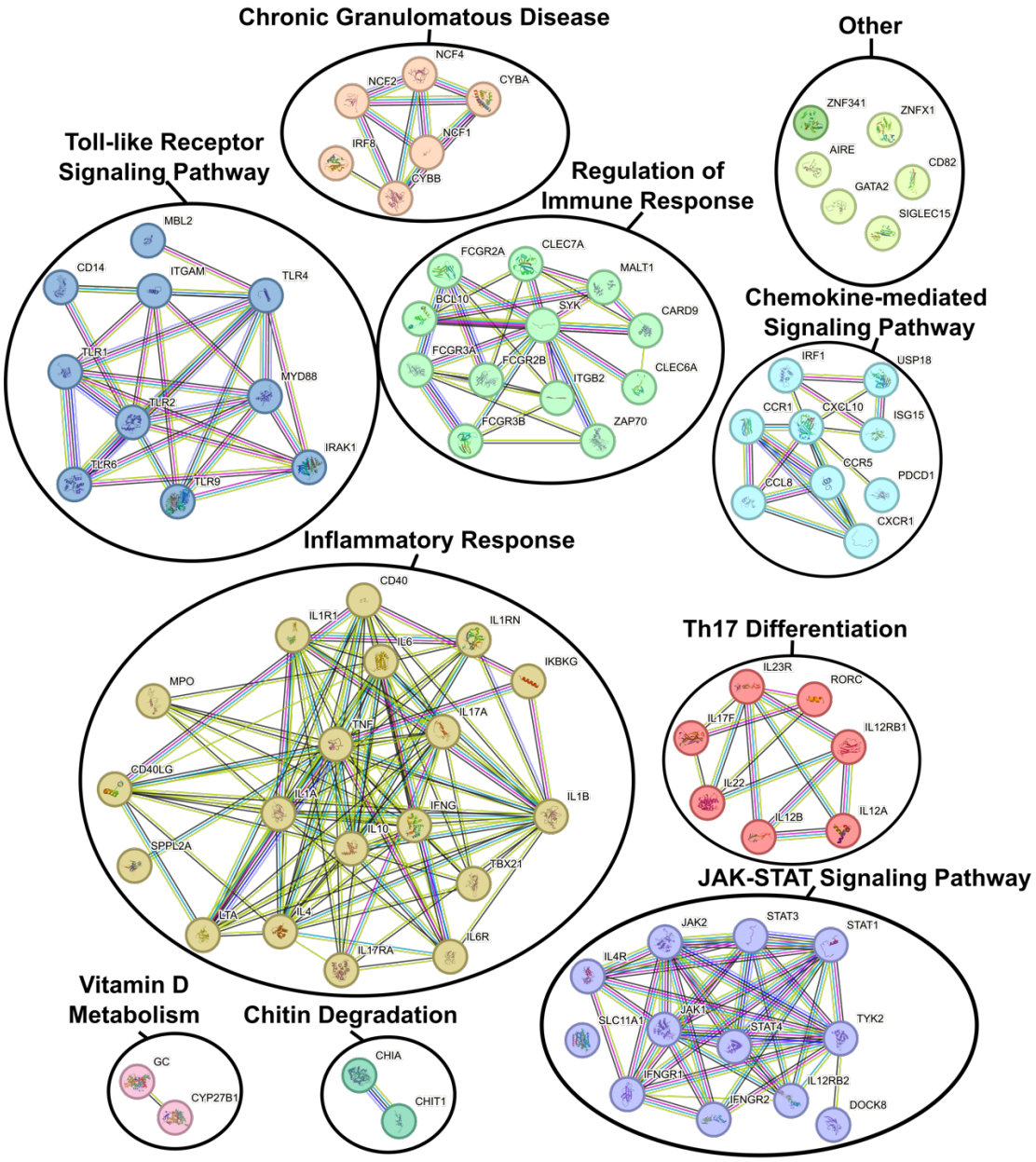

Figure 5. Candidate genes identified from the literature review clustered by STRING v. 12.0 with k-means clustering. Clusters were annotated according to STRING provided databases with the most relevant and enriched pathways.

3.4.2 Damaging Genetic Variant Analysis

The first step of our analysis was to identify any known or predicted damaging genetic variants that may explain the blastomycosis phenotype. Rare genetic variants with a 1000G all population MAF of less than 5% in the 86 candidate genes were assessed for damaging profiles using two methods: manual assessment of pLOF variants and bioinformatic assessment of non-synonymous deleteriousness. For the pLOF variants, 4 genetic variants in 4 separate genes were identified consisting of 1 stop gain, 2 splice site acceptor mutations, and 1 frameshift deletion (Table 15). None of these variants were statistically significant in a case-control analysis using the Fisher exact test. These variants were also assessed using publicly available databases and bioinformatic annotations to determine potential biological implication in influencing risk of infection and disease.

The *IFNG* and *STAT4* acceptor splice site variants are reported as benign with no or unlikely consequence on splicing in gnomAD (v. 3.1.2). The *CXCR1* G315X stop gain mutation is found to be heterozygous in 1 case. This mutation is not reported in gnomAD, 1000G, or ClinVar. The early stop codon is located at amino acid 315 near the end of the 350 amino acid protein (230). The *CCR5* S185fs*32 or CCR5Δ32 mutation was found in 1 case as a heterozygote.

Table 15. pLOF genetic variants in candidate genes.

Gene	Chr	Variant	Mutation	Case count (/18)	Control count (/9)	Fisher exact p-value
IFNG	12	c.115-2->T	Acceptor splice site	0	1	0.36
STAT4	2	c.129-2->T	Acceptor splice site	5	4	0.71
CXCR1	2	G315X	Stop gain	1	0	>0.99
CCR5	3	S185fs*32	Frameshift deletion	1	0	>0.99

We next assessed non-synonymous variation for predicted deleteriousness with SIFT, PolyPhen2, and CADD. Stringent deleteriousness thresholds were set requiring genetic variants to pass all three predictors as described in Section 2.7. A total of 24 genetic variants passed the threshold for deleteriousness (Table 16). Seventeen of these genetic variants had only one heterozygous individual. *IL12B* V298F had OR of 1 (95% CI: 0.08-11.8). These genetic variants did not show enrichment in cases or controls and were not investigated further. Six genetic variants had deleterious profiles with enrichment in cases or controls. *DOCK8* A597V had the highest OR of 2.74 (95% CI: 0.30-25.42) with 5 heterozygous cases and 1 heterozygous control. This variant is a tissue-specific expression quantitative trait locus (eQTL) in the pancreas ($p=2.6x10^{-6}$) reducing expression in the heterozygous state. ClinVar reports A597V as benign in combined immunodeficiency due to DOCK8 deficiency. The *TYK2* I684S variant had the highest CADD score of 32 and was found to be heterozygous in 4 cases and 1 control (OR = 2.13 (95% CI: 0.22-20.55). ClinVar annotates TYK2 as immunodeficiency 35 or familial atypical mycobacteriosis with AR inheritance but reports this variant as benign. The allele frequencies of I684S in 1000G and gnomAD are 2.8% and 5.7% with a total of 346 homozygotes reported. I684S is reported as an eQTL in GTEx with reduced expression in tibial nerve ($p=1.8x10^{-18}$), tibial artery ($p=1.5x10^{-14}$), and suprapubic skin ($p=1.5x10^{-12}$) in both heterozygous and homozygous forms. *IL17F* E126G was enriched in cases (OR = 2.13, 95% CI: 0.22-20.55) and V155I enriched in controls (OR = 0.23, 95% CI: 0.02-2.70). Both variants are reported as benign for candidiasis in ClinVar and no eQTLs in GTEx. The *TLR2* R753Q and *IL23R* R381Q variants were both found equally between cases and controls. *TLR2* R753Q is reported in ClinVar as a risk factor for tuberculosis. *IL23R* R381Q is reported in ClinVar as protective against inflammatory bowel disease and psoriasis.

Table 16. Putatively deleterious non-synonymous variants in candidate genes.

Gene	Variant	SIFT prediction	PolyPhen-2 prediction	CADD phred score	OR (95% CI)
DOCK8	A597V	D	D	24.2	2.74 (0.30-25.42)
TYK2	I684S	D	D	32	2.13 (0.22-20.55)
IL17F	E126G	D	D	29.4	2.13 (0.22-20.55)
TLR2	R753Q	D	D	29.4	0.49 (0.03-8.25)
IL23R	R381Q	D	D	26	0.47 (0.06-3.65)
IL17F	V155I	D	D	23.5	0.23 (0.02-2.70)
IL12B	V298F	D	P	22.7	1 (0.08-11.8)
AIRE	R471C	D	P	22.2	N/A*
CHIA	A57T	D	D	23.1	N/A*
CHIA	F216L	D	P	23.2	N/A*
CCR5	L55Q	D	D	24.3	N/A*
CCR5	A73V	D	D	25.8	N/A*
CCR5	L121R	D	D	27.4	N/A*
ITGAM	A479S	D	P	23.7	N/A*
ITGAM	A479D	D	D	24.3	N/A*
NCF2	R314W	D	P	27.3	N/A*
TLR1	I57M	D	D	22.5	N/A*
TLR2	P631H	D	D	24.7	N/A*
TLR2	R723C	D	D	24.3	N/A*
SPPL2A	A179E	D	P	26.9	N/A*
TYK2	A928V	D	D	32	N/A*
TYK2	R703W	D	P	22.4	N/A*
TYK2	A53T	D	D	24.6	N/A*
ZNF341	G149W	D	D	28.6	N/A*

*One heterozygous individual. CI, confidence interval; OR, odds ratio; D, deleterious; P, possibly damaging.

3.4.3 Single-variant Association Analysis

In the previous section, we described genetic variants in the candidate genes without testing for statistical association. We next evaluated the statistical association of candidate genetic variants with the blastomycosis phenotype. A single-variant association analysis was conducted with rare non-synonymous and pLOF variants in the candidate genes. There was a total of 103 variants in 42 candidate genes; 94 non-synonymous, 1 stop gain, 2 splice site acceptors, 2 non-frameshift deletions, and 4 frameshift indels (Table 17). Fisher exact test was used to test the association of each genetic variant. There were no statistically significant genetic variants in this analysis. The 103 genetic variants are reported in Table 17 with their Fisher exact test p-value, gene and variant annotation, rsID, case-control counts, and OR.

The top genetic variants in the single-variant association analysis were *GATA2* P161A, *IRAK1* T546M, *TYK2* G363S, *TLR1* R80T, and *IL17F* V155I (Table 17). *GATA2* P161A had the lowest p-value of 0.09 with 2 heterozygous controls. ClinVar reports this variant as conflicting interpretations of pathogenicity and no eQTLs were found in GTEx. The *TYK2* G363S non-synonymous variant is notably enriched in cases with 1 homozygote and 5 heterozygotes and 1 heterozygous control. This variant has the highest OR at 4.10 (95% CI: 0.5-36.27) and a Fisher exact p-value of 0.24. However, a heterozygous case and control are related and identity-by-descent analysis at the *TYK2* locus and surrounding 6 million base pairs on chromosome 19 resulted in a relatedness score of 0.83 across 1929 genetic variants. *TYK2* G363S is reported as benign in ClinVar, not predicted to be deleterious in SIFT, PolyPhen-2, or CADD, and an eQTL in GTEx with reduced expression in adrenal gland tissue in the heterozygous and homozygous states (p-value = 8.5×10^{-10}). *IL17F* V155I is previously reported in Section 3.4.2 as a putatively damaging variant. *TLR1* R80T and *IRAK1* T546M are reported in ClinVar as benign and no significant eQTLs in GTEx.

Table 17 shows that there were other interesting genetic variants with non-significant enrichment of alleles in cases compared to controls. There was a total of four other case enriched variants not in the top variants. The *TYK2* I684S, *DOCK8* A597V, and *IL17F* E126G variants were previously identified as putatively deleterious with ORs above two and described in Section 3.4.2 and Table 16. The *MALT1* R217G variant was found to be heterozygous in 4

cases with no alleles found in the controls. The 1000G and gnomAD allele frequency is 2.0% and 2.8%. SIFT and PolyPhen2 predictors indicate that this variant is benign and tolerated.

Table 17. Genetic variants in candidate genes.

Gene	Variant	Variant Type	rsID	Case Counts (/18)	Control Counts (/9)	OR (95% CI)	Fisher Exact p-value
GATA2	P161A	Non-synonymous	rs34799090	0	2	N/A	0.09
IRAK1	T546M	Non-synonymous	rs35638718	0	1*	N/A	0.11
TYK2	G363S	Non-synonymous	rs2304255	6*	1	4.10 (0.46-36.27)	0.24
TLR1	R80T	Non-synonymous	rs5743611	1	2	0.23 (0.02-2.70)	0.26
IL17F	V155I	Non-synonymous	rs11465553	1	2	0.23 (0.02-2.70)	0.26
SPPLA2	T475S	Non-synonymous	rs145209254	1	2	0.23 (0.02-2.70)	0.26
TLR9	G63V	Non-synonymous	-	0	1	N/A	0.29
MALT1	R217G	Non-synonymous	rs74847855	4	0	N/A	0.29
ITGAM	A1125T	Non-synonymous	-	0	1	N/A	0.31
FCGR2A	S314G	Non-synonymous	rs148465413	0	1	N/A	0.32
IL1R1	A124G	Non-synonymous	rs2228139	0	1	N/A	0.33
STAT4	E128V	Non-synonymous	rs140675301	0	1	N/A	0.33
TLR1	I57M	Non-synonymous	rs145135062	0	1	N/A	0.33
DOCK8	E169K	Non-synonymous	rs11789099	0	1	N/A	0.33
JAK2	R122C	Non-synonymous	rs147483622	0	1	N/A	0.33
CARD9	E270V	Non-synonymous	rs114895119	0	1	N/A	0.33
SPPLA2	A179E	Non-synonymous	rs200127156	0	1	N/A	0.33
IL4R	R185H	Non-synonymous	rs150955128	0	1	N/A	0.33
IL4R	P675S	Non-synonymous	rs3024678	0	1	N/A	0.33
IL4R	S786P	Non-synonymous	rs1805014	0	1	N/A	0.33
ITGAM	T1000N	Non-synonymous	rs41321249	0	1	N/A	0.33
TYK2	A928V	Non-synonymous	rs35018800	0	1	N/A	0.33
CD40	P175A	Non-synonymous	rs11086998	0	1	N/A	0.33
MPO	R604C	Non-synonymous	rs35670089	0	1	N/A	0.35
IFNG	c.115-2->T	Acceptor splice site	rs773507101	0	1	N/A	0.36
FCGR3A	L171H	Non-synonymous	rs10127939	3	3	0.45 (0.08-2.52)	0.39
CD40LG	G219R	Non-synonymous	rs148594123	1*	0	N/A	0.51
BCL10	A5S	Non-synonymous	rs12037217	3	0	N/A	0.54
FCGR3A	L171R	Non-synonymous	rs10127939	3	0	N/A	0.54
IL4R	S436L	Non-synonymous	rs1805013	3	0	N/A	0.54
IL12RB2	Q426H	Non-synonymous	rs2307145	2	0	N/A	0.55

IL6	D86V	Non-synonymous	rs2069860	2	0	N/A	0.55
CARD9	V385L	Non-synonymous	rs3124993	2	0	N/A	0.55
IL4R	E376del	Non-frameshift deletion	rs774578296	2	0	N/A	0.55
ZNF341	P95S	Non-synonymous	rs45577437	2	0	N/A	0.55
CD40LG	S132R	Non-synonymous	rs770773481	1*	0	N/A	0.55
IL23R	R381Q	Non-synonymous	rs11209026	2	2	0.47 (0.06-3.65)	0.59
DOCK8	A597V	Non-synonymous	rs17673268	5	1	2.74 (0.30-25.42)	0.65
IL17F	E126G	Non-synonymous	rs2397084	4	1	2.13 (0.22-20.55)	0.65
TYK2	I684S	Non-synonymous	rs12720356	4	1	2.13 (0.22-20.55)	0.65
FCGR3A	S170N	Non-synonymous	rs77144485	6	2	1.6 (0.29-8.86)	0.70
STAT4	c.129-2->T	Acceptor splice site	rs778032891	5	4	0.71 (0.16-3.18)	0.71
IL23R	V362I	Non-synonymous	rs41313262	1	0	N/A	>0.99
IL12RB2	G465D	Non-synonymous	rs2307153	1	0	N/A	>0.99
BCL10	A5V	Non-synonymous	-	1	0	N/A	>0.99
CHIA	A57T	Non-synonymous	rs182022651	1	0	N/A	>0.99
CHIA	F216L	Non-synonymous	rs36011905	1	0	N/A	>0.99
FCGR3A	F281V	Non-synonymous	rs396991	11*	6*	0.89 (0.28-2.85)	>0.99
FCGR3A	I211V	Non-synonymous	rs148181339	7	4	0.84 (0.21-3.37)	>0.99
FCGR2B	T88M	Non-synonymous	rs371903794	1	0	N/A	>0.99
NCF2	R314W	Non-synonymous	rs13306575	1	0	N/A	>0.99
CHIT1	T236S	Non-synonymous	rs61745299	1	0	N/A	>0.99
CHIT1	R40H	Non-synonymous	rs35920428	1	0	N/A	>0.99
CHIT1	N30S	Non-synonymous	rs146692911	1	0	N/A	>0.99
CXCR1	R335C	Non-synonymous	rs16858808	1	1	0.49 (0.03-8.25)	>0.99
CXCR1	G315X	Stop gain	-	1	0	N/A	>0.99
CXCR1	M31R	Non-synonymous	rs16858811	1	1	0.49 (0.03-8.25)	>0.99
CCR5	L55Q	Non-synonymous	rs1799863	1	0	N/A	>0.99
CCR5	A73V	Non-synonymous	rs56198941	1	0	N/A	>0.99
CCR5	L121R	Non-synonymous	rs150497029	1	0	N/A	>0.99
CCR5	S185fs*32	Frameshift deletion	rs333	1	0	N/A	>0.99
TLR1	H305L	Non-synonymous	rs3923647	1	1	0.49 (0.03-8.25	>0.99
TLR2	I556T	Non-synonymous	rs5743702	1	0	N/A	>0.99
TLR2	P631H	Non-synonymous	rs5743704	1	0	N/A	>0.99
TLR2	R650Q	Non-synonymous	rs200483398	1	0	N/A	>0.99

TLR2	R723C	Non-synonymous	rs371652530	1	0	N/A	>0.99
TLR2	R753Q	Non-synonymous	rs5743708	1	1	0.49 (0.03-8.25)	>0.99
ITK	H593Q	Non-synonymous	rs781268135	1	0	N/A	>0.99
IL12B	V298F	Non-synonymous	rs3213119	2	1	1 (0.08-11.8)	>0.99
IL6	D86E	Non-synonymous	rs13306435	1	1	0.5 (0.02-8.2)	>0.99
NCF1	G83R	Non-synonymous	rs139225348	1	0	N/A	>0.99
NCF1	S99G	Non-synonymous	rs10614	3	2	0.7 (0.11-4.8)	>0.99
TLR4	T199I	Non-synonymous	rs4986791	1	1	0.5 (0.02-8.2)	>0.99
CARD9	N524T	Non-synonymous	rs141310444	1	0	N/A	>0.99
CARD9	G171S	Non-synonymous	rs140508365	1	0	N/A	>0.99
SPPL2A	A200V	Non-synonymous	rs61752332	1	0	N/A	>0.99
SPPL2A	A200S	Non-synonymous	rs61752331	1	0	N/A	>0.99
SPPL2A	N126D	Non-synonymous	rs61751063	1	0	N/A	>0.99
IL4R	T9M	Non-synonymous	rs2234895	2	1	0.9 (0.07-10.5)	>0.99
ITGAM	P364H	Non-synonymous	-	1	0	N/A	>0.99
ITGAM	S367G	Non-synonymous	-	1	0	N/A	>0.99
ITGAM	A479S	Non-synonymous	-	1	0	N/A	>0.99
ITGAM	A479D	Non-synonymous	-	1	0	N/A	>0.99
MPO	I717V	Non-synonymous	rs2759	1	0	N/A	>0.99
MPO	I642L	Non-synonymous	rs112737382	1	0	N/A	>0.99
MPO	M251T	Non-synonymous	rs56378716	1	0	N/A	>0.99
TYK2	R703W	Non-synonymous	rs55882956	1	0	N/A	>0.99
TYK2	I60T	Non-synonymous	rs752152984	1	0	N/A	>0.99
TYK2	A53T	Non-synonymous	rs55762744	1	0	N/A	>0.99
IL12RB1	R528T	Non-synonymous	-	1	0	N/A	>0.99
IL12RB1	R283Q	Non-synonymous	rs117511121	1	1	0.5 (0.02-8.2)	>0.99
ZNF341	L148M	Non-synonymous	-	1	0	N/A	>0.99
ZNF341	G149W	Non-synonymous	-	1	0	N/A	>0.99
ZNF341	S406N	Non-synonymous	rs141578693	1	0	N/A	>0.99
CD40	G199R	Non-synonymous	rs41282788	1	0	N/A	>0.99
IFNGR2	A22del	Non-frameshift deletion	rs765468464	2	0	N/A	>0.99
AIRE	S338P	Non-synonymous	rs374555273	1	0	N/A	>0.99
AIRE	R471C	Non-synonymous	rs74203920	1	0	N/A	>0.99
ITGB2	R586W	Non-synonymous	rs5030672	1	0	N/A	>0.99

*Denotes homozygotes present. N/A, Genetic variants found only in cases or controls. “-“, No rsID available. CI, confidence interval; OR, odds ratio.

3.4.4 Gene-based Association Analysis

We conducted a gene-based association analysis combining genetic variants per gene to improve the statistical power to detect associations with the blastomycosis phenotype. These analyses are beneficial for small sample sizes and studies examining the association of rare genetic variants (171,178). Large sequencing studies have also applied these methods to successfully identify genetic susceptibility factors for COVID-19 (228) and inflammatory bowel disease (142). The gene-based association analysis was performed with the weighted binary SKAT-O test and an exploratory p-value threshold of 0.05 was used.

The first analysis consisted of testing rare pLOF genetic variants in candidate genes for statistical association. A total of 7 pLOF genetic variants in 6 different genes were assessed. No genes were found to be statistically significant in this analysis. *IFNG* and *CCR5* had the lowest p-values at 0.17 and 0.67, respectively. The remaining candidate genes containing pLOF variants had p-values of >0.99. Figure 6 shows the results of the pLOF SKAT-O analysis. The genetic variants comprising each candidate gene were described in Section 3.4.2. The *IRAK1* and *ITGAM* genes were annotated in ANNOVAR as containing genetic variants with frameshift indels. However, due to missing genotypes and variant parameter filtering, no individuals in our study were reported to carry these alleles.

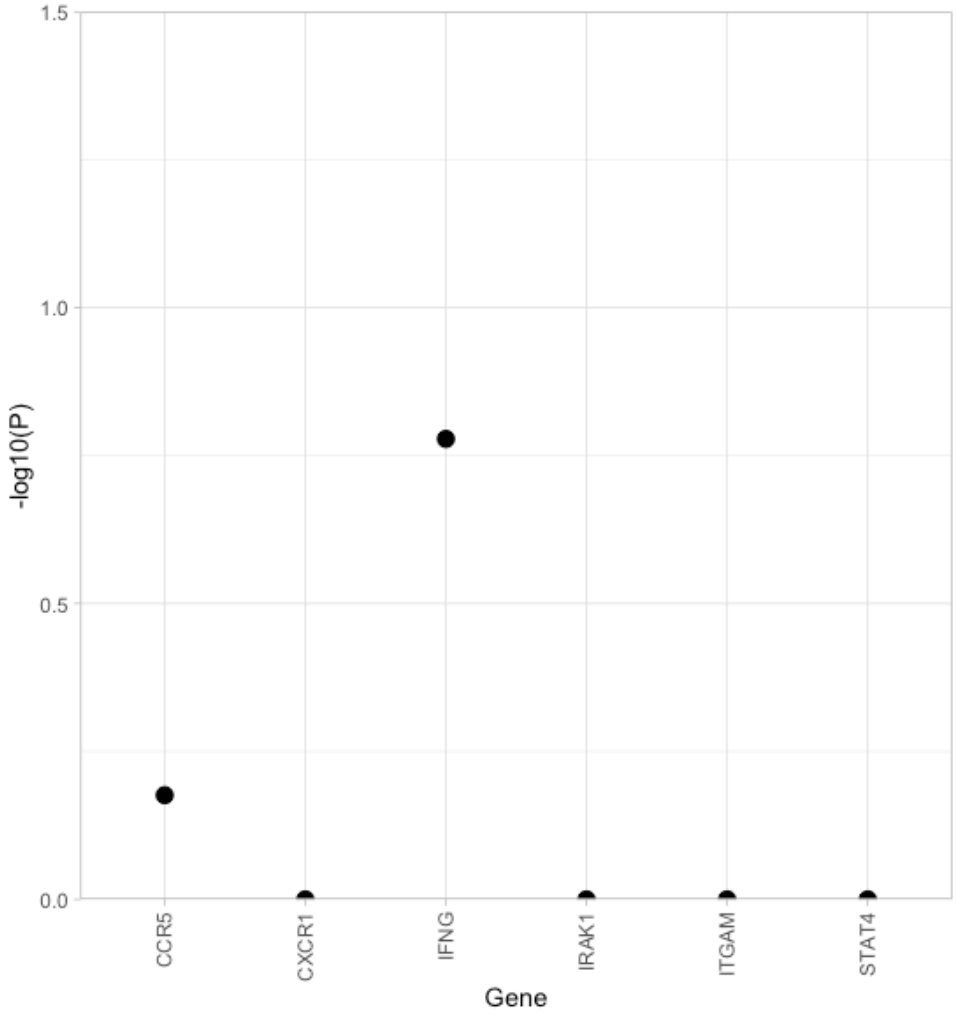

Figure 6. Weighted binary SKAT-O test results of candidate genes containing rare pLOF genetic variants.

The gene-based association analysis was expanded from only rare pLOF variants to include rare non-synonymous and non-frameshift indels. A total of 103 genetic variants were grouped into 42 candidate genes and analyzed with binary SKAT-O (Figure 7). None of the genes containing pLOF genetic variants were statistically significant after the addition of non-synonymous and non-frameshift indel variants. Two statistical signals were detected in candidate genes: *TLR1* and *GATA2*. The *TLR1* gene was nominally significant with a p-value was 0.024 and *GATA2* trended towards significance with a p-value of 0.051. The *TLR1* gene contained 3 rare non-synonymous genetic variants: I57M, R80T, and H305L. The I57M variant is annotated by *in silico* predictors as deleterious and damaging with a CADD score of 22.5. These

variants were enriched in blastomycosis controls, and all individuals were heterozygotes. I57M in two controls and one case, R80T in only one control, and H305L in one case and one control. Adjusting to the ratio of two cases per control, *TLR1* alleles were found more in controls than cases. The 1000G total population allele frequency was 0.1% for I57M, 2.3% for R80T, and 2.9% for H305L. The *GATA2* gene contained only 1 rare non-synonymous variant, P161A. The results for this genetic variant are described in Section 3.4.3.

The *TLR1* signal in rare variants is interesting as common variation has been reported to influence susceptibility to candidiasis and aspergillosis (90,91). Therefore, the binary SKAT-O statistical analysis was expanded to include both common and rare genetic variants by removing the MAF filter. There was a total of 172 genetic variants in 56 genes tested with binary SKAT-O. The *TLR1* signal was conserved with a p-value of 0.044 with the addition of two common variants, S602I and N248S (Figure 8). Both variants had nearly equal allele distribution in cases and controls although N248S was found more in cases while S602I was more in controls. The *GATA2* signal reached statistical significance with a p-value of 0.003 with the addition of the common genetic variant A164T (Figure 8). This common variant has a 1000G allele frequency of 23.3% and represented with 8 heterozygous cases and 1 homozygote and 5 heterozygous controls.

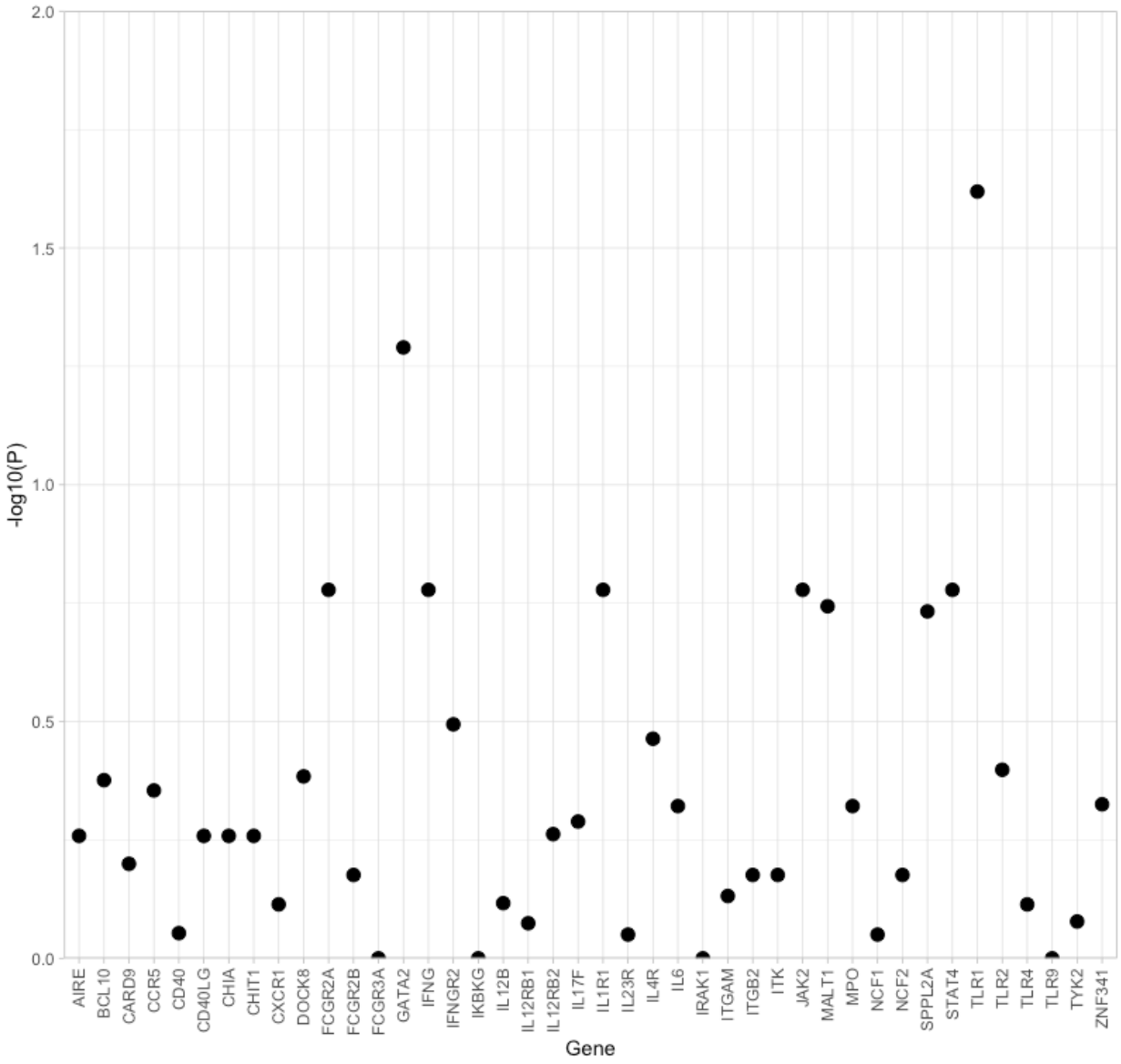

Figure 7. Weighted binary SKAT-O test results of candidate genes containing rare non-synonymous, non-frameshift deletions, and pLOF genetic variants.

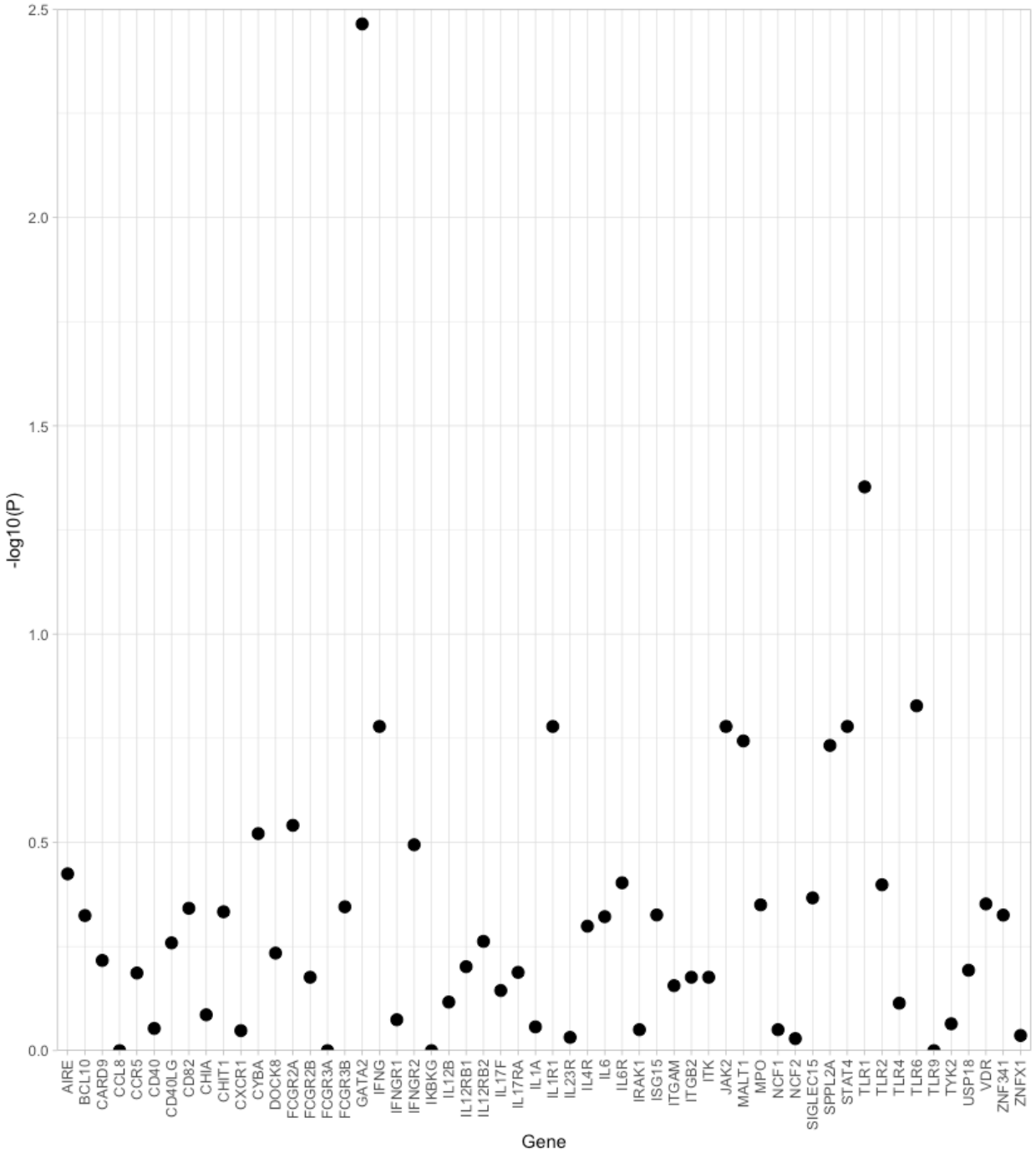

Figure 8. Weighted binary SKAT-O test results of candidate genes containing common and rare non-synonymous, non-frameshift deletions, and pLOF genetic variants.

3.4.5 Pathway Analysis

We next applied a similar variant aggregation approach to functionally related genes within pathways. This approach allows for the detection of pathways associated with the phenotype of interest (172). We used the candidate gene clusters generated using STRING to conduct the pathway analysis (Figure 5). Each of the 9 annotated gene clusters and the grouped singletons ("other") were analyzed with weighted binary SKAT-O. The plot in Figure 9 shows the JS or JAK-STAT signaling pathway with the lowest p-value of 0.39. This cluster is composed of the genetic variants in the following genes *TYK2*, *JAK2*, *STAT4*, *IL12RB1*, *IL12RB2*, *IFNGR2*, and *IL4R*.

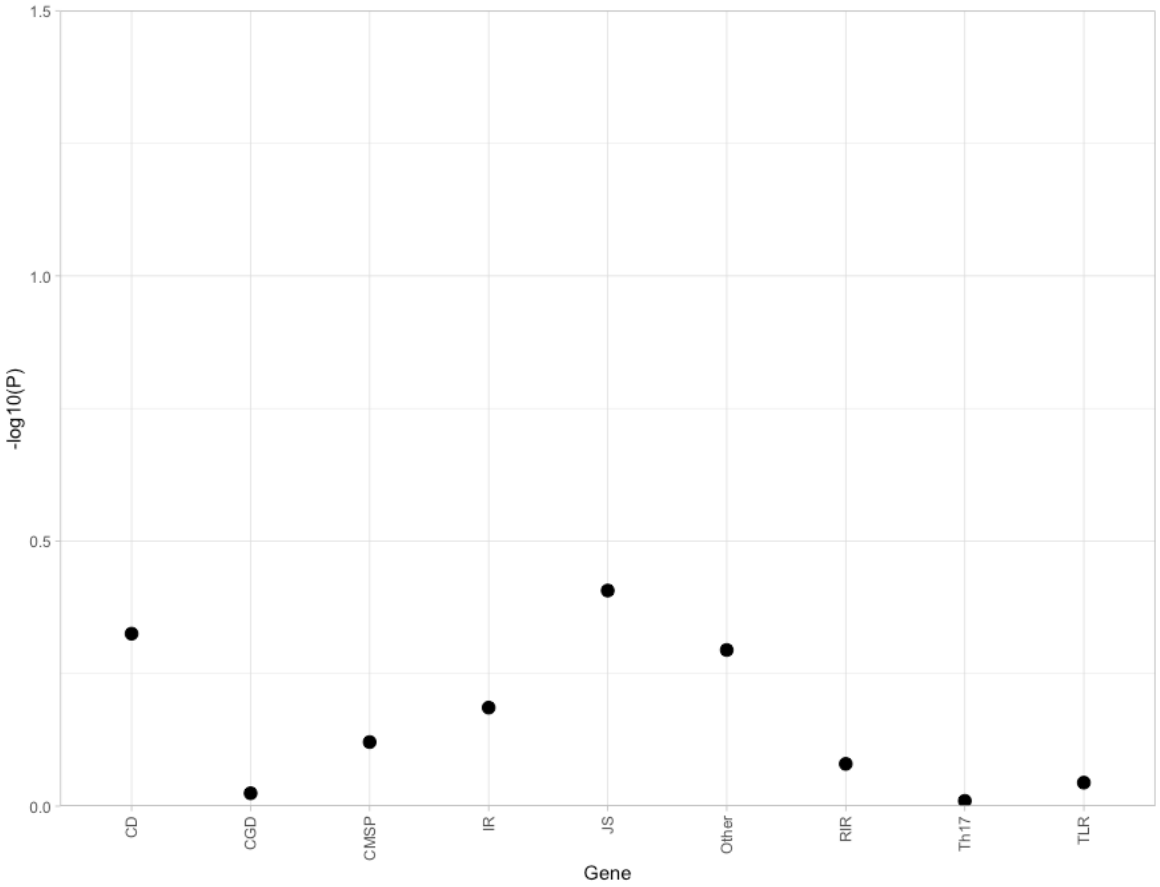

Figure 9. Weighted binary SKAT-O test results of STRING clustered pathways using rare moderate and pLOF genetic variants. The gene clusters are found in Figure 5. CD, chitin degradation; CGD, chronic granulomatous disease; CMSP, chemokine-mediated signaling pathway; IR, inflammatory response; JS, JAK-STAT signaling pathway; RIR, regulation of immune responses; Th17, Th17 differentiation; TLR, toll-like receptor signaling pathway.

3.5 Exome-wide Association Analysis

An exome-wide approach was conducted after the hypothesis-driven candidate gene approach to explore enrichment in any host genetic factors regardless of previous disease association. This approach allows for unbiased discovery of genetic signals that may have been excluded during candidate gene prioritization. This analysis tests if non-mycobacterial and non-fungal immunity-related genes may contribute to the blastomycosis phenotype.

3.5.1 Single-variant Association Analysis

We performed an exome-wide single-variant association analysis to determine if any host genetic signals were significantly enriched in cases or controls. All genetic variants that passed variant filtering (Section 2.5), including rare variants with MAF < 0.05, were selected for analysis. In total, 105,746 genetic variants were analyzed with PLINK v. 1.9 Fisher exact test and logistic regression under the allelic, dominant, and recessive models. The Manhattan plots of the association analyses in Figure 10 show that no genetic variants were statistically significant at the exome-wide threshold of $p<5x10^{-6}$. However, nine genetic variants in the Fisher exact test from seven unique genes were found below the exploratory $5x10^{-4}$ threshold (Figure 10A).

The top hits in the Fisher exact test are listed in Table 18. The variants were synonymous (3/9), intronic (2/9), non-synonymous (2/9), non-frameshift deletion (1/9), and non-coding RNA (1/9). All of the genetic variants were reported as benign in ClinVar (Table 18). The variant with the lowest statistically significant p-value is g.41958385A>C on chromosome 22 (OR = 18.18 (95% CI: 3.56-92.99), p-value = $9.92x10^{-5}$). This variant is positioned in the non-coding RNA *LINC00634* and the protein-coding gene *SMIM45* annotated as non-synonymous T57P. This variant is not reported in 1000G but reported in gnomAD with a total allele frequency of 46.3%. The *UBTFL1* G96R variant had the lowest gnomAD allele frequency at 7.8% with benign prediction with SIFT, PolyPhen-2, and CADD.

The single-variant association analysis yielded two genetic variants within genes associated with cancer (*WWC3*) and autoimmunity (*COL6A1*) at the exploratory threshold of $5x10^{-4}$. However, both variants are reported as benign with no publications available on associations with disease and no obvious link to antifungal or antimycobacterial immunity.

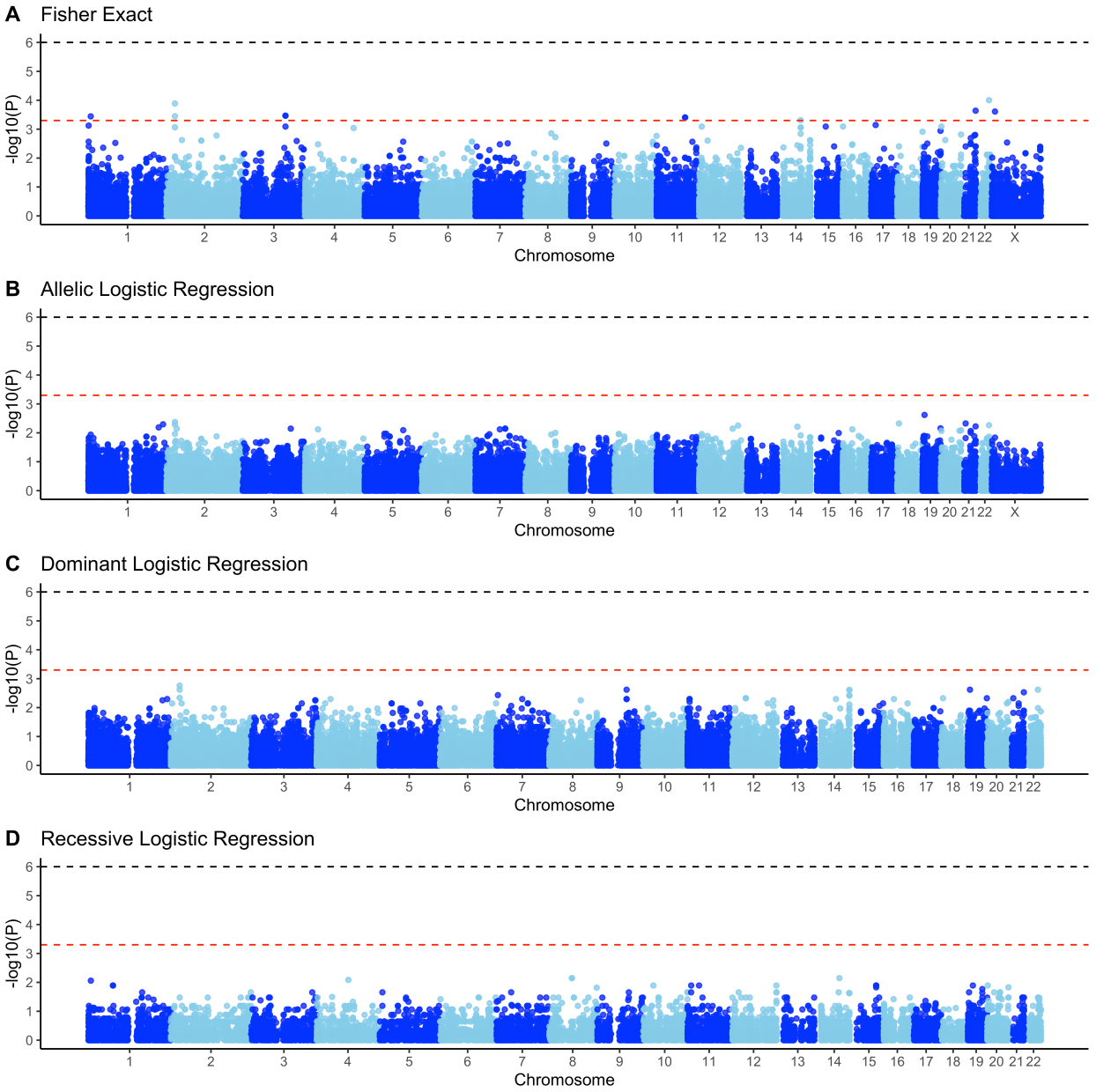

Figure 10. Manhattan plots of exome-wide variant association analyses. (A) Fisher exact test. (B) Allelic model logistic regression. (C) Dominant model logistic regression. (D) Recessive model logistic regression. Black line represents the $5x10^{-6}$ exome-wide significance threshold. Red line represents the $5x10^{-4}$ exploratory significance threshold.

Table 18. Top associated genetic variants in the exome-wide association analyses with the Fisher exact test.

Gene	Chr	Variant	OR (95% CI)	Fisher Exact P-value	Variant Consequence	gnomAD allele frequency	ClinVar
LINC00634	22	g.41958385A>C	18.18 (3.56-92.99)	9.92×10^{-5}	Non-coding RNA	46.3%	Benign
OTOF	2	V170V	16.00 (3.15-81.26)	1.30×10^{-4}	Synonymous	N/A	Benign
COL6A1	21	c.1461+41dupA>AG	0.03 (0.01-0.31)	2.30×10^{-4}	Intronic	73.7%	Benign
WWC3	X	c.508-9G>C	0.05 (0.01-0.29)	2.46×10^{-4}	Intronic	29.3%	Benign
PPP2R3A	3	D111D	N/A	3.42×10^{-4}	Synonymous	26.6%	Benign
PPP2R3A	3	S642G	N/A	3.61×10^{-4}	Non-synonymous	25.4%	Benign
OTOF	2	L914L	14.15 (2.80-71.50)	3.93×10^{-4}	Synonymous	N/A	Benign
PER3	1	A1016_S1033del	0.09 (0.02-0.34)	3.93×10^{-4}	Non-frameshift deletion	18.8%	Benign
UBTFL1	11	G96R	N/A	4.91×10^{-4}	Non-synonymous	7.8%	Benign

3.5.2 Gene-based Association Analysis

We next performed a genetic variant collapsing method to enhance power for association detection in the case where multiple variants in the same gene impact susceptibility to blastomycosis. Only rare pLOF, non-synonymous variants, and inframe indels were kept for analysis, consistent with candidate gene-based association analysis (Section 3.4.4), yielding 22,735 rare variants from the 105,746 total variants. These genetic variants were grouped according to their ANNOVAR gene annotation yielding 10,163 genes containing at least one variant. The genes were analyzed with the weighted binary SKAT-O statistical test (Section 2.6).

No genes were statistically significant after FDR p-value adjustment for multiple testing correction (Table 19). An exploratory threshold of 5×10^{-3} was considered to investigate top results for this analysis. A total of 8 genes were found below this threshold (Table 19 and Figure 11). The top hit was *THNSL1* with genetic variants enriched in controls in the heterozygous state and a p-value of 3.48×10^{-3}. The remaining genes were all enriched in the controls except for *LVRN*. A common variant not annotated in 1000G within *LVRN* skewed the results towards case enrichment. Removal of this variant shifts *LVRN* to control enrichment.

Table 19. Top genes from the exome-wide gene-based association analysis with weighted binary SKAT-O.

			No. heterozygous (no. homozygous) individuals			
Chr	Gene	No. variants	Cases (n = 18)	Controls (n = 9)	p value	Adjusted p value
10	*THNSL1*	5	1 (0)	7 (0)	3.48×10^{-3}	0.958
14	*PRSS55*	3	2 (1)	7 (1)	3.59×10^{-3}	0.958
19	*MISP*	3	0 (0)	4 (0)	3.59×10^{-3}	0.958
9	*MPDZ*	6	0 (0)	6 (0)	3.59×10^{-3}	0.958
16	*MT1E*	1	0 (0)	4 (0)	3.59×10^{-3}	0.958
19	*MVB12A*	2	0 (0)	5 (0)	3.59×10^{-3}	0.958
19	*ALKBH7*	1	1 (0)	5 (0)	4.11×10^{-3}	0.958
5	*LVRN*	9	7 (15)	8 (5)	4.27×10^{-3}	0.958

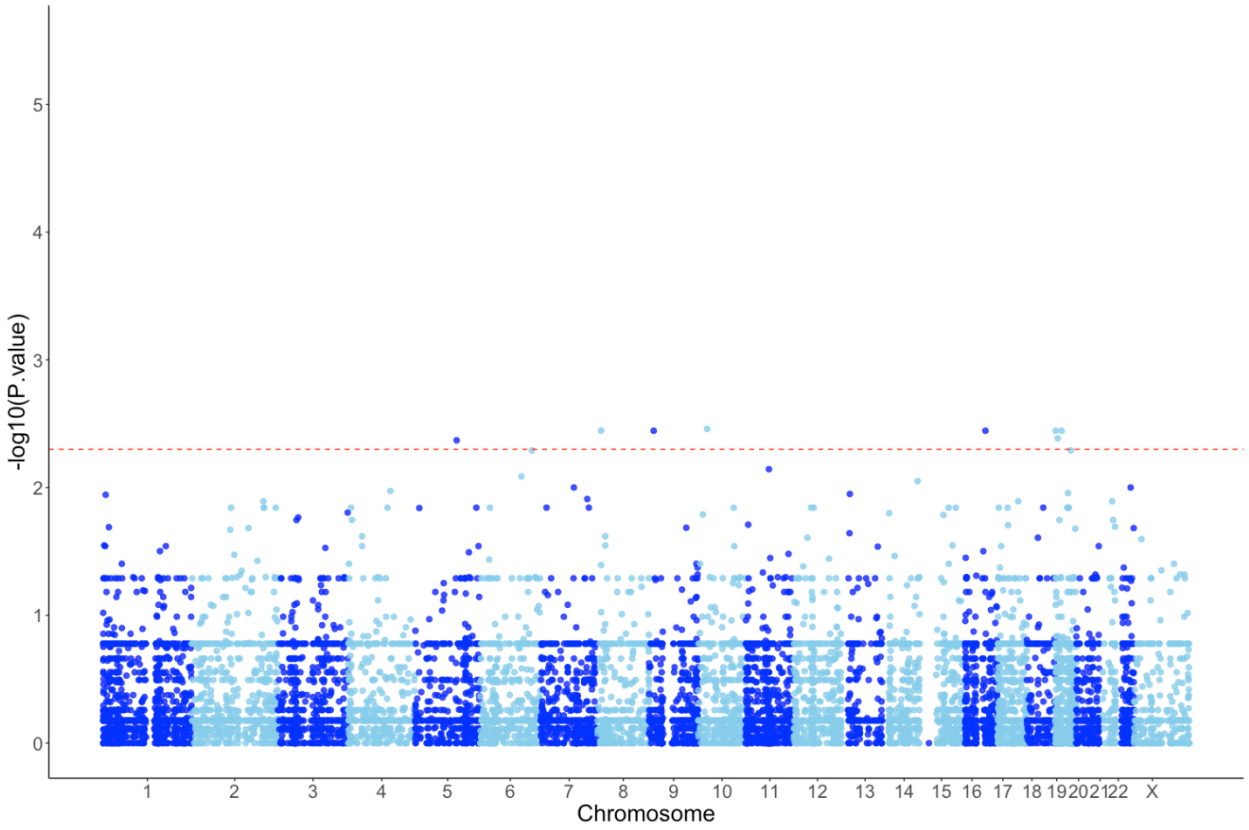

Figure 11. Manhattan plot of the exome-wide gene-based association analysis with weighted binary SKAT-O. The red line represents the exploratory 5×10^{-3} threshold.

3.5.3 Pathway Analysis

We next used the same collapsing approach to group variants within genes acting in the same pathways. As with the gene-based approach, this method enhances power for association discovery when multiple variants within a pathway impact the phenotype under study. We selected the NHC tool as a systematic method that can be applied exome wide to generate gene clusters for pathway-based association analysis (180). This tool generates small gene clusters with shared biological function to improve power for detecting genetic signals. The genes included for gene clustering contained genetic variants with deleterious CADD scores (CADD $\geq$ 20). A total of 78 gene clusters were generated with 7 gene clusters enriched in blastomycosis cases with $p < 0.05$. Three of these gene clusters were significantly enriched with an FDR less than 20% (Table 20).

The top gene cluster with the lowest FDR of 13.3% contained genes annotated by Reactome to be involved in chromatin remodeling and runt-related transcription factor 1 (RUNX1) interactions. The other two pathways each had an FDR of 13.8%. The first gene cluster was annotated by Reactome as "cross presentation of soluble exogenous antigens in endosomes". The genes contained in this gene cluster are involved in the proteasome and immunoproteasome complexes. The second gene cluster was annotated by Reactome as "intraflagellar transport" with genes involved in the intraflagellar transport (IFT) system for protein transport. The only significant gene cluster with known fungal susceptibility genes was the "CARD Domain Binding" gene cluster containing *CARD9* and *BCL10*. However, this gene cluster had an FDR of 26.5%, higher than the 20% threshold.

Our results show gene clusters with FDRs below 20% involved in chromatin remodeling, the proteosome, and IFT. The association of these gene clusters with blastomycosis may warrant further investigation to determine if they contribute to blastomycosis immunity.

Table 20. Exome-wide NHC pathway analysis with predicted deleterious genetic variants.

Pathway	Gene clusters	Number of genes	Number of cases	P-value	FDR
Chromatin remodeling, RUNX1 interactions	*ACTL6A, ACTL6B, INO80, NFRKB, PBRM1, SMARCC2, SMARCD3*	7	9	0.0022	0.133
Cross presentation of soluble exogenous antigens in endosomes	*PSMB5, PSMB6, PSMD9, PSMD13, PSME4*	5	8	0.0045	0.138
Intraflagellar transport	*IFT122, IFT140, TTC21B, WDR35*	4	8	0.0045	0.138
Regulation of commissural axon pathfinding by SLIT and ROBO	*ROBO1, ROBO2, SLIT3, SRGAP1*	4	6	0.0171	0.265
CARD Domain Binding	*BCL10, CARD10, CARD11, CARD9*	4	6	0.0171	0.265

CARD, caspase activation and recruitment domain, FDR, false discovery rate; ROBO, roundabout guidance receptor; RUNX1, runt-related transcription factor 1; SLIT, slit guidance ligand.

Chapter 4: Discussion

4.1 Host Susceptibility to Naturally Acquired Blastomycosis: A Scoping Review

We included a total of 58 articles on host susceptibility to naturally acquired blastomycosis using a scoping review approach. The included articles ranged from single patient to cohort studies and experimental approaches with mice and cell models. Most evidence of host susceptibility was derived from case reports and experimental studies. We identified four categories of evidence for host susceptibility: direct evidence of primary or secondary immunosuppression in blastomycosis, immunomodulatory therapy adverse effects, $CD4^+$ deficiency, and host targeting virulence factors. Direct evidence of blastomycosis susceptibility factors were initially sought for this scoping review as they provide an insight into the underlying immunological mechanisms required to protect against disease. Evidence of concurrent immunomodulatory therapies and blastomycosis in the database search led to further exploration of this literature. Specific breaches in immunity caused by these immunomodulators may predispose individuals to blastomycosis and inform potential host factors required for protective immunity. Similarly, we explored *Blastomyces* host targeting virulence factors as these targets can elucidate essential host factors for immunity. $CD4^+$ T cells are required for blastomycosis immunity and deficiency has been well described as a host susceptibility factor. Each of these evidence sources provide insights into blastomycosis immunity. The findings from each category were amalgamated to develop a better picture of the current landscape of host susceptibility to naturally acquired blastomycosis.

4.1.1 Physiological and Innate Immune Barriers

B. dermatitidis infection occurs most commonly through the inhalation of conidiospores (21). In the respiratory tract, the mucous layer entraps conidiospores and ciliated epithelial cells push the mucus and debris through mucociliary clearance. Dysfunction of mucociliary clearance impairs debris and conidiospore clearance from the respiratory tract (78). PCD is a rare primary immunodeficiency caused by genetic variation in the structure and function of cilia preventing effective mucus clearance and enhancing susceptibility to fungal, bacterial and viral infections (80). Guevara et al. report a case of severe pulmonary blastomycosis in a patient with PCD

successfully treated with amphotericin B and oral itraconazole (79). No genetic etiology was described for this patient. This is the only report of blastomycosis in a patient with PCD. CF is a genetic disorder of the CF transmembrane conductance receptor (CFTR) characterized by thick viscous mucus and impaired mucociliary clearance contributing to an increased risk of opportunistic bacterial and fungal infections (231). Gershan et al. report a case of CNS disseminated blastomycosis in a paediatric patient with CF successfully treated with amphotericin B (181). No pulmonary manifestations were observed although pulmonary histopathology was performed after treatment and resolution of CNS blastomycosis. Experimental evidence of mucus hypersecretion in canine and feline blastomycosis has been described mechanistically, but the effect on susceptibility was not determined (86). The dysfunction of mucus production and clearance may have predisposed these patients to blastomycosis, but further studies are required to confirm these findings.

4.1.2 Blastomyces Recognition

The recognition of *Blastomyces* is necessary for the initiation of protective immunity. Currently, only the soluble complement proteins C3 and C5 of the complement system have been implicated in host susceptibility to blastomycosis (195,225). The complement system functions in the recognition of pathogens by phagocytes, initiation of inflammation and chemoattraction, and formation of the membrane attack complex although the latter is not known to be effective against fungi (232). Complement activation occurs through three pathways: classical, alternative, and mannose-binding lectin pathways each converging and requiring C3 for downstream function. C3 is cleaved into C3a, C3b, and iC3b where C3a mediates inflammation and C3b and iC3b bind invading microbes and phagocyte complement receptors. C5 is similarly cleaved into C5a, a strong mediator of the inflammatory response, and C5b required for membrane attack complex formation (201). Zhang et al. show that BAD1 inhibits binding of C3 to *B. dermatitidis* surface β-glucan in an *in vitro* murine model (225). The inhibition of C3 binding may reduce CR3 recognition of C3 cleavage products C3b and iC3b in favour of BAD1-CR3 interaction and subsequent immunomodulation (233). The reduced virulence of *B. dermatitidis* in the absence of BAD1 is multifactorial but C3 inhibition may be a contributing

virulence mechanism for blastomycosis. Hirama et al. supports the requirement of the complement system in blastomycosis with a case of fatal pulmonary blastomycosis in the setting of lung transplantation and therapeutic eculizumab monoclonal antibody directed at C5 inhibition (195). This case had rapid onset pulmonary blastomycosis limited to the transplanted lung after eculizumab initiation. However, the contribution of concurrent immunosuppressive therapy to disease development cannot be dismissed. These findings are supported in other fungal infections with gene knockouts of murine C3 and C5 showing increased susceptibility to invasive aspergillosis and candidiasis (234,235).

No surface bound PRRs have been shown to be susceptibility factors for naturally acquired immunity to *Blastomyces*. Dectin-2 has been shown to recognize *Blastomyces* endoglucanase-2 (BI-Eng2) antigen for Th17-specific immune priming for vaccine-induced immunity but its role in susceptibility to naturally acquired blastomycosis is not known (236). NF-κB is an essential transcription factor downstream of PRR signaling for proinflammatory cytokine expression (98). In a murine model of *B. dermatitidis* infection, NF-κB in lung epithelial cells contributed to the initiation of IL-17A and GM-CSF production by natural Th17 cells (96). The knockout of *IKK2* to impair NF-κB function increased *B. dermatitidis* fungal burden and reduced survival compared to wild-type mice (96). TLR and CLR deficiencies have not yet been directly implicated in host susceptibility to naturally acquired blastomycosis. However, studies of vaccine-induced immunity suggest that MyD88 and CARD9 are essential for the Th17 response and protection from secondary *B. dermatitidis* infection (131,237).

4.1.3 Innate Immunity

The innate immune response is essential for the control of *B. dermatitidis* infection and the initiation of the adaptive immune response (21). Tissue resident alveolar macrophages are one of the front line innate immune cells to recognize and initiate innate immunity. The activation of alveolar macrophages requires GM-CSF to initiate antimicrobial function (238). GM-CSF also activates hematopoietic-derived macrophages, neutrophils, and DCs (238). Autoantibodies against GM-CSF are the primary cause of pulmonary alveolar proteinosis (PAP) characterized by impaired pulmonary surfactant clearance by alveolar macrophages (239). We identified four

studies implicating GM-CSF deficiencies with susceptibility to blastomycosis. Three case reports in human subjects show PAP and blastomycosis diagnoses (182–184). An experimental study in mice by Sterkel et al. demonstrated that GM-CSF receptor knockouts remarkably reduced survival in mice with high *B. dermatitidis* fungal burden after intratracheal infection (117). The inability of phagocytic cells to respond to GM-CSF contributed to the impaired immunity and reduced survival. Sterkel et al. also established that *B. dermatitidis* DppIVA directly targets and cleaves GM-CSF into an inactive form, contributing to fungal pathogenesis (117). This suggests an indispensable role in blastomycosis in both humans and mice as a host factor for protective immunity as well as a target for *B. dermatitidis* DppIVA to evade host immunity. However, future studies should evaluate and clarify the requirement of GM-CSF in human blastomycosis as the current evidence is limited to case reports.

Phagocytic cells also require TNF-α, a pleiotropic cytokine important in early innate immunity and the development of the Th1 response (84). TNF-α was the host susceptibility factor with the most supportive evidence for blastomycosis with 14 patients across 9 observational studies on TNF-α inhibitors and 3 experimental studies on TNF-α modulation by *B. dermatitidis* BAD1 (186,187,221,222,188–194,220). There is accumulating evidence that TNF-α deficiency contributes to blastomycosis as seen with histoplasmosis and coccidioidomycosis (240). Infliximab, adalimumab, etanercept, and certolizumab are mAb therapies that bind and inhibit TNF-α function in inflammation to treat autoimmune disorders (138). An adverse effect of reduced TNF-α is increased host susceptibility to primary infection, particularly histoplasmosis and coccidioidomycosis, but not reactivation of latent fungal infection (240). Blastomycosis most often occurs due to primary infection with only a few reports of latent reactivation (21,53,195). However, the incidence rate of blastomycosis in recipients of TNF-α inhibitors is not known. Further evidence of the requirement of TNF-α is derived from experimental studies of BAD1. Finkel-Jimenez et al. show in a mouse model that surface bound and soluble BAD1 bind CR3 to modulate TNF-α through TGF-β-dependent and -independent mechanisms, respectively (221,222). The downregulation of TNF-α was associated with reduced *B. dermatitidis* killing *in vitro* by macrophages (222). Brandhorst et al. later demonstrated the

binding of BAD1 to CR3 and CD14 to be involved in TNF-α suppression (220). This suggests that TNF-α modulation by BAD1 contributes to the pathogenesis of blastomycosis.

Vitamin D plays a role in innate immunity contributing to macrophage function similar to GM-CSF and TNF-α. The serum levels of 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) as well as host genetic variation in the vitamin D binding protein (VDBP) and vitamin D receptor (VDR) affect the function of innate immune cells and clearance of infectious diseases. Vitamin D insufficiency and polymorphisms in *VDBP* and *VDR* most notably influence human susceptibility to tuberculosis (241). Two studies have identified associations between vitamin D and blastomycosis (124,185). In a genetic association analysis of a human blastomycosis case-control cohort, Sainsbury et al. demonstrated the *VDBP* rs4588 genetic variant enriched in controls, suggesting a protective effect (124). Sainsbury et al. hypothesized that reduced VDBP affinity due to rs4588 increases 25OHD availability for macrophage induction of cathelicidin, a potent antimicrobial peptide (124). However, rs4588 is associated with lower serum levels of 25OHD, decreased macrophage activation, and greater susceptibility to tuberculosis (242). Monitoring vitamin D levels in blastomycosis has been performed by O'Brien et al. in dogs. This study determined that lower serum levels of 25OHD were associated with blastomycosis acquisition but not mortality (185). The direction of the association was not determined as 25OHD was measured only after blastomycosis diagnosis and therefore, vitamin D insufficiency may have been due to blastomycosis rather than a contributing factor. Overall, the findings of vitamin D in blastomycosis susceptibility are conflicting and require additional investigation to determine the role of genetic variation and serum metabolite levels. GM-CSF, TNF-α, and vitamin D are involved in innate immune cell antimicrobial function, particularly macrophages and neutrophils. Deficiency in each has been reported to increase blastomycosis susceptibility. The antimicrobial functions of macrophages, neutrophils, and DCs have been shown to be effective against killing *B. dermatitidis in vitro* and *in vivo* (243–245). The included studies further indicate a requirement for effective innate immune function early in infection.

The production of proinflammatory cytokines and chemokines from alveolar macrophages stimulates the recruitment of hematopoietic-derived immune cells. The

generation of these immune cells requires GATA2, a transcription factor important in hematopoietic stem cell gene expression and homeostasis (246). GATA2 haploinsufficiency and LOF causes monocytopenia and mycobacterial infections (MonoMAC), a primary immunodeficiency characterized by deficiency of monocytes, neutrophils, DCs, B cells, and NK cells (246). GATA2 deficiencies predispose individuals to a broad range of bacterial, fungal, and viral infections as hematopoietic-derived immune cells are important for the control of infectious diseases (247). Spinner et al. reports the only case of GATA2 deficiency and MonoMAC in blastomycosis caused by a LOF frameshift insertion in *GATA2* (75). The patient successfully recovered with liposomal amphotericin B followed by oral itraconazole. This sentinel case for GATA2 requirement in blastomycosis implicates the necessity of hematopoietic-derived immune cells for effective immunity in humans. The recruitment of these cells to the site of infection is another important step for their antimicrobial function in infection clearance. *B. dermatitidis* DppIVA functions similarly to human DppIV in cleaving N-terminus glutamate leucine arginine motif $(ELR)^+$ CXC chemokines. Lorenzini et al. demonstrated DppIVA cleavage of human CXCL2, or macrophage inflammatory protein 2, and murine CXCL1 leading to enhanced neutrophil recruitment with reduced stimulation of ROS production by cleaved CXCL2 (226). They hypothesized that these findings in combination with the cleavage of GM-CSF could explain the formation of pyogranulomatous inflammation with extensive neutrophil influx but reduced effectiveness of macrophage and neutrophil function. The BAD1 suppression of TNF-α and vitamin D deficiency may further impair macrophage function contributing to this clinical manifestation. Effective innate cell recruitment requires endothelial adhesion to move towards the site of infection (248). The inhibition of leukocyte function-associated antigen 1 (LFA-1) with efalizumab, a CD11a-binding monoclonal antibody, prevents leukocyte (monocytes, neutrophils, DCs, B, and T lymphocytes) adhesion to the vascular endothelium through the binding of intracellular adhesion molecule 1 (ICAM1) (205). A single case report of efalizumab and blastomycosis without concurrent immunosuppression was identified between FDA approval in 2003 and therapy withdrawal in 2009 (41,205). Atanaskova et al. report a case of cutaneous and pulmonary blastomycosis in a patient on efalizumab (41). The inhibition of T cell recruitment and activation by efalizumab was targeted

to treat psoriasis. However, the broad inhibition of immune cell adhesion may have contributed to the increased incidence of fungal infections and this case of blastomycosis (205). This is the only article describing vascular adhesion and migration for immune cells and blastomycosis. Additional studies are required to corroborate the findings from this case report.

Overall, studies of innate immunity in host susceptibility to blastomycosis have shown the importance of macrophage and neutrophil antimicrobial function and hematopoietic-derived immune cells at the site of infection. These findings are corroborated by experimental studies of macrophages and neutrophils demonstrating effective *B. dermatitidis* killing when stimulated with IFN-γ (244,245). Therefore, the identified innate host factors could influence susceptibility to blastomycosis. Future investigations should attempt to elucidate the role of these factors in blastomycosis host immunity.

4.1.4 Adaptive Immunity

Cell-mediated immunity, particularly $CD4^+$ T cells are essential for effective host immunity against blastomycosis. Blastomycosis in the setting of $CD4^+$ T cell deficiency has been described for persons living with HIV (9,14,211–217,15,53,54,73,206–208,210) and ICL (55,73,218). T cell deficiency has also been described in a case of severe malnutrition (209). The incidence rate of blastomycosis in $CD4^+$ deficiency is similar to the general population. Therefore, cell-mediated immunodeficiency is not thought to increase susceptibility to blastomycosis but rather exacerbate disease severity (21). This is in contrast to histoplasmosis, coccidioidomycosis, and cryptococcosis where incidence rates are higher for persons with $CD4^+$ deficiency than the general population (249). We report an updated table of the incidence of blastomycosis clinical manifestations and concurrent AIDS-defining illnesses in persons living with HIV and advanced HIV and ICL (Table 10). Persons living with advanced HIV with $CD4^+$ cell counts $\leq$200 cells/µL are at a higher risk for blastomycosis dissemination to the CNS and atypical sites at 45.2% and 38.7% compared to the general population rate of CNS dissemination around 3-10% of disseminated cases (53,250). The risk may also be greater for those with $CD4^+$ cell counts >200 cells/µL but only three patients were identified and only a single case presented with CNS and

atypical dissemination (206). Similarly, only 4 cases of ICL and severe malnutrition were available although two cases presented with CNS dissemination (55,218).

The differentiation of $CD4^+$ T cells into Th1 and Th17 cells is important for protective immunity against blastomycosis. The Th1 response was identified in three *in vivo* experimental studies in mouse models. Brummer et al. and Kethineni et al. both report recombinant IFN-γ supplementation improving survival in BALB/c and young CD-1 mice, respectively compared to saline-administered controls (128,129). These studies were the first to show a requirement of IFN-γ for murine survival. Previous studies of murine macrophage and neutrophil *in vivo* killing of *B. dermatitidis* first demonstrated enhanced antifungal capacity when stimulated with IFN-γ (244,245). The production of IFN-γ by $CD4^+$ Th1 cells is stimulated by IL-12 produced by phagocytic cells in response to PRR detection of *B. dermatitidis*. Brummer et al. demonstrated recombinant IL-12 supplementation improving survival in a BALB/c mouse model of blastomycosis (130). Recombinant IL-12 supplementation also improved survival in a C57BL/6 mouse model of histoplasmosis (251). Wüthrich et al. further demonstrate the importance of IL-12 and IFN-γ in an experimental study of BAD1 immunomodulation (223). They show that BAD1 decreases the production of IL-12 and IFN-γ and increases IL-10 production in BALB/c mice suggesting an additional virulence mechanism enhancing fungal pathogenesis (223). Only a single human case report has implicated the Th1 immune response through the JAK-STAT signal transduction pathway. Zeitler et al. report a case of pulmonary and cutaneous disseminated blastomycosis in a patient with polycythemia vera treated solely with ruxolitinib, a small molecule JAK inhibitor (199). The inhibition of JAK1 and JAK2 reduces phagocytic cell IFN-γ response by IFN-γR and has been shown to shift a predominant Th1 response to Th17 in the case of *STAT1* gain of function with resolution of CMC (252). Ruxolitinib could also potentially reduce the IL-12 and Th1 response through JAK2 and TYK2 inhibition contributing to the dissemination observed in the case of blastomycosis (253). This is supported by IL-12Rβ1 and IFN-γR1 loss of function variants predisposing individuals to both disseminated histoplasmosis and coccidioidomycosis (110,111,254). Further investigation of human blastomycosis cases could reveal insights into the requirement of Th1 to protect against disease dissemination. The Th17 response is essential in human blastomycosis with IL-6 identified as a

host susceptibility factor (72). Merkhofer et al. identified genetic variants in the *IL6* and *AS-IL6* gene loci significantly reducing Retinoic acid receptor-related orphan receptor gamma (RORγt) expression and IL-17 production, contributing to the increased incidence of blastomycosis in persons of Hmong ancestry (72). A previous study observed the disproportionately increased incidence of blastomycosis among persons of Asian and specifically Hmong descent suggesting a genetic predisposition (36). These findings are also consistent with IL-17A neutralization increasing *B. dermatitidis* burden in mice (96).

Additional host susceptibility factors involved in cell-mediated immunity include PD-1, CD52, and CD47. PD-1 is a receptor that inhibits T cell function and involved in T cell exhaustion. Monoclonal antibody inhibition of PD-1 with pembrolizumab restores T cell function for the treatment of cancer. Ferguson et al. report a case of disseminated blastomycosis in a patient on pembrolizumab to treat metastatic melanoma (197). The association of blastomycosis with PD-1 inhibition is paradoxical as inhibition is expected to improve the function of Th1 and Th17 cells (255). Accordingly, C57BL/6 mice with PD-1 knockout improved survival against experimental histoplasmosis compared to wild-type C57BL/6 mice (256). Alemtuzumab is a monoclonal antibody that binds CD52 to deplete CD52-expressing immune cells including $CD4^+$ T cells (257). Gerard et al. report a case of disseminated blastomycosis in a patient on alemtuzumab for hemophagocytic lymphohistiocytosis (198). This case had a heterozygous perforin (*PRF1*) variant of unknown significance that may have also contributed to blastomycosis. More cases are necessary to establish an association between alemtuzumab and blastomycosis. However, the reduction of T cells could exacerbate blastomycosis severity, similar to other $CD4^+$ deficiencies. Brandhorst et al. show BAD1 WxxWxxW tandem repeats bind to CD47 and inhibit T cell activation by proxy of decreased CD69 expression (224). This potential virulence mechanism further implicates the requirement of cell-mediated immunity in blastomycosis.

Two cases of blastomycosis were reported with concomitant immunomodulatory therapies targeting humoral immunity. Shingada et al. reported a patient with severe pulmonary blastomycosis on rituximab and a steroid taper for chronic kidney disease with perinuclear antineutrophil cytoplasmic antibody vasculitis. Rituximab targets CD20 on B cells to

treat B cell cancers and autoimmune disorders (202). Secondary infections including aspergillosis, *Pneumocystis jirovecii* pneumonia, and histoplasmosis have been reported in rituximab therapy but these findings were confounded by corticosteroid therapy and low $CD4^+$ cell counts (258). In a retrospective cohort study of 566 patients, Rogers et al. reported one patient with disseminated blastomycosis on ibrutinib, an irreversible BTK inhibitor, for a hematologic malignancy but without additional clinical information (200). The inhibition of BTK reduces B cell receptor signaling and inhibits B cell development for the treatment of B cell cancers (200). Genetic BTK immunodeficiency and agammaglobulinemia are not commonly associated with fungal infections. Yet, ibrutinib unexpectedly increases the risk of fungal infection with potential off-target effects on monocytes and macrophages (259). The current evidence does not suggest that humoral immunodeficiency plays a role in susceptibility to naturally acquired blastomycosis.

4.1.5 Limitations

There are several limitations for this scoping review. This scoping review was conducted by a single reviewer including the database search strategy, eligibility criteria, abstract review, and full-text assessment. We attempted to mitigate this issue through meetings about the search strategy and article inclusion for both the abstract review and full-text assessment to maintain consistency with the eligibility criteria. Forward and backward search of the references and citations yielded an additional 6 articles suggesting that our search strategy may not have fully captured all available literature on host susceptibility to naturally acquired blastomycosis. We suspect missing literature did not link a medical condition or treatment with the acquisition of blastomycosis but rather as a coincidental finding. We also only considered literature in English, French, and Spanish which may have missed relevant articles although blastomycosis is largely limited to North America. Another limitation was the inclusion of case reports, case series, and non-peer reviewed conference abstracts without study quality assessment. Case reports, series, and conference abstracts are low confidence studies and were interpreted with caution by providing supporting evidence and rationales, if available, for each susceptibility factor. The majority of the evidence for blastomycosis host susceptibility was identified in these study

types requiring us to include them to capture the current knowledge in the field. Given the rarity of blastomycosis and numerous predisposing conditions, we considered susceptibility factors with only a single evidence source as sentinel cases warranting further investigation to determine their role in blastomycosis.

4.1.6 Conclusions and Future Directions

This is the first scoping review to our knowledge on host susceptibility and immunity to naturally acquired blastomycosis. The systematic approach enabled us to construct the current landscape of host immunity to naturally acquired blastomycosis. Certain aspects of this review have been covered elsewhere: innate host immunity (16) and virulence factors (260). In this review, we included additional studies on innate host immunity and focus on the host targets of *Blastomyces* virulence factors. HIV $CD4^+$ deficiency has also been reviewed (249) but we provide here an update and include additional cases of HIV and non-HIV associated $CD4^+$ deficiency. The inclusion of articles on immunomodulatory therapies and blastomycosis in this review had not been previously conducted. This provided unique insights into blastomycosis as a secondary infection as well as revealing potential immune factors important for host immunity. We also considered evidence obtained from humans, mice, and dogs and compared findings across studies whenever possible. Gaps in findings across species also suggest additional avenues of research in this field.

In this scoping review, we identified several host susceptibility factors in naturally acquired blastomycosis. The innate immune response and particularly proinflammatory cytokines for macrophage and neutrophil function were identified in the included articles. TNF-α had the most evidence as a susceptibility factor in both humans and mice as well as a target for *Blastomyces* pathogenesis. So far, 14 patients on TNF-α inhibitors with blastomycosis have been identified. This evidence combined with the pathogen-targeted downregulation of TNF-α suggests this is an important early innate immune factor in blastomycosis. Surveillance of blastomycosis incidence rates among patients residing in endemic areas and whom are on TNF-α inhibitors should be undertaken to determine their impact on disease acquisition. Primary and secondary immunodeficiencies in GM-CSF receptor and GM-CSF autoantibodies were

identified in murine and human blastomycosis alongside *Blastomyces* DppIVA directly cleaving GM-CSF contributing to pathogenesis. This evidence suggests GM-CSF is essential for blastomycosis although additional human studies are needed to show the requirement of GM-CSF in protective immunity. Vitamin D was also identified as a potential susceptibility factor, but the evidence is conflicting, and more studies are required to elucidate its role in blastomycosis. Interestingly, the complement system was identified as a susceptibility factor, but no PRR has yet been shown, although evidence from vaccine-induced immunity implicates MyD88 and CARD9. For the adaptive immune system, cell-mediated immunity is essential for control of blastomycosis with $CD4^+$ deficiency increasing the severity of blastomycosis. There is accumulating evidence in mouse studies for the role of the Th1 immune response, but no human studies have been reported except a single case report on JAK inhibitors and blastomycosis. The Th17 immune response has been directly shown to be essential for blastomycosis immunity. Interesting sentinel cases included PCD, CF, GATA2 deficiency, efalizumab (LFA-1), and ruxolitinib (JAK1/JAK2) were identified meriting more awareness of their potential role in blastomycosis susceptibility.

Currently, the World Health Organization (WHO) does not recognize *Blastomyces* as a priority fungal pathogen. Yet, the mortality rate for blastomycosis is around 6.6%, increasing to 37% in those who are immunocompromised (52). Therapeutics are limited to a few antifungal agents with no reports of immunotherapies for blastomycosis. The insights into host susceptibility to blastomycosis can inform future clinical studies on the role of host deficiencies and the development and testing of host-directed therapeutics. However, more studies are needed to develop this field to justify the movement towards applied research and therapeutic discovery. We anticipate that this scoping review will raise awareness of blastomycosis and the benefits of understanding host immunity towards the improvement of disease outcomes.

4.2 Exome Sequencing Study Design for the Discovery of Host Genetic Risk Factors in Blastomycosis

We conducted the first exome sequencing study for blastomycosis in a case-control cohort from Manitoba and northwestern Ontario. The case-control cohort is composed of those diagnosed

and treated for blastomycosis and case-selected household controls. Similarities in environmental exposures across the cohort enabled the study of host genetic risk factors for the acquisition of blastomycosis. Given the dual purpose of the original case-control study of environmental and genetic risk factors, the cohort design is ideal for detecting associations for blastomycosis susceptibility. The unavailability of reliable and commercial serum testing also precluded the ability to test controls for previous subclinical infections.

Limited host genetic studies have been conducted for blastomycosis revealing genetic variants in *IL6*, *GATA2*, and *VDBP* influencing susceptibility and severity of disease (72,75,124). Our study attempted to identify additional host genes and polymorphisms that may influence susceptibility to blastomycosis. We sequenced and analyzed genetic variants from the exomes of 18 cases and 9 controls. We used the gold standard GATK best practices workflow for germline variant discovery including additional variant filtering to further improve dataset quality as we did not conduct confirmatory molecular investigations (140,148). The small sample size required us to use the recommended hard filtering for genetic variants as opposed to the variant quality score recalibration method in GATK. We conducted logistic regression and Fisher exact sample size calculations with the pwrss v. 0.3.1 R package using default settings (261). The Fisher exact power calculation was performed with the independent samples z test for two proportions. With our sample size of 27 and unbalanced case-control distribution of 2:1, we had 77.9% power to detect a large effect size with OR of 4 and p-value of 0.05. For the logistic regression power calculation, with our sample size of 27, we had 44.6% power to detect a large effect size with OR of 4 and p-value of 0.05. These sample size calculations did not factor in multiple testing correction. We could reasonably expect to see genetic signals with Fisher exact, but without multiple testing correction, the results would need to be contextualized with peer reviewed literature to support or oppose the findings. We used a combination of candidate gene prioritization and variant aggregation approaches to improve power for association detection by narrowing the search to known biologically relevant genes. The binary SKAT-O gene-based association analysis was optimal for our study as we do not have prior knowledge of the direction of association or the composition of causal and noncausal genetic variants for each gene (175). This was also applied to the STRING pathway aggregated

candidate gene variants in Section 3.4.1 although the approach did not yield any significant results. We also focused most of our analyses on rare genetic variation (MAF < 5%) as rare variants are more likely to be deleterious and negatively selected with large effect sizes (262). Common variants are more likely to have mild or no consequence on protein expression, structure, or function and therefore, limiting their utility in a small exploratory study (13). Similarly, the candidate gene analyses were conducted in a stepwise manner first using the most severe pLOF variants to prioritize variants with highest likelihood of having a sizeable effect on blastomycosis susceptibility. In the absence of overwhelmingly enriched pLOF variants, we next included non-synonymous and in-frame indels. The detection of genetic signals in the non-synonymous variant datasets precluded the next step of examining synonymous and non-coding variation. For the exome-wide gene cluster analysis with NHC, we used a different approach to prioritize likely damaging variants according to their CADD score. Genetic variants with damaging scores were retained to explore variation in biological processes enriched in the blastomycosis cases only. This method increased the likelihood that the included genetic variants impacted gene function enabling us to generate hypotheses about their potential function in blastomycosis susceptibility.

4.3 Discovery of Putative Host Genetic Risk Factors for Blastomycosis

4.3.1 Candidate Gene Analyses Identify TYK2, TLR1, and GATA2 as Potential Genetic Risk Factors for Blastomycosis

The prioritization of candidate genes with prior knowledge of susceptibility to fungal and mycobacterial diseases identified several genes and genetic variants for evaluation. We detected damaging and enriched variants in *TYK2*, *CXCR1*, *IFNG*, *STAT4*, *CCR5*, *DOCK8*, *MALT1*, *IL17F*, *IL23R*, and *TLR2*, as well as two nominally significant genes in the gene-based association analyses, *TLR1* and *GATA2*. No previously identified blastomycosis susceptibility variants were implicated in this study.

The *TYK2* variants I684S and G363S were observed in this study as enriched in blastomycosis cases in the damaging variant and single-variant association analyses, respectively (Section 3.4.2 and 3.4.3). *TYK2* LOF variants have been associated with reduced

IFN-γ, IL-17A, and IL-17F expression in T cells in response to IL-12 and IL-23 contributing to mycobacterial disease susceptibility (Appendix A) (143,263). LOF is also associated with increased susceptibility to viral infections, AR HIES, and protection from psoriasis (122,264,265). TYK2 is a tyrosine kinase involved in signaling from the cytokine receptors IL-12Rβ1 on T cells and interferon alpha and beta receptor subunit 1 (IFNAR1) on phagocytic cells (143). The IL-12Rβ1 receptor is involved in the Th1 response when heterodimerized with IL-12Rβ2 by recognizing and binding IL-12. The associated tyrosine kinases TYK2 and JAK2 (bound to IL-12Rβ2) mediate downstream signaling and phosphorylation of STAT4 for the upregulation of IFN-γ (266). IL-12Rβ1 and TYK2 are also involved in IL-23 cytokine receptor binding and signaling when heterodimerized to IL-23R. TYK2 mediates the signaling and activation of STAT3 and RORγt transcription factors and upregulation of Th17-specific cytokines (122). TYK2 is a 1187 amino acid protein consisting of four domains, protein 4.1, Ezrin, Radixin, Moesin (FERM), SH2, pseudokinase, and kinase domains involved in receptor binding (FERM and SH2), ATP and activity regulation (pseudokinase), and kinase function (kinase) (267). The *TYK2* I684S variant is located within the pseudokinase domain with a deleterious effect predicted by SIFT, PolyPhen-2, and CADD. Enerbäck et al. demonstrated that I684S reduced STAT4 phosphorylation in response to IL-12 in $CD4^+$ and $CD8^+$ T cells (264). Tomasson et al. further show that I684S reduces TYK2 protein expression with no effect on TYK2 phosphorylation (268). These findings are supported by Boisson-Dupuis et al. who found that I684S catalytic impairment could be rescued by TYK2 overexpression (263). Boisson-Dupuis et al. also show that phosphorylation of STAT3 in response to IL-23 was not impaired by I684S suggesting no impact on Th17 response (263). Further molecular investigations of *TYK2* I684S on blastomycosis susceptibility should determine its potential impact on the expression of IFN-γ and Th1 cytokines. The *TYK2* G363S variant had the highest OR in the candidate gene single-variant association analysis at 4.10 (95% CI: 0.46-36.27) but was not statistically significant in the Fisher exact test. G363S has previously been associated with reduced risk of acute myeloid leukemia but no molecular dysfunction was detected on TYK2 expression and function (268). The variant is located within the FERM domain suggesting a potential effect to be mediated by receptor binding. However, G363S is predicted to be benign by SIFT, PolyPhen-2, and CADD and reported in ClinVar as

benign for susceptibility to mycobacterial disease due to TYK2 deficiency. No associations were detected with autoimmunity (269). TYK2 had not been associated with blastomycosis susceptibility prior to our study. If TYK2 is involved in blastomycosis immunity, this could inform potential infectious risk of TYK2-targeting small kinase inhibitors such as deucravacitinib approved for the treatment of psoriasis.

TLR1 encodes the TLR1 PRR for the recognition of triacyl lipoproteins when heterodimerized with TLR2 (94). Previous studies have shown that genetic variants in *TLR1* are associated with increased susceptibility to candidemia and invasive aspergillosis (Appendix A) (90,91). Rare, non-synonymous *TLR1* variants were implicated in the candidate gene-based association analysis. The I57M, R80T, and H305L contributed to the significant enrichment of *TLR1* variants in the controls. The R80T variant has previously been shown to reduce proinflammatory cytokine production in response to the Pam3Cys TLR1/2 agonist and lipopolysaccharide (90). The I57M variant is predicted to be deleterious across SIFT, PolyPhen-2, and CADD suggesting a potential impact on TLR1 function, but reported as benign in ClinVar. Homozygosity for H305L is associated with protection from tuberculosis with increased IFN-γ expression but this is not seen in heterozygous carriers (270). No individuals in our study were homozygous for H305L. At the population level, there is positive selection of *TLR1* non-synonymous variants in European populations suggesting a generally beneficial effect (271). We also expanded the analysis to include the common *TLR1* N248S and I602S variants. The I602S variant drives reduced proinflammatory cytokine production (90,271). N248S is in close linkage disequilibrium with I602S which may lead to disease associations driven largely by I602S (271). The gene-based analysis for *TLR1* remained significant after the inclusion of the common I602S and N248S variants but neared the exploratory threshold. This suggests that the rare *TLR1* variants could be influencing resistance against blastomycosis, but additional validation cohorts would be required to confirm this association. Molecular investigations in lung epithelial cells have shown that IL-1R, but not TLRs, is required for PRR signaling for control of *Blastomyces* fungal burden in mice (96).

GATA2 is a zinc-finger transcription factor essential for hematopoietic stem cell gene expression regulation (246). Deficiency of GATA2 is recognized as a human IEI where damaging

genetic variants can cause MonoMAC and myelodysplastic syndrome as well as predispose individuals to leukemia and a broad range of fungal, bacterial, and viral infections (247). In our study, we found *GATA2* variants enriched in controls for both the rare and common candidate gene-based association analyses. The rare variant gene-based analysis was driven by *GATA2* P161A, a variant with conflicting interpretations of pathogenicity in ClinVar and damaging CADD score of 20.7. Publications on *GATA2* variation report P161A and P161S as benign and of uncertain significance but the functional effect of these variants was not determined (272,273). The common *GATA2* A164T variant pushed the signal towards nominal significance. The variant is reported as benign in ClinVar but patients with *GATA2* A164T have been reported with non-tuberculous mycobacterial disease, paracoccidioidomycosis, and histoplasmosis (274). There are also no reports of protective *GATA2* variants with about 50% of known variants reported to be pathogenic or likely pathogenic (272). Our findings are interesting as we would not expect controls to have *GATA2* variant enrichment. This is in contrast to a single case report of GATA2 deficiency and MonoMAC predisposing a patient to severe blastomycosis (75).

There were four genetic variants with severe gene consequences found in the candidate genes. The *CXCR1* G315X stop gain variant was found in one heterozygous case. There are no previous reports of this variant but in mice, CXCR1 LOF has been to increase *C. albicans* burden and mortality due to reduced neutrophil degranulation (101). In humans, the T276 (rs2234671) variant is associated with increased risk of disseminated candidiasis (101). A reduction in neutrophil degranulation would be suspected to increase the risk of blastomycosis as *B. dermatitidis* DppIVA cleavage of CXCL2 reduces macrophage and neutrophil antifungal function, enhancing fungal pathogenesis (226). However, the effect of heterozygous LOF for CXCR1 is not known and further molecular investigation would be required determine the effect. We found one blastomycosis case with heterozygous CCR5Δ32 in our study. The CCR5Δ32 variant is protective in the homozygous state against HIV infection and the heterozygous state has been recognized to decrease regulatory T cell function (275). CCR5 LOF is associated with improved control of fungal infection in a murine model of histoplasmosis suggesting a protective effect against dimorphic fungi (276). This is in contrast with our finding of one heterozygous case. The *IFNG* c.115-2->T and *STAT4* c.129-2->T variants were annotated in ANNOVAR v. 2019October24

as acceptor splice site variants but gnomAD v. 3.1.2 correctly annotates theses variants outside the dinucleotide acceptor splice sequence as *IFNG* c.115-3dupA and *STAT4* c.129-3dupA. Neither variant is predicted to affect splicing according to the *in silico* predictor SpliceAI. ClinVar annotates these variants as benign with no publications suggesting deleterious effects.

The *DOCK8* A597V variant is enriched in cases and predicted to be damaging by SIFT, PolyPhen-2, and CADD. DOCK8 deficiency can cause AR HIES and susceptibility to fungal infections (122). However, heterozygosity for the A597V variant was found in the cases and reported as benign in ClinVar for combined immunodeficiency due to DOCK8 deficiency. *MALT1* R217G was found entirely in cases with four heterozygous individuals. R217G is not predicted to be damaging and is benign for MALT1 combined immunodeficiency in ClinVar. *IL17F* E126G and V155I were both predicted to be damaging in the damaging variant analysis with case and control enrichment, respectively. *IL17F* E126G has previously been associated with microbial keratitis but this was not statistically significant (277). There are conflicting reports of V115I as a risk factor for rheumatoid arthritis (278). For fungal infections, E126G and V155I are reported as benign for candidiasis in ClinVar. The *TLR2* R753Q variant was equally distributed with one case and control but associated with controls due to the 2:1 case-control ratio. R753Q is a known risk factor for tuberculosis susceptibility by functionally impacting TLR2 dimerization with TLR6, MyD88 recruitment, and impairing NF-κB activation (279,280). The role of TLR2 in blastomycosis is not well understood, but simultaneous knockout of TLR2, TLR3, TLR4, TLR7, and TLR9 elevated fungal burden in mice, although this was not statistically significant (96). The *IL23R* R381Q variant was similarly distributed with 2 heterozygous cases and 2 controls resulting in an association in controls due to the imbalanced sample. R381Q is associated with protection from Crohn's disease and ulcerative colitis with reduction of proinflammatory IL-17 production speculated to contribute to the protective phenotype (281). The reduction of IL-17 expression by IL-17 autoantibodies as well as IL-23 monoclonal antibody inhibitors increases the risk of mucosal *Candida* infection (89,116). We cannot draw conclusions from the *TLR2* and *IL23R* variants as they were found equally between cases and controls. Additionally, both variants are expected to increase susceptibility to fungal infections suggesting an opposite trend to the findings here.

4.3.2 No Genetic Variants or Genes were Associated with Blastomycosis Susceptibility in the Exome-wide Association Analyses

We conducted an exome-wide association analysis consistent with the methodology for GWAS. We did not find any statistically significant genetic variants in this analysis but given the small sample, this is not unexpected. The application of genetic variant aggregation by gene and analysis with weighted binary SKAT-O was sought to improve association detection. Again, no genes were significantly associated with the blastomycosis phenotype at the exome-wide threshold. We applied exploratory thresholds for both analyses to investigate the top results of each analysis. The top hit from the single-variant association analysis was the non-coding RNA *LINC00634* g.41958385A>C variant also located within *SMIM45* as the non-synonymous variant T57P. There was enrichment of this genetic variant in cases with an OR of 18.18 (95% CI: 3.56-92.99). This variant is common with a gnomAD frequency of 46.3% and reported as benign in ClinVar (Table 18). *LINC00634* is upregulated in esophageal squamous cell carcinoma and colorectal carcinoma but not reported to be involved in antifungal immunity (282,283). In the gene-based association analysis, *THNSL1* was the most significantly associated gene with five genetic variants enriched in controls. *THNSL1* encodes a threonine synthase-like protein likely involved in threonine biosynthesis similar to *THNSL2* (284). However, the function of THNSL1 in humans has not been elucidated. Neither *LINC00634* nor *THNSL1* were considered further in this study due to the absence of associations with antifungal immunity. *ALKBH7*, *COL6A1*, *LVRN*, *MISP*, *MPDZ*, *MT1E*, *MVB12A*, *OTOF*, *PER3*, *PPP2R3A*, *PRSS55*, *UBTFL1* and *WWC3* were also identified as top hits in the exome-wide single-variant and gene-based association analyses. However, these genes are not known to have any associations with antifungal immunity.

4.3.3 Chromatin Remodeling, Proteosome, and Intraflagellar Transport as Hypothetical Pathways for Blastomycosis Susceptibility

The NHC analysis yielded three pathways significantly enriched in blastomycosis cases with an FDR below 20%. The most significant pathway identified involved genes in the switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex (*ACTL6A, ACTL6B, PBRM1,*

SMARCC2, SMARCD3) and the INO80 chromatin remodeling complex (*INO80*, *NFRKB*). These are ATP-dependent chromatin remodelers composed of several subunits that vary by cell and tissue type (285). Both SWI/SNF and INO80 are involved in the remodeling of chromatin to regulate gene promoters through repressing or facilitating transcription factor binding. Chromatin remodelers, particularly SWI/SNF, are involved in hematopoietic stem cell differentiation and the regulation of immune gene promoters through the RUNX1 transcription factor (285). For example, the SWI/SNF complex remodels chromatin in the promoter of *CSF1* (encoding GM-CSF), regulating expression by enabling binding of the RUNX1 transcription factor (286). The *PBRM1* or BAF180 subunit of SWI/SNF is important for the expression of IFN-γ-induced genes in phagocytic cells. *PBRM1* LOF reduces expression and phosphorylation of STAT1 through the loss of chromatin remodeling at the *IFNGR2* promoter, inhibiting its expression (287). These findings have direct implications for blastomycosis susceptibility since IFN-γ and GM-CSF are essential for effective murine immunity and survival (117,128,129). However, the contribution of polymorphisms in the SWI/SNF complex to the blastomycosis phenotype are multifactorial and can vary between individuals.

The cross presentation of soluble exogenous antigens in endosomes gene cluster was composed of genes encoding subunits (*PSMB5*, *PSMB6, PSMD9, PSMD13*) and a regulator (*PSME4*) of the proteasome. The function of the proteasome is mainly in the degradation of ubiquitinated proteins (288). The substitution of subunits in the 20S proteasome complex forms the immunoproteasome involved in foreign antigen processing for presentation on MHC class I molecules and detection by cytotoxic $CD8^+$ T cells (289). However, no variants in genes encoding subunits of the immunoproteasome were found in our dataset. The PSMD9 subunit of the proteasome contributes to the activation of NF-κB by degrading IκBα, the alpha subunit of the IκB kinase and inhibitor of NF-κB nuclear translocation (290). PSMD9 overexpression increased NF-κB activity suggesting a possible role of *PSMD9* variation influencing NF-κB immune responses (290).

The IFT system was found to be enriched in blastomycosis cases with an FDR below 20%. Putatively damaging genetic variants in IFT complex A (*IFT122, IFT140, TTC21B,* and *WDR35*) were found in eight cases (Table 20) (291,292). The IFT system, composed of IFT ATP-

dependent motors and complex A and B, transports proteins along cilial microtubules from the base of the cilia towards the ciliary tip (anterograde) and back towards the base (retrograde) (292). LOF variants in complex A genes and *WDR35* result in ciliopathies, most frequently cranioectodermal dysplasia (*WDR35*, *IFT122*, and *IFT140*) and early onset chronic kidney diseases (*TTC21B*) (293,294). Patients with cranioectodermal dysplasia due to *WDR35* and *IFT122* LOF variants have been noted to have increased rates of respiratory illnesses (293,295,296). A patient with homozygous LOF *TCC21B* variants was also reported to have PCD alongside kidney disease (294). No genetic variants in our dataset were found to be pathogenic for severe ciliopathies but the damaging CADD score indicates potential variability in ciliary function in blastomycosis. PCD and CF have been reported in cases of blastomycosis potentially implicating impaired mucus clearance with susceptibility to disease (79,181). This suggests that pulmonary cilia could be essential for clearance of *Blastomyces* conidiospores and impairment could reduce the initial fungal load necessary to establish infection.

The CARD domain binding gene cluster contained relevant antifungal genes but had an FDR of 26.5%, above the 20% threshold set for FDR. *CARD9* and *BCL10* belong to the CARD9-BCL10-MALT1 signalosome that is important for CLR signaling in antifungal immunity (297). CARD9 is also essential for vaccine-induced blastomycosis immunity (131). However, we cannot conclude a role of the CARD9-BCL10-MALT1 signalosome based on our results. Future studies should attempt to elucidate the requirement of CARD9 in susceptibility to naturally acquired blastomycosis.

Overall, the exome-wide pathway analysis approach with NHC identified several pathways with potential roles in susceptibility to naturally acquired blastomycosis. These findings are not sufficient to implicate these pathways in susceptibility to naturally acquired blastomycosis, but they are beneficial for generating hypotheses for future studies. We believe that the IFT system and the potential role of pulmonary cilia in innate barrier defense may be an important innate defense against blastomycosis. The SWI/SNF and INO80 chromatin remodeling systems regulate a multitude of genes and the complexity challenges further study in blastomycosis. However, the regulation of essential blastomycosis immunity genes suggests a potential role of chromatin remodeling and associated pathways with susceptibility. The

proteasome is important in NF-κB regulation through PSMD9, identified in our study. However, proteasome dysfunction has been found to influence risk of viral but not fungal infections (288). Taken together these results provide several intriguing avenues for future research into blastomycosis immunity.

4.4 Limitations of the Blastomycosis Case-Control Exome Sequencing Study

There were several limitations for the blastomycosis case-control exome sequencing study. The main limitations were the small sample size and variable exposure between cases and controls which may have limited the ability to detect genetic differences. However, there are many difficulties with recruitment of individuals with blastomycosis as there only a few cases per year, a lack of awareness, and no routine diagnostic tools which can lead to misdiagnoses and missed cases. Environmental and clinical factors can also influence the risk of infection and severity of disease (68,73). We were unable to eliminate environmental and clinical factors as confounding variables in this study due to the unavailability of clinical data. There were also limitations in the case-control selection, namely the unbalanced recruitment of cases and controls due to unavailability of controls to participate in the study. There was also the possibility of cases and controls being related as we requested a household or neighborhood control since they should have similar risk factors for infection. Again, this design improved the ability to study risk factors for acquisition of blastomycosis where diagnostics are not readily available. From the 27 exome sequences, we had 18 cases and 9 controls for a 2:1 case to control ratio. In three instances, we observed relatedness between the cases and controls leading to the finding that a related case and control share the *TYK2* G363S genetic variant.

The exome analyses also had several limitations. The choice of exome sequencing limited the ability to detect genetic variants in non-coding regions which could have an impact on blastomycosis susceptibility. We also filtered out remaining non-coding as well as synonymous variants for our analyses. A previous study identified variants in the regulatory non-coding region of *IL6* conferring susceptibility to blastomycosis with whole genome sequencing (72). However, for our study, the exome sequencing approach was the most cost effective and a suitable method for detecting damaging variants that could influence the

blastomycosis susceptibility phenotype (262). The inclusion of non-coding variation could also further reduce the statistical power to detect associations in an already underpowered study. Another limitation was that we were unable to use the variant quality score recalibration method in the GATK best practices due to sample size constraints, which may have improved dataset quality. For the candidate gene analysis, the literature review was not conducted systematically meaning that we could have missed antifungal and antimycobacterial genes that could impact blastomycosis susceptibility. The exome-wide analysis was conducted to detect associations that may have been missed by the candidate gene analysis. However, the sample size limited the statistical power to detect associations in the exome-wide dataset (Section 4.2). There were also limitations with the weighting method for SKAT-O. The 1000G MAF for all populations was used to generate the weights but not all variants are represented in this database. We assumed the missing variants were rare with weights corresponding to the lowest observed MAF. Therefore, the weighting could be heavier than reality and potentially affect our results. We also assumed that the deleteriousness predictions (SIFT, PolyPhen-2, and CADD) were accurate for our analyses. The conservative threshold for damaging variant analysis may have missed potential damaging variants. However, manual curation improved interpretation and evaluation of candidate variants. The CADD $\geq$ 20 filter for the gene cluster analysis captured predicted damaging variants as we examined pathways enriched in blastomycosis cases. We cannot be certain that the included variants are damaging but the filter improves the ability to detect potential pathways influencing blastomycosis susceptibility. Overall, we identified putative associations between genetic variants, genes, and pathways with blastomycosis susceptibility. Without additional validation cohorts or molecular investigation, we could not conclude any causal links that directly affected susceptibility.

4.5 Conclusions and Future Directions for the Study of Host Genetic Risk Factors in Blastomycosis

4.5.1 Conclusions

We found putative genetic signals for susceptibility to blastomycosis in exome sequences from a blastomycosis case-control cohort. These signals generated hypotheses for molecular

investigations and future cohort studies of blastomycosis susceptibility. We examined genetic variation using a candidate gene approach to prioritize genes and rare variant aggregation methods to detect these genetic associations. We provide *in silico* evidence of both TYK2 and TLR1 possibly influencing susceptibility to blastomycosis. Further validation cohorts and molecular studies are needed to determine the impact of *TYK2* and *TLR1* variation in blastomycosis. The enrichment of *GATA2* variants in controls was not expected as majority of genetic variation is pathogenic. We are uncertain if the benign variants identified in this study are protective as a previous case report demonstrated blastomycosis susceptibility in a patient with GATA2 deficiency (75). We also conducted an exome-wide gene cluster analysis that yielded several genes in biological processes enriched in blastomycosis cases that may be valuable for generating hypotheses for future research. The SWI/SNF and INO80 chromatin remodeling gene cluster was the most significantly enriched pathway, followed by proteasome subunits and regulators and intraflagellar transport genes. These pathways are interesting for blastomycosis although chromatin remodeling and proteasome are complex requiring larger cohorts to determine a role for genetic susceptibility to blastomycosis.

The genetic signals found in this study are novel for blastomycosis and provide additional information to develop more studies on host susceptibility and immunity to blastomycosis. Our findings can help to inform and improve future clinical, cohort, and experimental studies. Further validation of the genetic signals identified will determine the role of each gene and pathway and provide insights into future host-directed therapeutic targets and potentially genetic risk factor screening. Determining the essentiality of TYK2 could also inform a potential risk of fungal infections for patients on TYK2 inhibitors. Currently, blastomycosis is a low priority fungal pathogen according to the WHO priority fungal pathogen list and few studies are conducted for this disease (298,299). We hope this thesis can help to increase awareness and inform future research to improve outcomes of blastomycosis.

4.5.2 Future Directions

Based on the findings of this study, there are a few immediate directions that should be followed up on. Firstly, a knockout of *TYK2* and generation of the I684S and G363S genetic

variants should be evaluated *in vitro* for their effects on the IL-12 and IL-23 responses in T cells. Clinical presentations of HIES and blastomycosis could also suggest TYK2 deficiency warranting additional follow up and reporting (122). Active surveillance of blastomycosis among individuals residing in endemic regions upon administration of TYK2 small molecule inhibitors may uncover a predisposition. Rare non-synonymous *TLR1* variants were significantly enriched in blastomycosis controls. Future studies should attempt to replicate this finding or investigate the impact of the rare genetic variants on TLR1-TLR2 function. The significant *GATA2* signal in the controls is not expected to afford resistance to blastomycosis. Future cohort studies should consider *GATA2* in an attempt to replicate these findings. However, we expect that damaging *GATA2* variants should increase susceptibility to blastomycosis. From the exome-wide gene cluster analysis, significant enrichment of IFT genes was observed suggesting a potential role in blastomycosis susceptibility. Genetic screening of blastomycosis cases with ciliopathies could provide further evidence of genetic predisposition for infection.

Future studies of host genetic susceptibility to blastomycosis should consider the recruitment of larger case-control cohorts, comparing mild or moderate disease versus severe blastomycosis, and incorporating clinical information into the analyses. Larger cohorts and improved case-control definitions could improve the odds of observing genetic associations with blastomycosis susceptibility and severity. Other considerations include the sequencing of *Blastomyces* species and development of serological diagnostics to improve cohort design. We anticipate that future studies will yield additional genes and primary immunodeficiencies associated with blastomycosis. However, the low number of annual cases and the rarity of genetic immunodeficiencies reduces the probability of observing both simultaneously. Clinical awareness of blastomycosis and primary immunodeficiencies can improve the chances of catching such cases. Experimental studies based on our findings may yield molecular mechanisms for disease susceptibility. By finding genetic predisposition and mechanisms of disease, we develop an improved understanding of the host requirements for protective immunity. The translation of this knowledge can inform clinical practice for precision medicine, the development of fungal vaccine candidates, host-directed therapeutics, and potential adverse effects of immunomodulatory therapies.

Chapter 5: References

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Appendix

Appendix A: Candidate Gene List

Gene	Infectious Agent	Dysfunction	Reference
AIRE	*Candida*, *Mycobacterium*	Dysfunction of memory cell regulation increasing risk of autoantibodies and risk of autocytokine autoantibodies	Puel et al. 2010 (116)
BCL10	*Candida*	Impaired CARD9-BCL10-MALT1 signalosome activation of NF-κB	Gross et al. 2006 (297)
CARD9	*Candida*	Impaired CARD9-BCL10-MALT1 signalosome activation of NF-κB	Gross et al. 2006 (297)
CCR1	*Candida albicans*	Neutrophilia in mice kidneys associated with protection	Lionakis et al. 2012 (300)
CCR5	*Cryptococcus*; HIV	Co-receptor with CD4 for HIV entry. Increased susceptibility to Cryptococcal infections in mice	Original Study Design, Huffnagle et al. 1999 (301)
CCL8	*Candida*	Genetic association with candidiasis	Smeekens et al. 2013 (302)
CD11b	*B. dermatitidis*	* Type 3 complement receptor used by soluble *B. dermatitidis* virulence factor BAD-1 to suppress TNF-α	Brandhorst et al. 2004 (220)
CD14	*B. dermatitidis*	* Macrophage surface antigen used by soluble *B. dermatitidis* virulence factor BAD-1 to suppress TNF-α	Brandhorst et al. 2004 (220)
CD18/ ITGB2	*B. dermatitidis*	* Type 3 complement receptor used by soluble *B. dermatitidis* virulence factor BAD-1 to suppress TNF-α	Brandhorst et al. 2004 (220)
CD40	*B. dermatitidis*, *M. tuberculosis*	Reduced IL-12/IFN-γ axis activation. Binding with CD40LG product CD40L	Wuthrich et al. 2006 (303)
CD40LG	*Paracoccidioides, P. jirovecii, C. albicans*	Reduced IL-12/IFN-γ axis activation and impaired humoral recombinant class switching (X-linked hyper IgM syndrome)	Cabral-Marques et al. 2012, Hostoffer et al. 1994 (125,304)
CD82	*Candida*	Reduced CD82 production decreasing dectin-1 signaling	Merkhofer and Klein 2020 (16)
CHIA	Fungi	* Degradation of chitin	Vega and Kalkum 2012 (305)
CHIT1	*C. albicans*	CHIT-1 deficiency associated with reduced fungal burden	Schmitz et al. 2021, Vega and Kalkum 2012 (305,306)
CLEC6A	*C. albicans*	Impaired recognition of hyphal antigens and α-mannan	Bi et al. 2010 (85)
CLEC7A	*Coccidioides, Candida, Aspergillus*	Impaired recognition of fungal β-glucan antigen	Bi et al. 2010, Hsu et al. 2022 (85,307)
CXCR1	*C. albicans*	Impaired neutrophil degranulation mechanism	Swamydas et al. 2016 (101)
CXCL10	*Aspergillus*	Reduced expression of CXCL10 and impaired Th1 response	Merkhofer and Klein 2020 (16)
CYBA	*Mycobacterium, Aspergillus*	Chronic granulomatous disease. Dysfunction in phagocytic oxidative burst.	Holland 2013, Boisson-Dupuis et al. 2015 (102,103)
CYBB	*Mycobacterium, Aspergillus*	Chronic granulomatous disease. Dysfunction in phagocytic oxidative burst.	Holland 2013, Boisson-Dupuis et al. 2015 (102,103)
CYP27B1	*Mycobacterium*	*Conversion of circulating 25(OHD) to VDR ligand	Kongsbak et al. 2013 (308)

		$1,25(OH)_2D$	
DOCK8	*Candida, Aspergillus, Cryptococcus, Histoplasma*	HIES; Impaired Th17 differentiation	Zhang et al. 2020 (122)
FCGR2A	*Cryptococcus*	Associated with increased susceptibility to fungal infection. Possible differences in IgG affinity	Original Study Design, Hu et al. 2012 (229)
FCGR2B	*Cryptococcus*	Associated with increased susceptibility to fungal infection. Possible differences in IgG affinity	Original Study Design, Hu et al. 2012 (229)
FCGR3A	*Cryptococcus*	Associated with increased susceptibility to fungal infection. Possible differences in IgG affinity	Original Study Design, Hu et al. 2012 (229)
FCGR3B	*Cryptococcus*	Alleles associated with influencing susceptibility to cryptococcal infection. Possible differences in IgG affinity	Original Study Design, Hu et al. 2012 (229)
GATA2	*B. dermatitidis*	Severe pulmonary blastomycosis in setting of MonoMAC syndrome due to *GATA2* frameshift insertion (c.871+2_3insT) mutation	Spinner et al. 2016 (75)
IFNG	*Mycobacterium*	Abolished IFN-γ signaling	Kerner et al. 2020 (309)
IFNGR1	*Mycobacterium, Histoplasma, Coccidioides*	Impaired response to IFN-γ	Vinh et al. 2009, Zerbe and Holland 2005 (110,111)
IFNGR2	*Mycobacterium*	Impaired response to IFN-γ	Bustamante et al. 2014 (126)
IKBKG	*Mycobacterium*	Impaired NF-κB signaling	Bustamante et al. 2014 (126)
IL1A	*B. dermatitidis*	Reduced Th17 cells and increased fungal burden	Original Study Design, Hernandez-Santos et al. 2018 (96)
IL1B	*Aspergillus*	Increased risk of invasive aspergillosis	Original Study Design, Merkhofer and Klein 2020 (16)
IL1R1	*B. dermatitidis*	Reduced Th17 cells and increased fungal burden	Hernandez-Santos et al. 2018 (96)
IL1RN	Autoimmunity	Polymorphisms associated with systemic lupus erythematosus	Original Study Design, Blakemore et al. 1994 (310)
IL10	*Aspergillus*	Polymorphisms associated with increased risk of invasive pulmonary aspergillosis	Original Study Design, Maskarinec et al. 2016 (92)
IL12A	N/A	* Heterodimer formation with IL12B to form IL-12 cytokine	Bustamante et al. 2014 (126)
IL12B	*Mycobacterium*	Reduction or absence of IL-12 in leukocytes and EBV-B cells	Bustamante et al. 2014 (126)
IL12RB1	*Mycobacterium, Coccidioides, Paracoccidioides*	Impaired or abolished response to IL-12 and IL-23	Vinh et al. 2011, Moraes-Vasconcelos et al. 2005 (108,109)

IL12RB2	*Mycobacterium*	Impaired IL-12 response. Heterodimer with IL12RB1 in IL-12 response	Olbrich and Vinh 2023 (311)
IL17A	*Candida*	Impaired Th17 response	Puel et al. 2010 (116)
IL17F	*Candida*	Impaired Th17 response	Puel et al. 2010, Puel et al. 2011 (116,118)
IL17RA	*Candida*	Impaired Th17 response	Puel et al. 2011 (118)
IL22	*Candida*	Impaired Th17 response	Puel et al. 2010 (116)
IL23R	*Mycobacterium, Candida*	Deficiency in IFN-γ production (mycobacterial disease) and MAIT T cell specific IL-17A/F-dependent immune deficiency (CMC)	Philippot et al. 2023 (119)
IL4	*Candida*	Reduced IL4 associated with protection from chronic disseminated candidiasis	Merkhofer and Klein 2020 (16)
IL4R	*Aspergillus*	Polymorphisms associated with allergic bronchopulmonary aspergillosis	Carvalho et al. 2010 (312)
IL6	*B. dermatitidis*	Impaired Th17 response	Merkhofer et al. 2019 (72)
IL6R	N/A	* Iatrogenic immunosuppression of IL-6 response with monoclonal therapies (e.g., toxilizumab)	Vinh et al. 2021 (313)
IRAK1	N/A	Impaired toll-like receptor response in fibroblasts	Mina et al. 2017 (314)
IRF1	*Mycobacterium*	Reduced IFN-γ response	Ogishi et al. 2023 (315)
IRF8	*Mycobacterium*	Loss of DCs and impaired IL-12 production	Boisson-Dupuis et al. 2015, Bustamante et al. 2014 (102,126)
ISG15	*Mycobacterium*	Loss of ISG15 protein; Reduced IFN-γ production by lymphocytes	Bogunovic et al. 2012 (316)
JAK1	*Mycobacterium*	Impaired IFN-γ signaling	Rosain et al. 2019 (127)
JAK2	N/A	* Downstream signaling for IFNGR2 and IL12RB2	Lionakis et al. 2014 (317)
LTA	Autoimmunity	* Role in rheumatoid arthritis	Original Study Design, Calmon-Hamaty et al. 2011 (318)
MALT1	*Candida*	Impaired CARD9-BCL10-MALT1 signalosome activation of NF-κB	Gross et al. 2006 (297)
MBL	*B. dermatitidis, Cryptococcus neoformans*	MBL deficiency associated with Cryptococcal meningitis. MBL inhibiting TNF-α in mice infected with *B. dermatitidis*	Koneti et al. 2006, Ou et al. 2011 (319,320)
MPO	*B. dermatitidis*, *C. albicans*	Deficiency in HOCl generation in neutrophils. *B. dermatitidis* is resistant to myeloperoxidase	Original Study Design, Merkhofer and Klein 2020, Brummer et al. 1992 (16,104)
MYD88	*B. dermatitidis*	Dysfunction of TLR downstream signaling	Wang et al. 2016 (237)
NCF1	*Mycobacterium, Aspergillus*	Chronic granulomatous disease. Dysfunction in phagocytic oxidative burst	Holland 2013, Boisson-Dupuis et al. 2015 (102,103)
NCF2	*Mycobacterium, Aspergillus*	Chronic granulomatous disease. Dysfunction in phagocytic oxidative burst	Holland 2013, Boisson-Dupuis et al. 2015 (102,103)

NCF4	*Mycobacterium, Aspergillus*	Chronic granulomatous disease. Dysfunction in phagocytic oxidative burst	Holland 2013, Boisson-Dupuis et al. 2015 (102,103)
SLC11A1/ NRAMP1	*M. tuberculosis*	Increased IL-10 anti-inflammatory cytokine production.	Original Study Design, Awomoyi et al. 2002 (321)
PDCD1	*H. capsulatum*	* Pdcd1-/- knockout improved mouse survival	Lazar-Molnar et al. 2008 (256)
RORC	*Mycobacterium, Candida*	Impaired IL-17A/F and IL-22 production; Impaired IFN-γ in γδ T cells and Th1 cells	Okada et al. 2015 (322)
SIGLEC15	*Candida*	Loss of sialic acid detection	Jaeger et al. 2019 (323)
SPPL2a	*Mycobacterium*	Conventional type 2 DC deficiency with resulting defective IFN-γ secretion in $CD4^+$ T cell subsets	Rosain et al. 2019 (127)
STAT1	*Mycobacterium, Coccidioides, Histoplasma, Candida*	Impaired or abolished IFN-γ and IFN-α response	Sampaio et al. 2013, Smeekens et al. 2013, Bustamante et al. 2014 (126,302,324)
STAT3	*Candida*	Th17 cell deficiency; HIES	
STAT4	*Paracoccidioides*	Impaired IL-12 response and IFN-y production	Schimke et al. 2017 (112)
SYK	*Candida*	Dysfunction of tyrosine kinase downstream of dectin-1 and dectin-2	Bi et al. 2010 (85)
TBX21	*Mycobacterium*	Impaired IFN-γ production in innate (iNKT, MAIT) and adaptive (Th1) lymphocytes	Yang et al. 2020 (325)
TLR1	*Aspergillus, Candida*	Impaired cytokine response	Plantinga et al. 2012, Kesh et al. 2005 (91,326)
TLR2	*Candida*	Reduced IFN-γ and IL-8	Maskarinec et al. 2016 (92)
TLR4	*Candida*	Increased IL-10 anti-inflammatory cytokine production.	Maskarinec et al. 2016 (92)
TLR6	*Aspergillus*	Impaired recognition to fungal antigens	Kesh et al. 2005 (91)
TLR9	*C. albicans*	Impaired recognition of fungal CpG DNA	Miyazato et al. 2009 (93)
TNF	Dimorphic fungi	Reduction of TNF-α (TNF-α inhibitors) associated with increased risk of fungal infections	Castillo et al. 2016 (21)
TYK2	*Mycobacterium*	AR-HIES. Impaired IFN-γ, IL-17A, and IL-17F expression in T cells in response to IL-12 and IL-23	Kreins et al. 2015, Boisson-Dupuis et al. 2018 (143,263)
USP18	N/A	Pseudo-TORCH syndrome (type I interferonopathy). Loss of ISG15-dependent IFN-α/β signaling inhibition	Bucciol et al. 2019 (327)
VDBP	*B. dermatitidis*	Availability of vitamin D for immune function	Sainsbury et al. 2014 (124)
VDR	*Mycobacterium*	Less available vitamin D metabolites is speculated	Roy et al. 1999 (328)
ZAP70	*Mycobacterium*	Severe combined immunodeficiency; Loss of T cell activation in response to antigens	Santos et al. 2010 (329)
ZNF341	*Candida*	AR-HIES. Reduced expression of STAT3 and impaired Th17 response	Beziat et al. 2018 (120)
ZNFX1	*Mycobacterium*	Unknown - monocytosis observed	Le Voyer et al. 2021 (330)

*No dysfunction or inborn error of immunity discovered but shared function with included gene.