IDENTIFYING GENETIC SUSCEPTIBILITY TO FUNGAL INFECTIOUS DISEASES IN COLOMBIAN PATIENTS

Presented by:

Carlos Andres Arango Franco, Microbiologist

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Tutor

Andrés Augusto Arias Sierra, Bacteriologist, MSc, PhD.

TUTORIAL COMMITTEE

José Luis Franco, MD, MSc, PhD

Oscar D. Cano, MD

Anne Puel, MSc, PhD

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LIST OF ABREVIATIONS

AA, amino acids
AFB, acid-fast bacilli
AD, autosomal dominant
ADCC, antibody-directed cell toxicity
AF, Allele frequency
AIDS, acquired immune deficiency syndrome
AIR, auto-inhibitory region
APECED, autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy
AR, autosomal recessive
BCG, bacilli Calmette-Guérin
CADD score, Combined annotation dependent depletion
CARD9, caspase recruitment domain-containing protein 9
CBC, complete blood cell
CGD, chronic granulomatous disease
CIB, Corporación para Investigaciones Biológicas, Medellín
CLR, C-type lectin receptors
CMC, chronic mucocutaneous candidiasis
CMCD, chronic mucocutaneous candidiasis disease
CMV, cytomegalovirus
CRP, C-reactive protein
DAVID, The Database for Annotation, Visualization and Integrated Discovery
DHR, Dihydrorhodamine assay
EM, Enviromental mycobacterial
EBV-B Cells, Epstein Barr Viruses- transformed B Cells
ESBL, extended-spectrum beta-lactamase
ExAC, Exome Aggregation
FAD, flavin adenine dinucleotide
FACS, Fluorescence-activated cell sorting,
fMLF, formyl-methionyl-leucyl-phenylalanine
GAFFI, Global Action Fund for Fungal Infections
GDI, gene damage index
GIT, gastro intestinal tube
GM-CSF, granulocyte macrophage colony–stimulating factor
GMS, Gomori's Methenamine Silver
Hg19, referencial human genome 19
HGMD, Human Gene Mutation Data Bases
HHV8, human herpes virus 8,
HIES, hyper IgE syndrome
HIV, Human Immunodeficiency virus
IFIs, Invasive fungal infections
IFNγ, Interferon gamma
ITAM, immunoreceptor tyrosine-based activation motif (ITAM)
IUIS, international union of immunological societies
KPCs, Klebsiella pneumoniae carbapenemases producers
MAF, Minimal Allele Frequency
MRI, Magnetic resonance imaging
MSC Mutation Significance Cutoff
MSMD, mendelian susceptibility to mycobacterial diseases
NADPH, nicotinamide adenine dinucleotide phosphate oxidase
NCF4, Neutrophil Cytosolic Factor 4
NETs, Neutrophil extracellular traps
NGS, next generation sequencing
OMIM, online mendelian inheritance in man
PAI, Colombian Official Immunization Program
PAS, Periodic Acid–Schiff
PB1, Phox and Bem1
PBL, peripheral blood lymphocytes
PBMCs, peripheral blood mononuclear cells
PC, phox and Cdc24 domain
PCR, polymerase chain reaction
phox, phagocytic oxidase
PIDEC, primary immunodeficiency expert committee
PID, primary immunodeficiency diseases
PIDNet, Primary Immunodeficiency Network
PMA, Phorbol 12-myristate 13-acetate
PMC, paracoccidioidomycosis
PMN, polymorphonuclear neutrophil
Polyphen2, Polymorphism Phenotyping v2
PPD, purified protein derivative
P-RR, proline rich region
PRRs, innate pattern recognition receptors
PRV-3, parainfluenza virus type 3
PtdIns(3)P, phosphatidylinositol-3-phosphate
PX, Phox homology; PRR, proline-rich region
RD, rare diseases
RLU, relative light unit
ROS, reactive oxygen species
RPMI, medio Roswell park memorial institute
RSV, respiratory syncitial virus
RTº, room temperature
SH3, Src homology 3
SIFT, Sortig Intolerant from Tolerant
TB, tuberculosis
TLC, total lymphocyte counts
TLR, toll like receptors
TNF, tumor necrosis factor
VZV, varicella zoster virus
WBC, white blood cell counts
WES, whole exome sequencing
WT, wild type
XL, X-linked

ABSTRACT

Introduction: The landscape of human genes involved in the immune response is increasingly clear. Most mutations are associated with conventional Primary Immunodeficiency Diseases (PIDs), which confers a Mendelian predisposition to multiple infectious diseases; including fungal infections where each type of infection is highly suggestive of a specific type PID. However, the genetic susceptibility to fungal infections such as *Paracoccidioides brasiliensis, Histoplasma capsulatum* and *Corynespora cassiicola* is not fully undertood.

Objective: To identify the molecular defects in a cohort of five unrelated Colombian patients with invasive fungal infections caused by *Paracoccidiodes brasiliensis*, *Histoplasma capsulatum* and *Corynespora cassiicola.*

Methods: This is a descriptive study that presents a molecular characterization of five Colombian patients with confirmed invasive fungal infections (IFIs). Patient 1 (P1) presented with *Corynespora cassiicola* infection, P2 had disseminated histoplasmosis, and three more patients (P3, P4 and P5) presented juvenile Paracoccidioidomycosis (PMC). All patients were children with less than 15 years of age and all were HIV-negative. We reviewed medical records and performed PCR, tissues staining, western blotting, flow cytometry, immunological evaluation and whole exome sequencing (WES) in samples from all patients. Sanger sequencing was also used to confirm the genetic variants in patients and relatives.

Results: Genetic analysis of *CARD9* in patient P1 showed compound heterozygosity for a frameshift mutation with premature stop codon in exon 2 (c.23_29del; p.Asp8Alafs10X) and a nonsense mutation in exon 6 (c.C865T; p.Q289X). The p. Asp8Alafs10X has not been previously reported in the literature. CARD9 protein expression was absent in peripheral blood mononuclear cells (PBMCs) from the patient. Genetic analysis of patient P2, identified in *NCF4* a homozygous genetic variation in exon 3 (c.C172T; p.R58C). Immunoblot analysis demonstrated a significant reduction of $p40^{phox}$ and $p67^{phox}$ proteins in Epstein Barr Viruses (EBV) transformed B cells line and neutrophils from the patient. His neutrophils and EBV-

B cells showed a significant defect in superoxide production during phagocytosis and fMLF stimulation, whereas extracellular release of superoxide elicited by phorbol ester was unaffected. Finally, in patients P3, P4 and P5 affected with juvenile PMC, WES revealed candidate variants in *IL17RA*, *IL18R1*, *NLRP2* and *PIK3CA* were i*n silico* studies predicted to be deleterious or pathogenic. We are currently investigating the impact of these variations at the protein expression and functional levels.

Conclusion: We studied five unrelated Colombian patients with severe IFIs: A patient with severe phaeohyphomycosis (P1) by *C. cassiicola* and compound heterozygous mutations, including the novel variant (p.Asp8Alafs10X) in the *CARD9* gene. In a patient with disseminated histoplasmosis (P2), we identified a novel homozygous mutation in the *NCF4* gene conferring susceptibility to microorganism, suggesting that $p40^{phox}$ protein plays an essential role during oxidative responses especially in fungal clearance. In the three patients with Juvenile PMC (P3, P4 and P5) although several candidate gene variants were selected to be good candidate genes according our analysis criteria, further re-analysis and functional assays are necessary.

This information represents the first molecular characterization of Colombian patients with severe IFIs. This information can be useful to employ the specific and proper treatment and to promote in near future new therapeutic strategies in order to improve the life quality of these patients.

Key words: Rare diseases, Primary immunodeficiency diseases, compound heterozygous, phaeohyphomycosis, Juvenile PMC, Invasive fungal diseases, genetic theory of life-threatening infectious diseases, germline mutations, Chronic Mucocutaneous Candidiasis, dimorphic fungus, deep dermatophytosis, opportunistic pathogens, phytopathogen, Human connectome, pan-fungal PCR, protein domain, consanguinity, NADPH oxidase, protein–protein interaction, reactive oxygen species, Whole exome sequencing.

1. INTRODUCTION

RARE DISEASES AND PRIMARY IMMUNODEFICIENCY DISEASES

Rare diseases (RD), conform a broad group of diseases characterized by low prevalence (1/2000) and high impact on the patients' quality of life and their families. Worldwide, around 7000 rare diseases have been described, they together represent between 6% and 8% of diseases in the population, affecting between 420 million to 560 million people and imposing a significant global burden that became a serious public health issue (1-5).

Around 80% of RD are genetic in origin and a half of these diseases are present in children less than 5 years-old. RD may cause loss of life and impose a huge physical, psychological, and socioeconomic burden on patients and their families (6). The genetic defects associated with these diseases are recognized as three main types: single gene disorders, chromosome disorders and multifactorial disorders (7, 8).

Primary Immunodeficiency Diseases (PIDs) are part of the single gene disorders; they are inborn errors of immunity that selectively affect the production of cells and/or molecules involved in the immune response, therefore leading to abnormalities in the ontogeny, differentiation or effector function of the immune system (9, 10). Although there are only few published reports addressing the prevalence of PIDs, in 2013 Bousfiha et al and the PID experts estimate that 6 million people may be living with a PID worldwide, whereas only 27,000–60,000 have been identified (11); therefore, these disorders may be more common than initially expected. In the United States the prevalence is 1 in 2,000 in children, and 1 in 1,200 in adults (12).

The heterogeneous errors of immunity observed in PIDs result in enhanced susceptibility to infectious diseases, autoimmunity, and malignancies (13, 14); most PIDs are monogenetic with variable expressivity and often exhibit incomplete penetrance (9, 14).

PIDs currently represent an excellent model to describe the importance and function of the immune system. In many cases, the study of PIDs has led to the identification of new genes that play a crucial role either in the immune homeostasis or as part of the immune response including specific effector functions or immune cell development (15). These PIDs are considered "nature experiments" and for the last 60 years of study more than 200 PIDs have been associated with a specific gene alteration. Moreover, the Primary Immunodeficiency Expert Committee (PIDEC) of the International Union of Immunological Societies (IUIS) published a report in 2015 with more than 280 disease-causing genes, leading to different clinical phenotypes of PIDs including chronic inflammation, infections, autoimmunity, lymphoproliferation, allergic manifestations and organized these diseases by categorizing them into 9 major groups: 1) affecting cellular and humoral immunity, 2) combined immunodeficiencies with associated or syndromic feature, 3) predominantly antibody deficiencies, 4) diseases of immune dysregulation, 5) congenital defects of phagocyte number, function or both, 6) defects in intrinsic and innate immunity, 7) Autoinflammatory disorders, 8) Complement deficiencies and 9) Phenocopies of PIDs (9).

For the interest of this text, we have emphasized in PIDs associated with genetic susceptibility to microorganisms which are included in the group of defects in intrinsic innate immunity and congenital defects of phagocyte number, function, or both.

1.1. GENETICS IMMUNE DEFECTS CONFERING SUSCEPTIBILITY TO MICROORGANISM, AN APPROACH FROM FUNGAL INFECTIONS

Advances in the field of Human Genetics of infectious diseases has provided support to the mendelian genetic theory of life-threatening infectious diseases. This theory suggests that germline mutations affecting the human immune system genes may confer susceptibility to a large group of microorganisms (fungi, bacteria, protozoa and virus). Such defects mainly manifest during childhood as monogenetic inborn errors of innate immunity (one gene, one disease) (13, 16). Such knowledge has been beneficial in towards a better understanding of the clinical variability observed in the clinical setting since only a minority of infected patients develop severe clinical manifestations. Nowadays, developments in molecular and genetic tools such as new generation mass sequencing (9) has facilitated the discovery of new disease-causing genes, allowing the molecular characterization, phenotype-genotype correlation and diagnosis of an important number of patients with errors in the innate monogenic immunity. It has also aid on therapeutic approaches to the point of even generating novel alternatives such as personalized medicine, gene therapy, and pharmacogenomics among others (9, 17).

Our genome contains at least 1854 gene products involved in immune responses. Of these, 1540 genes have been associated with innate immune responses and around 515 genes were associated with adaptive immune responses (including 201 genes that were associated with both responses (18). Until today, defects on 32 of these genes have been associated with dysfunction of the innate immune system and 31 genes with congenital defects of phagocytes

conferring human diseases (9). We will describe the more important characteristics of some of these diseases according with the Primary Immunodeficiency Expert Committee of the IUIS.

Mendelian susceptibility to mycobacterial diseases

Mendelian Susceptibility to Mycobacterial Diseases (MSMD), (OMIM # 209950), is a rare inherited condition characterized by selective predisposition to clinical disease caused by weakly virulent mycobacteria, such as bacillus Calmette-Guérin (BCG), vaccines and non-tuberculous environmental mycobacteria (EM), in otherwise healthy patients with no overt abnormalities in routine hematological and immunological tests (19). The patients are also vulnerable to the more virulent *Mycobacterium tuberculosi*s. About half of them also suffer from clinical disease caused by non-typhoidal or, more rarely, typhoidal *Salmonella*. MSMD designation does not recapitulate all the clinical features, as patients are also prone to salmonellosis, candidiasis and tuberculosis, and more rarely to infections with other intramacrophagic bacteria (listeriosis, nocardiosis, klebsiellosis), fungi (candidiasis, histoplasmosis, PMC, coccidioidomycosis), or parasites (leishmaniasis, toxoplasmosis) (19). Viral infections have also been reported, including diseases caused by cytomegalovirus (CMV), human herpes virus 8 (HHV8), parainfluenza virus type 3 (PRV-3), respiratory syncytial virus (RSV) and varicella zoster virus (VZV).

The genetic study of MSMD patients has allowed the identification of defects in 11 genes (*IL12RB1, IL12B, AR-IFNGR1, AD-IFNGR1, IFNGR2, STAT1, CYBB, IRF8, TYK2, ISG15, RORC and NEMO*). The high level of allelic heterogeneity has already led to the definition of 19 different clinical disorders affecting principally the IFNγ-mediated immunity (**figure 1 and table 1**) (19, 20). All these data confirm that the human IFNγ-mediated immunity is

essential for the control of mycobacterial infections. The study of MSMD led to the discovery of autoantibodies against IFNγ with late-onset mycobacterial diseases as phenocopies of MSMD, mimicking inborn errors of IFN-γ immunity (21-24).

Chronic granulomatous disease (CGD)

Superoxide (O_2^-), as a precursor of reactive oxygen species (ROS), it is produced by the leukocyte NADPH oxidase an enzyme that consists of two membrane-bound components forming a flavocytochrome b558, gp91^{phox} (*phox* from phagocyte oxidase) and p22^{phox}, and three cytosolic proteins called p40^{phox}, p47^{phox} and p67^{phox} (**figure 2**) (25-28). The gp91^{phox} protein is the catalytic subunit; it contains a NADPH and FAD binding site and two heme prosthetic groups. The p22^{phox} subunit mediates the bind of a trimeric complex of p47^{phox}, p67^{phox} and p40^{phox}. The p67^{phox} subunit interacts with flavocytochrome b558 to stimulate electron transfer between NADPH and FAD (29, 30). The p47^{phox} and p40^{phox} subunits act as regulated adaptor proteins that optimize the assembly and activity of the enzyme complex. p47^{phox} contains tandem SH3 domains for binding its target PxxP motif in p22^{phox} as well as a PX (*phox* homology) domain that interacts with phospholipids; both are masked in the resting state. The p40^{phox} subunit has a PX domain, with high specificity for phosphatidylinositol-3-phosphate PtdIns(3)P, which is also masked in resting cells (30-35). In resting cells, the cytosolic NADPH oxidase components (p47^{phox}, p67^{phox}, and p40^{phox}) are associated in a trimer complex with a 1:1:1 stoichiometry through specific domains (30, 36); the complex change their conformation and translocate to the plasma membrane complex (gp91^{phox}/p22^{phox}) upon cell activation, e.g. binding of particles, bacteria, fungi or soluble inflammatory mediators to specific receptors on the phagocyte cell surface (30, 37); p47^{phox} is in a 'closed conformation', as its auto-inhibitory region (AIR) (aa 292-340) interacts with

its two SH3 domains to keep the protein 'locked' (38, 39). Priming induced by TNFα or GM-CSF induces p47^{phox} phosphorylation which produce conformational changes and its binding to the proline rich region (P-RR) of the p22^{phox} and NADPH complex formation (40-42). After that, the NADPH binding site of gp91^{phox} becomes available for NADPH in the cytosol. NADPH donates two electrons to gp91^{phox}, which are then transported within the protein to FAD, thereafter to the prosthetic group hemes, and finally to molecular oxygen at the other side of the membrane. In this way, O_2^- is generated inside the phagosome or on the cell surface, in close proximity to the phagocytized or attached microorganisms (30, 43-45).

Genetic defects in the NADPH oxidase complex and a failure of superoxide generation leads to a rare syndrome called Chronic Granulomatous Disease (CGD) characterized by early onset of recurrent and severe infections affecting the lung (pneumonia), lymph nodes (lymphadenitis), liver (abscess), bone (osteomyelitis), gastrointestinal tract and skin (abscesses or cellulitis) (46, 47). CGD represents a heterogeneous group of disorders characterized by defective generation of respiratory burst in human phagocytes (neutrophils, mononuclear cells, macrophages, and eosinophils). The clinical manifestations of CGD typically begin in infancy or early childhood. CGD patients are particularly susceptible to *Staphylococcus aureus, Pseudomonas spp, Nocardia spp, Burkholderia cepasia, Escherichia coli, Listeria spp, Serratia marcescens, Salmonella spp* and *Klebsiella spp*. Furthermore, the dysregulated inflammatory response results in granuloma formation and other inflammatory disorders, which are a distinctive hallmark of this disorder (47-50). Fungal infections also occur, most common being caused by filamentous molds as invasive aspergillosis due to *Aspergillus fumigatus* followed by *Aspergillus nidulans* (51) while, dimorphic fungus

infections such as histoplasmosis and blastomycosis do not occur in CGD, nor does cryptococcosis (48, 51). Approximately two-thirds of CGD cases result from defects in the X-linked gene *CYBB* encoding the gp91^{phox} (OMIM # 306400). The other forms of CGD are autosomal recessive (AR), characterized by mutations in *CYBA* (OMIM # 233690), *NCF1* (OMIM # 233700) and *NCF2* (OMIM # 233710) encoding p22^{phox}, p47^{phox} and p67^{phox}, respectively. Until now, only one case of AR-CGD has been described affecting the *NCF4* gene (OMIM # 613960), encoding p40^{phox}, (**table 2)** (9, 17, 47, 52-55).

The *NCF4* (Neutrophil Cytosolic Factor 4) gene on 22q13.1 chromosome encodes p40^{phox}, the last NADPH oxidase subunit to be identified (56). *NCF4* contains 10 exons spanning approximately 18 kb encoding a predicted 339-amino acid protein with a calculated molecular mass of 37 kDa (35, 57). p40^{phox} is predominantly expressed in bone marrow cells: neutrophils, monocytes, basophils, eosinophils, mast cells, megakaryocytes, B and T cells (57-59). This protein has 3 well-defined domains, listed in order from the N-terminus: a PX (*phox*) domain (aa 19 – 140) (60-62), an SH3 domain (aa 175 – 225) (58) and a PC (*phox* and cdc24p) domain (aa 285–306), also known as the PB1 (35, 61). The PX domain of p40^{phox} interacts specifically with membranes enriched in phosphatidyl inositol 3-phosphate (PtdIns(3)P) (31-34). The SH3 domain of p40^{phox} has also been suggested to interact with the proline-rich region (PRR) of p47^{phox} and thereby link p47^{phox} and p67^{phox} (36, 63). The interaction between p40^{phox} and p67^{phox} is mediated by their respective PB1 domains (64, 65).

Genetic immune defects conferring susceptibility to fungal infections

A fungus (plural: fungi or funguses) is any member of the group of eukaryotic organisms that includes unicellular microorganisms such as yeasts and molds, as well as multicellular fungi that produce familiar fruiting forms known as mushrooms. These organisms are classified as a fungi kingdom. Fungi are ubiquitous in the environment, as they are found on every continent including the artic soil, in the deep sea, and in the air. Mammals have evolved in the presence of diverse fungi, and our immune system has adapted to their presence (66). Humans are constantly exposed to fungi from birth, however few mycotic infections affect them, which evidenced an adequate immune response to the fungi. Microorganism encounters require a coordinated host innate and adaptive immune response to successfully eradicate the fungus and promote long-lived immunological memory of the exposure (67).

We have germline genes for innate pattern recognition receptors (PRRs) in our genomes that provide defenses against fungal infection (Dectin-1, Dectin-2, Dectin-3, DC-SIGN, Langerin, Mannose receptor, TLR2, TLR4, Galectin-3, CD36, complement receptor, Manosse-binding lectin and Pentraxin-3). The innate response controls most fungi, with oxygen radicals, inflammation, cytokines production and grown factors that modulate de acquired immune response (66).

Cellular mediated immunity represents the main adaptive immune response to control fungal diseases as noted in HIV+/AIDS patients who has a progressive decline in the CD4+ T Cells count and have high susceptibility to mycosis by opportunistic fungus and develop diseases such as candidiasis, neumocystosis, criptococosis and histoplasmosis. The T cell population Th1 and Th17 control fungal infections. Th1 through IFNγ and TNFα production, activate other cells as macrophages to eliminate fungi by fungicidal or fungistatic mechanisms. Th17

cells activate and recruit PMNs mainly in the mucosal membrane, to control fungal infections (**figure 3**) (68-70)**.** However Th17 is not the only pathway involved in PMNs recruitment in fungal infections. For example, it has been shown the early recruitment of PMNs in response to Aspergillus is mediated via IL1R/MyD88/Card9 dependent production for CXCR2 ligands that finally will enhance the chemotaxis to PMNs. These data revealed a coordinated regulation of chemokine induction and neutrophil recruitment by IL-1R/MyD88 and CLR/CARD9 signaling pathways that operate in a biphasic manner and in epithelial and hematopoietic compartments to orchestrate sterilizing immunity against *A. fumigatus*. In the early phase of the infection dependent CXC chemokine production is driven via IL-1R/Myd88 and late phase CXC chemokine production is leading by Card9 hematopoietic cells (71-73).

The antibody-mediated immunity with protective effects that recognize fungi has been classified into direct and indirect mechanisms. The direct mechanisms results in the inhibition of growth or microbicidal activity through the direct binding of immunoglobulins to the pathogen (74), indirect mechanisms on the other hand, refers to the immunoglobulin mediated resolution of infection by enhancing the microbicidal potential of effector cells (68). Opsonization, activation of complement pathway, and antibody-directed cell toxicity (ADCC) are associated with indirect effects of antibodies during infection (67-69, 75).

The immune response against fungi are robust and heterogeneous, however according to the Global Action Fund for Fungal Infections (GAFFI), globally, over 300 million people of all ages suffer from a serious fungal infection every year, with over 1.66 million people estimated to die. From a public health perspective, the incidence of invasive fungal infections increases over time with the rise in at-risk populations (76-78).

Medically important fungi include dimorphic fungi (*Coccidioides immitis* or *posadii*, *Paracoccidioides brasiliensis* and *Histoplasma capsulatum),* and dermatophytes *(Trycophyton violaceus, Trycophyton rubrum, Trycophyton mentagrophytes*). Of relevance are dematiaceous fungi (*Phialophora verrucosum, Exophiala spp, Corynespora cassiicola),* and IFIs, such as candidiasis, aspergillosis, pneumocytosis and cryptococcosis, all considered a major health problem (70). *Candida spp*. not only is medically important but also causes severe chronic mucocutaneous candidiasis (CMC).

Chronic mucocutaneous candidiasis (CMC)

Chronic mucocutaneous candidiasis (CMC) is characterized by recurrent or persistent symptomatic mucocutaneous infections caused by fungi of the genus *Candida*, mostly the commensal *Candida albicans*, affecting nails, skin, and the oral and genital mucosa (79). CMC is frequent, and associated with other infections caused by a broad spectrum of microorganisms, it is also present in the context of acquired conditions including HIV infection, immunosuppressive therapies, prolonged antibiotic therapies, and diabetes mellitus, as well as with various inherited primary T-cell immunodeficiencies (70). CMC may also be categorized as syndromic CMC in patients with autosomal-dominant (AD) hyper IgE syndrome (HIES), AD signal transducer and activator of transcription 1 (STAT1) gain-of-function mutations, autosomal-recessive (AR) deficiencies in interleukin (IL)-12 receptor β1 (IL-12Rβ1), IL-12p40, caspase recruitment domain-containing protein 9 (CARD9), retinoic acid-related orphan receptor γT (RORγT) or AR autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED)(79) .

Chronic mucocutaneous candidiasis disease (CMCD) is defined as CMC in patients with no other prominent clinical signs and with none of the genetic defects causing other PID.

Currently, 4 genetic defects have been described as etiology of CMCD (*IL-17RA, IL-17RC, IL-17F* and *ACT1)*. All these genes are involved in communication between the innate and adaptive immune system towards the cellular mediated immunity composed by subpopulations of Th17 and Th1 lymphocytes, which confirms the role of IL-17 in mucocutaneous immunity against *Candida* infections (70, 79-87).

Invasive fungal diseases

Invasive fungal diseases (IFDs) are life-threatening infections that require rapid diagnostic and frequently occur in patients with immunodeficiencies (IDs), either iatrogenic, acquired, or primary (PIDs) (88-90). IFDs, such as candidiasis, aspergillosis, pneumocytosis and cryptococcosis in particular, have become a major health problem. They represent an important public health problem with a rapidly growing at-risk population of patients, and the appearance of fungal infections that are resistant to treatment. Therefore, IFDs generate considerable morbidity and mortality worldwide, and as they frequently develop in patients with multiple conditions its pathogenesis remains poorly understood (91). They are also related with an excessive economic burden, owing to extended hospitalization, the need for exhaustive supportive care, and the use of costly antifungal agents (88, 91). In rare cases, however, IFDs occur in otherwise healthy patients without any known risk factors (70) and occasionally can be produced by 'rare yeasts' that are encountered as frequent colonizers of human skin, mucosal surfaces, in food items or in the environment (92). These "idiopathic" IFDs represent a unique chance to gain insight into IFD pathogenesis (70). (89, 93), there are some described PIDs associated with IFDs (16, 70, 93)

Deep dermatophytosis

Dermatophytes are cosmopolite filamentous fungi who affect keratinized tissues such as hair, skin and nails. They frequently cause benign infections such as tinea capitis, tinea corporis and/or onychomycosis. By contrast, deep dermatophytosis is a rare condition defined by the invasion of the dermis and hypodermis by dermatophytes, sometimes associated with lymph node, brain, digestive tract or bone involvement. In patients with HIV or immunosuppressive therapy sometimes behavior as life-threatening infection (94) AR CARD9 deficiency has been found in patients suffering from an expanded spectrum of fungi as *Trichophyton violaceum, Trichophyton rubrum and Trichophyton* mentagrophytes (95, 96).

Deep Mycosis (systemic fungi and subcutaneous fungi)

Deep mycoses are uncommon infections caused by fungi; they are divided into subcutaneous and systemic mycoses caused by primary pathogenic and opportunistic fungal pathogens. The primary systemic pathogenic fungi (*Coccidioides immitis, Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Paracoccidioides brasiliensis*) are able to establish infection in a normal host whereas, opportunistic pathogens require an immune compromised host (e.g., cancer, organ transplantation, surgery, and AIDS) in order to establish infection as in *Cryptococcus neoformans, Candida spp., Aspergillus spp., Talaromyces marneffei (*called before *Penicillium marneffei)* the Zygomycetes, *Trichosporon beigelii*, *Fusarium* spp and environmental fungus infections (97, 98).

Clinically, cases of primary deep mycoses are asymptomatic or clinically mild infections occurring in apparently healthy individuals living or traveling in endemic areas. However,

patients exposed to a high inoculum of organisms or those with altered host defenses may suffer life-threatening progression or reactivation of latent foci of infection (97, 98) .

Deep systemic mycoses by dimorphic fungi (*Histoplasma capsulatum* and *Paracoccidioides brasiliensis)* are present as hyphae in the environment and as yeasts in tissues, the transition between these two forms being triggered by temperature changes. Infection occurs by inhalation of the infecting particles followed by their deposition in the lung. Although usually self-limiting, some fungi exposures can be potentially fatal in individuals with a failure of the immune system, therefore acting as an opportunist infection (99). Lanternier et al, described that patients with disseminated dimorphic infections should therefore be explored for defects of the IFNγ/IL-12 circuit and those patients should be considered at risk of infection with endemic fungi (70).

Histoplasma capsulatum is a thermally dimorphic soil inhabitant, causal agent of histoplasmosis, with worldwide distribution. Endemic areas have been defined in the United States especially in the Ohio and Mississippi river valleys, as well as in Central and South American countries, including Colombia (100-105). It is usually asymptomatic but may occasionally results in severe illness. *H. capsulatum* is normally restrained to the lung, however in patients with progressive extrapulmonary infection its clinically manifested as disseminated histoplasmosis (99, 106, 107). Hematogenous dissemination probably occurs in most patients during the acute infection before cellular immunity develops. Disseminated disease occurs in approximately 1 in 2000 patients with acute infection (100, 106)

The clinical presentations of histoplasmosis include: subclinical asymptomatic, acute pulmonary, chronic pulmonary, progressive disseminated and African histoplasmosis *(Histoplasma capsulatum var duboisii*). Pulmonary infections are the primary manifestation of histoplasmosis, varying from mild pneumonitis to severe acute respiratory distress syndrome. Dissemination of *H. capsulatum* within macrophages is common and becomes symptomatic primarily in patients with defects in cellular immunity. The spectrum of disseminated infection includes acute, severe, life-threatening sepsis and chronic, as well as slowly progressive infection with involvement of the central nervous system, endocardium, gastrointestinal tract and less frequently other intra-abdominal organs (108-110). The disseminated histoplasmosis is often associated with compromised host defenses due to innate immunodeficiencies and acquired immunodeficiency syndromes such as HIV infection, hematologic disorders including leukemias and lymphomas, as well as secondary to immunosuppressant effects due to solid organ or stem cell transplantation, and treatment of chronic inflammatory diseases (101, 111-115).

The immune response against *H. capsulatum* is mainly associated with both, innate and adaptive immune response mechanisms, to neutralize the pathogen and withstand infection. Macrophages and dendritic cells have major roles in the activation of cellular pathways (116). This response starts after recognition by Dectin-1, C-type lectin receptors and toll like receptors, which are essential for initial cytokine production and/or fungi phagocytosis (116, 117). There is clinical evidence supporting the importance of IFNγ produced by Th1 cells and TNFα in host defenses to this pathogen. The Th17 response is important for controlling

many fungal infections and may be beneficial, although not essential, to control the *H. capsulatum* (116, 118).

There are reports in patients suffering PID with mutations in the IFNγ axis who develop disseminated infections by *H. capsulatum*, *Coccidioides spp*, *Mycobacteria* and *Salmonella* (119-121). Also, patients with molecular defects in GATA2, STAT1 and STAT3 have been reported with histoplasmosis (69, 122).

Paracoccidioidomycosis (PCM) is a systemic and endemic mycosis restricted to tropical and subtropical areas of Latin America, including Colombia (123-129). The infection is caused by the thermal dimorphic fungi *Paracoccidioides brasiliensis* and *Paracoccidioides lutzi* (124), PMC is the most common systemic mycosis in Latin America where up to ten million people are infected and 80% of the cases reported are from Brazil, Colombia and Venezuela (130). More than 15,000 cases of paracoccidioidomycosis have been reported between 1930 and 2012, a number that only partially reflects the prevalence of the disease in Latin America (131).

The clinical presentation of PCM include**:** subclinic- asymptomatic, acute/juvenile, chronic/adult (128, 132) and in some papers also include residual form with fibrosis (133-135). Only a small percentage of individuals infected by *P. brasiliensis* develop clinical PCM possibly in part because of genetically determined inter-individual variability of host immunity (108, 136, 137).

Usually the primary infection is subclinical or asymptomatic in majority of immunocompetent individuals and can be eventually reactivate with chronic manifestations involving several tissues (138). The acute/juvenile form commonly affects children and

young adults who tend to present more disseminated lesions. Frequently they present generalized or intra-abdominal lymphadenomegaly associated by lesions of the skin, bones, the oral and intestinal mucosa and the bone, and hepatosplenomegaly. Whereas, the chronic/adult form affect 80% to 95% of total cases, is more common in adult men and frequently is limited to lesions involving the lungs and oropharyngeal or nasal region, the skin and oral mucosa adjacent to the mouth and nose., palatal ulceration extending to the gums and tongue (128, 129, 131, 132).

Regard to residual form, the abnormalities are present mainly after therapy and leads to several grades of fibrosis, characterized by alveolar and interstitial opacities, enlarged and calcified hilar and mediastinal lymph nodes, distortion of the pulmonary parenchyma, and pseudotumoral masses (135).

There are few reports of associated genetic defects cause susceptibility to PCM in humans. In 2005, disseminated infection by *Paracoccidioides brasiliensis* was reported in two patients; one patient had an inherited deficiency of the β1 subunit of the Interleukin *12 /* Interleukin *23* receptor *(IL12RB1/IL23R)* (139), and the second patient with a CD40 ligand *(CD40L*) defect (140). There are two additional reports in patients with genetic defect in STAT4 (141) and GATA2 deficiency (142).

The immune response against *Paracoccidioides brasiliensis* starts when pattern-recognition receptors (PRRs) identify the cell wall of this fungus. The recognition occurs via β-glucan present on the conidial surface as well as the dectin-1 and CR3 receptors, or other receptors present on the surface of the phagocytic cells; whereas in the case of *P. brasiliensis* yeasts whose cell walls are composed mainly of α-glucan, recognition of yeast cells could be

through a receptor other than dectin-1 or CR3, for example Dectin-2 (67, 143). *P. brasiliensis* yeasts also induces NET structures through a mechanism that is partially dependent on ROS production while the conidia induce NET formation through a ROS-independent mechanism (143).

Neutrophils have been described as the major cells involved in the inflammatory response against *P. brasiliensis*. One of the defense strategies is ROS production through superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•); also, PMNs produce Neutrophil Extracellular Traps (NETs) which could be dependent or independent of reactive oxygen species; this reaction may occur against both the conidia and yeast morphotypes of *P. brasiliensis* (143). About the acquired immunity to control the *P. brasiliensis* infection, there is a cellular mediated response resulting in activation of phagocytes due to IL-12/IFNγ as well as a predominant secretion of IgG2a antibodies; in contrast, progressive disease are characterized by the production of low levels of IFNγ, early secretion of high levels of IL-5 and IL-10, eosinophilia, and polyclonal activation of B cells, as well as a preferential secretion of IgG2b and IgA isotypes (132, 144, 145). Recently studies in mice, have found that TLR-4 cooperates with Dectin-1 and mannose receptors to expand Th17 cells induced by *P. brasiliensis* stimulated dendritic cells, which aids the fungus control (146).

Deep subcutaneous mycoses comprise several clinical entities caused by invasion of the skin and subcutaneous tissue by saprophytic fungi that live in soil and vegetation (147). The main subcutaneous mycoses include: sporotrichosis, chromoblastomycosis, mycetoma, phaeohyphomycosis, hyalohyphomycosis, and lacaziosis. An example of these deep

subcutaneous mycoses are causing by deamaticeas fungus causing phaeohyphomycosis which referes to a spectrum of rare mucocutaneous to systemic infections in humans, caused by dark, melanin pigmented dematiaceous fungi including the genus *Alternaria*, *Bipolaris*, *Curvularia, Cladosporium*, *Exserohilum*, *Phialophora*, *Exophiala* and *Corynespora cassiicola (148).* *Corynespora cassiicola* is a ubiquitous fungus member of *Ascomycota* phylum and *Pleosporales* order with tropical and subtropical geographic distribution (149). This fungus is a phytopathogen that causes target spot on leaves, stems, roots and flowers of more than 280 plant species, including many economically important crops in over 70 countries (149-151). It rarely causes human infections, however there are four reports in adults causing phaeohyphomycosis affecting mainly subcutaneous and soft tissue, one of these cases reported a subcutaneous phaeohyphomycosis caused by *C. cassiicola* with prominent tissue necrosis in a woman associated to a possible heterozygous mutation in the Caspase Recruitment Domain Family Member *(CARD9)* gene (**table 3**) (87, 152-154). Mutations in *CARD9* gene have been described causing phaeohyphomycosis by several fungal infections such as *Phialophora verrucosum*, *Exophiala dermatitidis*, *Exophiala spinifera* and *Corynespora cassiicola* (85, 87, 155)

The *CARD9* (Caspase Recruitment Domain Family Member) gene on 9q34.3 chromosome encodes the CARD9 protein. CARD9 contain 536 aa in 13 exons. This protein has 2 well-defined domains, CARD domain (aa 6 - 98) and two coiled coil domain segments (aa 117-277 and 332-419). Coiled coils are built by two or more alpha-helices that wind around each other to form a supercoil. CARD domain is a homotypic protein interaction module composed of a bundle of six alpha-helices and is involved in apoptosis through their regulation of caspases that contain CARDs in their N-terminal. The CARD domain typically

associates with other CARD-containing proteins, forming either dimers or trimers involved in inflammation through their regulation of NFκB, was identified by its selective association with the CARD domain of BCL10, a positive regulator of apoptosis and NFκB activation, and is thought to function as a molecular scaffold for the assembly of a BCL10 signaling complex that activates the NFκB activation pathway (156, 157).

According with Lionakis et al, *CARD9* deficiency are characterized by an adult-onset fungal disease, susceptibility is restricted to fungi without concurrent bacterial or viral infections and finally, is the only PID in which both mucosal and systemic fungal disease develop (158). *CARD9* gene mutations have been described in patients with CMC and CMCD, invasive candidiasis causing meningoencephalitis, vertebral osteomyelitis and colitis, deep and superficial dermatophytosis, Extrapulmonay Aspergillosis and invasive phaeohyphomycosis. All these reports demonstrate the critical dependence on CARD9 in human antifungal immunity (158, 159) It has been found that in CMCD patients, CARD9 is required for adaptive but not innate immunity response to oral and mucosal *Candida albicans* infections; showing that CARD9 is essential for the development and subsequent differentiation of Th17 cells (9, 84, 155, 160-163).

Few studies related to PIDs have been done in Colombian population; most of them carried out by the group of Primary Immunodeficiencies of the University of Antioquia. At the present time where we are studing a cohort of more than 40 patients with clinical diagnosis including disseminated BCG, tuberculosis and disseminated infections such as candidiasis, histoplasmosis, cryptococcosis, paracoccidioidomicosis and salmonellosis. With the use of WES technologies, we have identified patients with complete AR IL-12RB1 (partial

recessive (PR)-IFN-GR1 and AD-STAT1-GOF deficiencies (unpublished data), and also the patients we studied and reported here. We consider that the molecular characterization of new Colombian patients allows us to understand the molecular mechanism by which these defects lead to phenotypic expression in humans and facilitate the possible development of new therapeutic alternatives for these pathologies including personalized medicine, gene therapy, and pharmacogenomics as well as genetic counseling to patients and their families.

1. HYPOTESIS:

The clinical phenotype exhibited in patients with invasive fungal infections is a consequence of a single-gene inborn errors of immunity.

2. OBJECTIVES

2.1. GENERAL AIM

To identify new defects in genes known or unknown to be involved in susceptibility to fungal diseases in a cohort of five Colombian patients with invasive fungal infections caused by *Corynespora cassiicola*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis.*

2.2. SPECIFIC AIMS

- To describe the clinical and immunological characterization of the cohort.
- To identify the single gene mutations underlying molecular defects identified by Whole Exome Sequencing.
- To characterize the mutation at the molecular level, establishing the cellular and immunological effect of the genetic defects under studied by functional test

3. MATERIAL AND METHODS

Patients and ethical issues

The cohort included five Colombian patients affected with severe invasive fungal infections recruited through the Primary Immunodeficiency Network (PIDNet) from different geographic areas in Colombia, South America (**figure 4**). Patient 1 (P1) is a female who presented a *Corynespora cassiicola* infection when she was 6 years old, patient 2 (P2) is a male presenting a disseminated histoplasmosis infection at the age of 2 years old, finally, last three patients (P3, P4, P5) are two females and one male with juvenile disseminated PCM, at the age of 14, 12 and 10 years old, respectively (**table 4**). All patients included were HIV negative without other PID diagnosed before.

We reviewed the patients' medical records to obtain relevant clinical information, five families were interviewed in order to complete the familial pedigrees and later stablish the allele segregation which was possible in four from five families included in the present study. This study was approved by the local review boards (F8790-07-0010) and all patients and their relatives signed the informed consents according to the "Scientific Standards for Technical and Administrative Health Research" established by the Colombian Ministry of Health Resolution 008430 of 1993.

Whole blood samples were collected from patients (probands), relatives and healthy controls employing the venipuncture collection method in 4 mL tubes coated with K_2EDTA anticoagulant or Sodium Heparin (BD). We used these samples for whole-blood activation, DNA extraction, plasma collection, the isolation of peripheral blood mononuclear cells (PBMCs) for B-lymphocyte immortalization to be used for the *in vitro* experiments.

Cell purification and culture

Blood samples were taken from the proband and from their parents and siblings. Human PBMCs were isolated from whole blood (16 mL) subjected to Ficoll density gradient separation (Histopaque®-1077 Sigma-Aldrich), and centrifuged 20 minutes at 400g/22ºC, the interphase was collected and cells washed twice with PBS. To obtain Epstein Barr Viruses B Cells (EBV-B Cells), cells were suspended in an appropriate volume of PBS and counted them using a hemocytometer. Cell cultures were grown using 48-well flat bottom tissue culture plates (corning incorporated) at a density of 1×10^6 cells/mL in RPMI (Gibco, Thermo Fisher Scientific); supplemented with 10% FBS (Gibco, Thermo Fisher Scientific), 1x penicillin/streptomycin (Gibco, Thermo Fisher Scientific), 0,5 ug/mL cyclosporine A (Sigma) for infection with the EBV supernatant (the marmoset cell line, B95-8 supernatant). Finally, all cells were cultured at 37°C, under an atmosphere containing 5% CO_2. EBV-transformed B-cell lines (EBV-B) and PBMCs were cryopreserved for later use.

Human Neutrophils purification

Cells were obtained from peripheral venous blood taken in EDTA tubes. Neutrophils were purified by density gradient by means of the commercial kit (Polymorphprep ™ AXIS-SHIELD, Norway). For this procedure, 4 mL of the Polymorphprep were added in a 15 mL tube, then 4 mL of blood was slowly added through the wall of the tube in a 1: 1 ratio. The mixture was centrifuged at 500g for 40 min at room temperature, with brake and acceleration of 1. Subsequently, the neutrophil layer was removed according to the manufacturer's instructions and taken to a fresh 15 ml tube. Cells were washed with HBSS buffer at 400g for 10 min. The supernatant was then discarded and 1 mL of RPMI was added. From this solution the cellular viability was determined to perform the different procedures

DNA and RNA extraction

Human genomic DNA (gDNA) was isolated from 1 mL whole blood or cell lines. The Red blood cells were lysed in extraction buffer (10 mM Tris, pH 7.4, 0.1 M EDTA, 0.5% SDS, and 10 mg/mL proteinase K) and centrifuged 3200rpm/15min/4ºC by twice. To the white pellet, we added 1 mL of cell lysis solution (QIAGEN) and incubated at 37°C. gDNA was isolated by phenol/chloroform extraction, precipitated in ethanol, and suspended in 10 mM Tris, pH 7.4; 1 mM EDTA (Puregene, Gentra Systems).

RNA was isolated from EBV-B cell lines using the TRIzol™ Reagent (Thermo Fisher Scientific), after chloroform was added and precipitated the RNA from the aqueous layer with isopropanol. Reverse transcriptase PCR (RT-PCR) was performed using the SuperScript III first-strand synthesis system with oligo-dT primers according to the manufacturer's protocol (Thermo Fisher Scientific). The cDNA was stored at −20°C.

Whole Exome Sequence (WES) and bioinformatic analysis

Genomic DNA was extracted from whole blood using the DNA Purification Kit (Puregene, Gentra Systems) or QIAmp DNA mini kit (QIAGEN). DNA was used in whole exome sequencing (WES). Briefly, Exome capture was performed with the Agilent SureSelect V4/V5 (Agilent Technologies). Paired-end sequencing was performed on a Hiseq4000 platform (Illumina) generating 100-base reads. The reads were aligned to the reference human genome GRCh37/Hg19 using the Burrows-Wheeler Alignment tool (BWA v 0.7.12-r1039)(164). Then, the .sam file generated was converted to a .bam file using SAMtools view (165). Duplicate reads were marked and mate-pair fixed with Picard tools (v1.119) (http://picard.sourceforge.net). Then, Indel realignment and base recalibration calls were made with GATK (Genome Analysis Toolkit, Version 3.5)(166) to improve the base qualities

to obtain more accurate quality scores. Then, SAMtools mpileup and BCFtools (165) were used to identify SNVs and Indels. Annotations of variants were made using web wANNOVAR (http://wannovar.usc.edu/).

Approaches to select candidate

To identify the single gene inborn errors underlying molecular defects we performed two strategies, a hypothesis based candidate gene approach and a hypothesis-generating genome-wide strategy (167).

Hypothesis based candidate-gene approach: using this approach, we tested the hypothesis that the proteins related with the immune response to fungus are affected in some patients with intrinsic and innate immunity defects. This approach is based on human data obtained from patient's samples (experiments *in vitro* and *in vivo*) as well as data from animal models to probe new defects on genes previously described as PID causing-gene, with emphasis on those involved mainly in the Th17 related pathway (*CARD9, IL17RA, IL17RC, IL17F, AD-STAT1, ACT1*), and the IL-12/IFNγ circuit (*IL12B, IL12RB1, IFNGR1, IFNGR2, CYBB, ISG15, STAT1, NEMO, IRF8*).

Hypothesis-generating, genome-wide approach based on whole exome sequencing:

Whole exome sequencing was performed for the discovery of known or unknown genes involved in susceptibility to invasive fungal infections. This approach generates massive amounts of parallel data, which must be analyzed through specific criteria and filters in order

to identify the candidate genes; in this case, related with defects in intrinsic and innate immunity.

Filtering and selecting appropriate variants

By WES, we identify between 50,000 and 400,000 high-quality variants. To select candidate variants for our study, we prioritized variants in disease-causing genes by applying a system of criteria both at the variant level and the gene level.

At the variant level criteria:

- **Frequency:** A variant with allele frequency (AF) below 1% (<1%) was considered a rare variation, while a variant with AF >1% was considered a common allele. The minimal allele frequency was determined with the use of different data bases, including: 1000 genomes (http://www.internationalgenome.org), Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org), Human Gene Mutation Data Bases (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php).
- **Potential functional effect:** To estimate the potential functional effect several *in silico* predictors were used, including the Polymorphism Phenotyping v2 (PolyPhen 2), Sorting Intolerant from Tolerant (SIFT), Combinated annotation-dependent depletion (CADD score) and MSC (Mutation Significance Cutoff) (168-172).

At the gene level:

- **Gene function:** The gene encodes a protein that is involved in a pathway relevant to the phenotype. We used bioinformatic tools like the Human Connectome Server (http://hgc.rockefeller.edu) which calculates the biologic distance between proteins

according to relevant functions and features (173).

Gene expression: First, we must know where are the gene expressed, cell type or tissue of relevance to the phenotype. We evaluated the expression pattern of every single gene in multiple online software including: The Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov), the Human Gene Database (Genecards) (http://www.genecards.org). Alamut Visual Software (Interactive-Biosoftware) was used to analyze the mutations and as a link to multiple data bases and visualize the WES results (9).

- **Strong purifying selection:** the relevance of a gene to human disease depends on the knowledge from genetics population. The gene damage index (GDI) (http://lab.rockefeller.edu/casanova/GDI) is a tool to correlate the evolution pressure, complexity protein, molecular weight and number of protein paralogues. It is an efficient method for filtering out false-positive variants (168, 174)**.**

Experimental validation of the genetic findings

In the validation of WES findings, functional studies are necessary to correlated the new identified genotype with the clinical phenotype (175). Experimental validation must prove that the variant or 2 variants (in cases of compound heterozygous) destroys, affects or alters the expression and / or function of the gene product (9, 175). All mutations in the selected candidate genes were characterized at the molecular level by testing the function of the mutated allele (null, hypomorphic, hypermorphic). RT-PCR, FACS or western blotting were used as a first step in the *in vitro* functional characterization, where we assessed the

expression at both, the transcriptional and translational levels of the variant in different cell types from the patient. Once new genes were identified, we collaborated with experienced researchers working in the specific gene identified. Then, depending on the gene and the function of the protein, we developed various functional tests.

Sanger sequencing

Genomic DNA samples of patients and their family members were used as a template for PCR amplification using site-specific oligonucleotides to target 300-600 bp regions encompassing the mutation. PCR was performed from each set of primers using Invitrogen ® Taq polymerase. Amplicons were sequenced using BigDye terminator technology with a Genetic Analyzer Sequencer 3500XL (Applied Biosystem ®). Subsequently, the data was collected and aligned with the reference sequence of the NCBI data bank, using the software Snapgene (GSL Biotech LLC) and four picks software (Nucleobytes B.V. Gerberastraat 117 1431 RA Aalsmeer, The Netherlands. KvK Amsterdam). Once the mutation was confirmed in the patients, we stablished the complete genotypification of the family and design the pedigree, showing allele segregation to confirm the AR or AD model.

Immunoblot Analysis of Protein Expression

Protein extraction from PBMC, human neutrophils (PMNs) and EBV-B cells was performed using a mix of RIPA cell lysis buffer 10x (#9803, Cell Signaling Technology), supplemented with Protease Cocktail inhibitors (M222, AMRESCO) and Phenyl Methyl Sulfonyl Fluoride (P-7626, Sigma). In 200 uL of lysis buffer we added 2 uL of PMSF and 2 uL of cocktail inhibitors. Then we added 50 uL/ 5 x 10^6 cells of the mixture (buffer + inhibitors) in a 1.5 mL vial. The vials were kept on ice for 30 minutes and vortexed every 5 minutes for 20

seconds, until 30 minutes have elapsed. Finally, protein concentration was measure using Bradford protein assay (500-0205, Biorad).

Protein extract (30 µg) was loaded into Laemmli buffer (Fermentas #R0891) and separated by electrophoresis (10% SDS PAGE, 100v/90minutes), the gel was transferred into a PVDF (Millipore Immobilon-P Membrane, PVDF, 0.45 µm) membrane using standard migration (100v/60 minutes) and then the membrane was blocked during 2 hours with 5% Milk–TBST buffer. After blocking, the membrane was washed three times with TBST for 5 minutes and incubated with primary antibody overnight. In following day membranes were washed three times with TBST and incubated with secondary antibody and probed as required with the following: rabbit anti-CARD9 protein (Protein Tech Polyclonal 10669-1-AP), rabbit anti-GAPDH (Santa Cruz sc-25778), rabbit anti-p40phox (Upstate-Merck 07-503), rabbit anti-p67^{phox} (Merck 07-002) anti-gp91^{phox} (Santa Cruz sc-130543), rabbit anti-p47phox (Merck 07-001), mouse anti-p22^{phox} (Santa Cruz sc-130550), rabbit anti-GAPDH (Santa Cruz sc-25778), rabbit anti-GAPDH (Sigma G9545); Secondary Ab Goat Anti-Rabbit IgG HRP (1706515) and secondary Ab Goat Anti-Rabbit (Sigma A9169). Final results were revealed by chemiluminescence on X-ray film (Santa Cruz) using the kit from Clarity™ Western ECL Substrate (Biorad 1705060).

Flow cytometry Immunotyping

To assess the expression of the subunits of NADPH oxidase, EBV-B cell lines were washed in phosphate-buffered saline (PBS) and dispensed into 96-wells plate for labeling. Cells were permeabilized with Cytofix-Cytoperm (BD 554722) for 20minutes at RTº, after first antibodies were used: mouse IgG2a anti-p22^{phox} (Santa Cruz sc-130550), mouse IgG1 anti-p67^{phox} (Santa Cruz sc-374510), mouse IgG1 anti-gp91^{phox} (Santa Cruz sc-130543,) mouse

IgG1 anti-p47^{phox} (Santa Cruz sc-17845). Secondary Ab goat anti-mouse (Invitrogen A11029). Data was analyzed with a FACSCANTO II Flow Cytometer (BD, Franklin Lakes, NJ) in Flowjo X (TreeStar Inc, Ashland).

Determination of phagocyte NADPH oxidase activity

Amplex Red. H_2O_2 was evaluated the release using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (ThermoFisher Scientific, USA) in EBV-B cells from healthy controls and patients according to the instructions of the kit manufacturer. Cells were washed in Krebs-Ringer's phosphate solution and then left inactivated or activated by incubation for 30 minutes with PMA (400ng/mL) at 37 °C. The H_2O_2 released was quantified using a Victor X4 PerkinElmer (176).

Dihydrorhodamine 123 assay (DHR). Peripheral blood leukocytes (PBL) from patients and controls were incubated for 5 minutes at 37 °C with Dihydrorhodamine 123 (Sigma-Aldrich) in the presence of catalase (1,300 IU/mL; Sigma-Aldrich), and next was the activation for 30 minutes with PMA (400 ng/mL) or 20 minutes with formyl-Met-Leu-Phe (80 μg/mL) (Sigma-Aldrich). Events were collected on a FACSCanto II flow cytometer (Becton Dickinson) and analyzed using the Flowjo Software (Treestar Inc) (176).

Luminol assay. Cells NADPH oxidase activity was assessed using luminol enhanced chemiluminescence was used in PMNs and EBV-B cells. In PMNS, $2x10^5$ cells were suspended in PBS with glucose (PBS plus 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM dextrose), Luminol (50 μM), plated into each well of a 96-wells plate in the presence or absence of

SOD (75 ug/mL). Cells were preheated at 37°C for 10 minutes before addition of PMA (400 ng/mL), heat killed *Candida albicans* or $1x10^6$ heat killed *Histoplasma capsulatum*. In EBV-B, we use Superoxide Anion Assay Kit (Sigma,CS1000). Briefly, $7x10^5$ cells were suspended in PBS with glucose (PBS plus 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM dextrose) and Luminol, plated into each well of a 96-wells plate in the presence or absence of SOD (4 units/uL). Cells were preheated at 37°C for 10 minutes before addition of PMA (400 ng/mL). Relative light unit (RLUs) were monitored at 60- to 90-seconds intervals for 30 or 50 minutes by the long kinetic module in an Lmax microplate luminometer (Molecular Devices). Integrated RLU values were calculated by SoftMax software (Molecular Devices), and background chemiluminescence (measured in wells that included all reagents but no cells) was subtracted to report total ROS production (17).

Studies performed primarily by the student

Patients and ethical issues: patient's recruitment, review medical charts, build familial pedigrees, take blood samples, were performed by the student.

Cell purification and culture: was performed by the student

Human Neutrophils purification: was performed by the student

DNA and RNA extraction: DNA Extraction was performed by the student in Medellin-Colombia, RNA extraction was performed together with Alejandro Nieto help in the laboratory of Human Genetics of Human Infections diseases in Paris, during Carlos internship on September 26 – December 7 of 2016.

Whole Exome Sequence (WES) and bioinformatics analysis: DNA was extracted by the student, after we sent 3ug to MACROGENE company in Korea, they performed the Exome

capture with the Agilent SureSelect V4/V5 (Agilent Technologies) and paired-end sequencing on a Hiseq4000 platform (Illumina) generating 100-base read. After that we get Raw fastQ files and we applied a pipeline from our University with help from Centro Nacional de Secuenciación (Raw FastQ Files, FatXToolKit, Map to HG19, Sort and Mark Duplicates, Indel realignment, Base Quality Recalibration, variant calling (GATK, SNP – INDEL database)) After that, Student performed a variant filtering by WebAnnoVar in order to get genes of interest.

Approaches to select candidate: was performed by the student

Filtering and selecting appropriate variants: was performed by the student

Sanger sequencing: P1 and P3, were performed by the student in Medellín –Colombia, P2 and P5 were performed by the student in the laboratory of Human Gentics of Human Infections diseases in Paris, during Carlos internship on September 26 – December 7th of 2016.

Immunoblot Analysis of Protein Expression: P1 Western blot assays were performed by the students in Medellín – Colombia and western blot for P2 were performed together by the student and Alejandro Nieto help in the laboratory of Human Genetics of Human Infections diseases in Paris, during Carlos internship on September 26 – December 7th of 2016.

Flow cytometry Immunotyping: NADPH subunits expression were performed together by the student and Alejandro Nieto help in the laboratory of Human Genetics of Human Infections diseases in Paris, during Carlos internship on September 26 – December 7th of 2016.

Dihydrorhodamine 123 assay (DHR): were performed together by the student and Jesús Alvarez help in Medelli-Colombia

Luminol assay: were performed by the student

RESULTS

In this work, we studied the genetic susceptibility of five Colombian patients to a severe fungal infection. To do that, we evaluated the clinical information, cellular phenotype, gDNA, mRNA and protein levels of these patients and their families. Patient 1 (P1) presented a *Corynespora cassiicola* infection, patient 2 (P2) a disseminate Histoplasmosis and patients 3, 4 and 5 (P3, P4, P5) presented a juvenile disseminated Paracoccidioidomycosis (**Table 4**).

PATIENT 1

Clinical history

Patient 1 (P1) is a female born in 2006 in the rural area of El Difícil (Magdalena, north of Colombia, South America). She was born from the third uncomplicated pregnancy of parents with apparently no consanguineous parents. She has 3 three younger healthy brothers. She shared the living space with domestic animals and her father is a farmer. She apparently received all required vaccination by the Colombian Official Immunization Program (PAI). At the age of 9-years-old she consulted with epistaxis and nasal nodule of three years duration that had progressed to perforate the nasal bridge, hard palate and cheek (**figure 5A and 5B*).*** A biopsy was performed where mycotic granulomas were identified. Initially, fungal cultures were reported as *Mucor*, so she was treated with Amphotericin B, but subsequently this was changed to Voriconazole (10mg/kg/day) because *Aspergillu*s infection was suspected. The patient showed clinical improvement after 10 weeks of treatment, and only the nasal septal and palatal perforation remained. Eighteen months later, she returns for medical attention due to progression of the skin lesion, with more facial involvement extending to the left eye.

New samples were obtained which were sent to a specialized mycology laboratory, where pan-fungal polymerase chain reaction (PCR) was performed, identifying the fungal isolate as *Corynespora cassiicola*. After that, she was hospitalized and antifungal therapy was initiated with Liposomal Amphotericin B (5mg/k/day) and Posaconazole for 1 month. Following clinical improvement, the patient was discharged with oral Posaconazole and Terbinafine.

Although sustained clinical improvement was reported, the treatment was abandoned due to social barriers with worsening and progression of the facial lesions. Six months later she consulted, at this time, she was hospitalized in a tertiary hospital in Medellin (Colombia) and received multispecialty care. Upon admission, a large, indurated, foul smelling and verrucous ulcerated lesion was observed on the left side of the face, with extensive necrosis and crusting. The lesion extended to the left ear and beneath the mouth, it also presented an almost complete loss of tissue of the dorsum of the nose and complete perforation of the nasal bridge and palate (**figure 5C**). MRI imaging of the face, excluded cerebral or ophthalmic involvement, but confirmed extensive involvement of the cranial bones (**figure 5D**). Both acute necrosis and chronic granulomatous inflammation were observed in tissue biopsies, with numerous mycotic structures showing angioinvasion (**figures 6A and 6B**).

No isolates were recovered from aerobic or anaerobic bacterial cultures, but numerous hairy, gray colonies were obtained in fungal cultures. Pan-fungal PCR identified the isolated as *C. cassiicola* (**figure 6C**) with antifungal susceptibility testing that confirmed susceptibility to Posaconazole, Amphotericin B and Voriconazole. Treatment was again initiated with lyposomal Amphotericin B (5 mg/kg/day) and Posaconazole (20 mg/kg/day). Caspofungin (50 mg/m2/day) was added due to inadequate clinical response after one month of antifungal therapy.

At this point, the patient continued to deteriorate leading to the clinical suspicion of an underlying Primary Immunodeficiency upon assessment by the pediatric infectious diseases specialist and PID experts.

Complete white blood cell counts (WBC) during multiple hospitalization showed normal total lymphocyte counts (TLC), monocytes and platelets (**table 1-S**). Serology and viral load for HIV were negative. Serum immunoglobulins were within normal values for reference. although IgG was close to the upper limit of reference while IgE was moderately elevated in comparison to reference thresholds (**table 2-S**).

Immunophenotyping of the patient revealed normal numbers and compartmentalization of cell subsets, NK, B cells, and monocytes (**table 3-S).** Extended B cell analysis by FACS showed a B subset decompensation with increase of Naïve B cells ($IgD^{+}/CD27^{-}$) and decrease of marginal zone ($IgD^{+}/CD27^{+}$), switched memory ($IgD^{-}/CD27^{+}$) and transitional ($CD24^{++}/CD38^{++}$) B cells **(table 4-S).** Circulating T extended subsets cells were normal, except by the T helper lymphocytes effectors and terminally differentiated ($CD4^{+}CCR7^{-}$ $CD45RA^{+}$ subsets) which were elevated in percentage, as well as, in total numbers **(table 5-S).**

After 6 months of antifungal treatment and aggressive wound care, the patient had adequate clinical response with almost complete healing of the facial lesion. She was discharged with oral Posaconazole and Terbinafine, but one month later, she was readmitted because of progression of the facial lesion. Liposomal Amphotericin B was restarted and a multidisciplinary medical Staff concluded that due to the extensive involvement of the patient, a surgical approach was not feasible to control fungal spread. The patient was discharged with oral antifungal therapy.

Unfortunately, the patient has had multiple readmissions to her local hospital because of progression of her fungal infection, for which she has received rescue therapy with Amphotericin B, along with recombinant human granulocyte colony-stimulating factor (G-CSF) (10 ug/kg/day) with no clinical response.

Genetic analysis of *CARD9*

According to the hypothesis-generating, genome-wide approach and the clinical phenotype of patient 1, we performed whole exome sequencing (WES) and variants were confirmed by Sanger sequencing on genomic DNA (**figure 7A**). We identified 2 different mutant *CARD9* alleles in the patient's gDNA showing compound heterozygosity (**table 5**). Based on the cDNA sequence, the first variant was a deletion of ACGAGTG nucleotides (c.23_29del) in exon 2. This deletion was predicted to causes a frame shift mutation by a change of Aspartic acid 8 (Asp8) to Alanine creating a new premature stop 10 codons later (p. Asp8Alafs*10). The frameshift predicts a CARD9 protein with a theoretical molecular weight of 1.7 kDa, a truncated CARD domain and absence of both *coiled coil* domains (**figure 7C**). Testing was performed in the family members revealing that any other members in the family were heterozygous for this allele (**figure 7B**).

The second *CARD9* allele was a substitution (c.C865T) in exon 6 that predicts a nonsense mutation which replaced Glutamine in position 289 with a premature stop codon (p.Q289X). This mutation also predicted a CARD9 protein with a theoretical molecular weight of 33.6 kDa, a truncated protein with an absent of the second coiled coil domain (**figure 7C**). The patient, her mother, and two younger brothers were heterozygous for this allele (**figure 7B**). We could not sequence the gDNA of her father. With these findings and the known

association between the *CARD9* gene and genetic susceptibility to fungal diseases we performed *in silico* studies to test the impact of these variants in this candidate gene.

CARD9 in silico studies

The effect of the variants identified in the CARD9 gene was assessed by *in-silico* studies of their potential biological impact at the protein level. These *CARD9* variants were predicted to have both a damaging and deleterious effect by PolyPhen (170, 171) and SIFT scores (172), respectively (**table 5**). The CADD score (168) of the c.C865T variant identified in *CARD9* (score= 35) was well above the mutation cutoff (MSC) of 0.699 (**table 5 and figure 8**). Also, this substitution was previously reported in the literature as homozygous (c.C865T / c.C865T) in Algerian and Tunisian patients with deep dermatophytosis caused by *T. violaceum* and *T. rubrum* (86).

Interestingly, based on frequency databases (HGMD, Ensembl, NHLBI, Exome Sequencing Project, 1000 Genomes Project, and ExAC), the c.23_29del variant is not present in healthy individuals. Furthermore, its absence in other pathogenic data bases indicated that this variant has not been previously reported in other patients; it could then possibly be private to this kindred since to the best of our knowledge, it is likely a novel causing allele associated with autosomal recessive AR phaeohyphomycosis.

Expression of the CARD9 protein

In order to evaluate the protein level effect of the deletion (c.23_29del) and substitution (c.C865T) variants, western blot analysis of *CARD9* was performed from protein extracts from Peripheral Blood Mononuclear Cells (PBMCs), reveling the absence of CARD9 protein

in the patient when compared to control (**figure 7D**). This suggests us that mutations in the patient may be responsible of the susceptibility to invasive fungal infection.

PATIENT 2

Clinical history

Patient 2 (P2) is a 7-year-old boy, born in 2010 from apparently non-consanguineous Colombian parents living in a rural area of Colombia (San José de Apartadó, Antioquia). His father is a farmer and the family live in a wooden house and share the habitat with wild and domestic animals such us pigeons, bats, hens, cows, and dogs. He has four healthy siblings, two sisters and two brothers. There is family history of miscarriage and in early childhood deaths with one infant who died at 14-months-old, twin brothers who died during childbirth and a spontaneous abortion at 4 months of pregnancy. The cause of these events where reported as unknown. The patient received all the vaccines according to the Colombian Official Immunization Program (PAI), including bacilli Calmette-Guérin (BCG) at birth, without complications.

Symptoms began at the age of 2 years when he consulted at his local clinical center due to recurrent fever, chronic diarrhea and dysentery-like symptoms for the past 20 days. Disseminated tuberculosis (TB) was considered and the patient was referred to a high complexity hospital where he was found malnourished and presenting cervical and inguinal lymphadenopathy, oral candidiasis, hepatosplenomegaly and severe lung involvement. Complete Blood Cell (CBC) counts at admission showed a pancytopenia (Platelets < 64000 mm^3 and hemoglobin < 8.4 g/dL) and mild leukopenia (Leucocytes < 6000 cells/uL) (**table 6-S**) according with the age-matched reference values (177-179). Hypoalbuminaemia (1.9

g/dL; ref: 3.8-5.4 g/dL), hypocalcaemia (7.1 mg/dL; ref: 8.4-10.2 mg/dL), hypophosphatemia (2.7 mmol/L; ref: 3.5-5.1 mmol/L) and high serum C-reactive protein (CRP) (16.44 mg/dL; ref: 0.01-0.82 mg/dL) were also noticed. Serum gamma-glutamyl transferase (GGT), AST and ALT as well as serology and viral load for HIV and PPD skin test were negative. Coprological examination showed abundant leucocytes, erythrocytes and pseudomicelles, while negative for *C. difficile* A and B toxins, *Cryptosporidium spp*, *Salmonella spp*, *Shigella spp*, rotavirus, adenovirus and other intestinal parasites. A cervical lymph node biopsy revealed abundant necrotizing granulomatous chronic inflammation with phagocytic accumulations with intraphagocytic round structures and multinucleated giant cells. A bone marrow biopsy study showed normal cellularity with maturing granulopoiesis and erythropoiesis, and adequate megakaryocytopoiesis. Aggregations of histiocytes and microgranulomas formation were also observed.

In both biopsies, Gomori's Methenamine Silver (GMS) and Periodic Acid–Schiff (PAS) stain were positive for fungal structures. *Histoplasma capsulatum* was identified in multiple samples and tests including: PCR from a cervical lymph node biopsy, serology, urine antigen, complement fixation for fungi and blood, also in cultures from lymph node and bone marrow samples. Three gastric aspirates and mycobacterial cultures were negative for acid-fast bacilli (AFB) mycobacterium. Culture of a transtracheal aspiration sample was positive for extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumonie.*

The patient had an appropriate response to the treatment stablished with liposomal amphotericin B, itraconazole, piperacillin/tazobactam, fluconazole, metronidazole, meropenem, and linezolid with appropriate response. Unfortunately, twenty days later, a gastro intestinal tube (GIT) infection was confirmed with a culture and isolation of *Klebsiella*

pneumoniae carbapenemases (KPCs) and was treated successfully with clindamycin, vancomycin and ciprofloxacin.

The finding of disseminated histoplasmosis and bacterial infection in patient prompted further investigation for an underlying PID. At the age of 3-years-old, flow cytometry of lymphocyte populations was performed in peripheral blood lymphocytes (PBL) showing that his complete blood count and differential T-, B-, and NK cell populations were normal (**table 7-S**). The records of serum immunoglobulins were evaluated reveling high IgE, normal IgG and IgA, and mildly elevated IgM (**table 8-S**). At the age of 4-years-old, a phorbol ester–stimulated neutrophil oxidant production detected by dihydrorhodamine 123 (DHR123) assay did not show a partial reduction of intracellular oxidant production after PMA stimuli (**figure 9**).

Genetic analysis of *NCF4*

The *NCF4* gene is a member of the phagocyte NADPH oxidase system and is associated to a form of Chronic Granulomatous Disease (CGD)(17). According to genome wide approach and the clinical phenotype of the patient we performed whole exome sequencing (WES) and variants were confirmed by Sanger sequencing on genomic DNA. We identified a homozygous transition c.C172T in exon 3 of the *NCF4* gene that predicts a missense mutation that replacing Arginine by Cysteine in position 58 (R58) (p.R58C) at the PX domain of the $p40^{phox}$ protein (**figure 10A, C and E**). The patient's parents, and one sister were found heterozygous for this allele. Also, two brothers and one sister were homozygous for this allele (**figure 10B**). No mutations in X-Linked *CYBB,* or in autosomal *NCF1*, *NCF2*, or *CYBA* genes related with CGD were found.

***NCF4 in-silico* studies**

The *in-silico* evaluation of the potential biological impact of the c.C172T variant identified in *NCF4*, revealed both a damaging and deleterious effect by PolyPhen (170, 171) and SIFT scores (172), respectively **(table 5).** The CADD score (168) of the variant identified in *NCF4* (score= 35) was well above the corresponding *NCF4* specific mutation significance cutoff (MSC) of 25 (**table 5, figure 10 D**)**.** On the other hand, non-homozygous individuals with the variant c.C172T were not previously reported in any of the public databases (HGMD, Ensembl, NHLBI, Exome Sequencing Project, 1000 Genomes Project, and ExAC), suggesting that this variant is extremely rare, possibly private to these kindred. Arg58 amino acid residue of p40^{phox} shown high evolutionary conservative among orthologues species (**figure 10E**).

NCF4 mRNA and p40^{phox} protein expression

In order to evaluate the effect of the c.C172T variant at the level of *NCF4* mRNA and p40^{phox} protein we performed qPCR, immunoblot and FACS. Both *NCF4* mRNA (evaluated by quantitative PCR) **(figure 11)** and p40^{phox} protein expression (evaluated by Western blotting and FACS) were decreased in the patient's Epstein-Barr Virus (EBV)-immortalized B cells (**figure 12 A, B, C).** We also observed a reduction in the p67^{phox} protein expression in the patient compared with the control. All these data suggested that the *NCF4* mutation is likely to impair expression at the mRNA and protein levels.

EBV-B cells and neutrophil studies

To further characterize the p40^{phox} defect and the effect in NADPH oxidase caused by the c.C172T variant in this patient, we assayed EBV-B cells and neutrophil oxidant production in response to phorbol ester or formyl-methionyl-leucyl-phenylalanine (fMLF). As shown in

figure 12, NADPH oxidase activity elicited by PMA in EBV-B cells was significantly impaired compared with controls **(figure 13A and B).** Also, intracellular oxidant production during neutrophil phagocytosis of serum-opsonized *C. albicans* and *H. capsulatum* was markedly reduced, although PMA stimulated superoxide release was normal **(figure 14A, B y C).** In addition, we assessed functional respiratory burst activity by flow cytometry with dihydrorhodamine 123 to measure intracellular H_2O_2 production. PMNs from the patient, his father (heterozygous) and brother (homozygous) were stimulated with PMA and found quantitatively normal. However, the PMNs from the patient and his homozygous brother for the mutation c.C172T did not respond normally to activation involving priming with low concentrations of tumor necrosis factor (TNF) followed by stimulation with fMLF **(figure 15).**

These genetic and cellular findings strongly support the pathogenicity of the homozygous transition c.C172T in exon 3 of the *NCF4* gene. To the best of our knowledge, this is a novel causing allele in *NCF4* associated with autosomal recessive CGD in a boy who presented disseminated histoplasmosis.

PATIENTS P3, P4 AND P5

Clinical history

Patient 3 (P3) is a female born in an urban area of Colombia (Neiva, Huila). She comes from consanguineous parents and has one brother. She is negative for HIV test. The patient received all the vaccines according to the Colombian Official Immunization Program (PAI), including bacilli Calmette-Guérin (BCG) vaccine at birth, without complications. The patient had a history of recurrent infections since childhood and consulted at 14-years-old presenting

vomit, acute diarrhea, weight loss, colitis and granulomatous changes in the gastrointestinal tract. PCM was diagnosed by microorganism isolation in a colon biopsy and resection of a small bowel segment was required due to intestinal obstruction. At the age of 16 years she developed again multiple adenopaties; non-suppurative abscess on the face, cervical, axillary and inguinal lymph nodes were noted and the microscopy analysis revealed *Paracoccidioides.* At the age of 20-years-old she was evaluated for immunodiffusion and complement fixation founding non-reactive for histoplasmin, paracoccidin, aspergilin and antigen of *H. capsulatum*. A recurrent onychomycosis due to *Candida albicans.*

Patient 4 (P4) is a 12-year-old female from an urban area of Colombian (Bogota, Cundinamarca) previously reported in the literature (180). She was born from parents with not history of consanguinity and has 1 healthy brother. The patient is HIV negative and received all the vaccines according to the Colombian Official Immunization Program (PAI), including bacilli Calmette-Guérin (BCG) vaccine at birth, without complications. She consulted for fever, weight loss, hepatosplenomegaly and generalized lymph nodes over the last 4 weeks (**figure 16A**). Upon admission, the fever persisted and she started developing sking lesions in the extremities and abdomen. A skin biopsy was reported as chronic granulomatous inflammation with the presence of mycotic structures; furthermore, visualization of typical fungal structures were noted in a lymph node biopsy (**Figure 16B and 16C**). Confirmatory molecular studies performed. DNA was extracted from lymph node tissue, using a pan-fungal PCR and subsequently, the amplified fungus was sequenced, compared to the data banks and identified as *P. brasiliensis*.

Patient 5 (P5) is a 11-years-old male born in 2005, coming from a rural area of the central west of Colombia (Yocotó, Valle del Cauca). The patient is HIV negative. His parents have history of consanguinity and presented multiple cervical adenopaties associated with fever

and discreet hepatomegaly, including a 7 cm neck mass. Inguinal and abdominal lymphadenopathy were also noted. A biopsy from one of the cervical lesions reported *P. brasiliensis*.

Genetic analysis

Whole exome sequencing (WES) analysis was performed in P3, P4 and P5 according to the clinical phenotype of Juvenile PCM. With the massive data obtained, we applied different system of criteria in order to select several candidate gene variants as possible disease-causing genes, however these variants already continue under study.

Patient 3 P3 had a homozygous transition variant (c.T958C) in exon 11 of the *IL17RA* gene that predicts, a missense mutation that replaced tryptophan in position 320 (W320) with an Arginine (W320R) 57 amino acids residues before SEFIR domain of IL-17RA protein (**table 5).** This variant was confirmed by Sanger sequencing on genomic DNA **(figure 17A**). In the family members only the patient was homozygous for this allele, while parents were heterozygous (**figure 17B**)**.** *in-silico* analysis indicated that this variant is predicted to cause a damaging effect in the protein by PolyPhen and a deleterious effect in the protein by SIFT2. Also, CADD score was high comparable with the MSC CADD (**figure 17C** and **table 5**).

We found in p**atient 4 P4** two interesting candidate variants. First, was a heterozygous variant in *IL18R1* (c.1514C>A, p.Ser505Tyr), and two heterozygous variants in *NLRP2* (c.938C>T, p.Pro313Leu and c.1664A>G, p.Tyr555Cys). *In silico* analysis predicted a damaging effect (by PolyPhen) and a deleterious effect (by SIFT) for the variant identified in *IL18R1* and one of the variants in *NLRP2* (c.938C>T, p.Pro313Leu). Also, the CADD score vs the MSC CADD showed a high impact of variation. The second heterozygous

variant in *NLRP2* (c.1664A>G, p.Tyr555Cys) was predicted to be tolerant by SIFT and damaging by PolyPhen. The CADD score vs the MSC CADD did not showed a high impact of variation (**table 5 and figure 1S**).

All data was confirmed by Sanger sequencing on genomic DNA (**figure 18A**) and only the patient and his father where heterozygous for variant p.Ser505Tyr in IL-18R1 and p.Pro313Leu in the *NLRP2* gene (**figure 18B and 18C**).

P**atient 5 P5** (**figure 19**) after WES analysis, we found heterozygous variants in four possible candidate genes (*PIK3CA, CCR5, RAG2* and *ACIN1)*. All these candidate variants presented damaging prognostic, predicted by CADD score vs MSC CADD analysis (**Table 6**). The variant in the *PIK3CA* gene is a nonsense mutation producing a stop codon at the Arg612 residue (c.1834C>T ; p.Arg612*), this variant presented a very high CADD score (**figure 2S).**

The previous results in patients P3, P4 and P5 are preliminary, therefore more studies are being carried out in order to select possible disease causing gene in these patients.

DISCUSSION

In the present study, we presented a Colombian child with an autosomal recessive *CARD9* deficiency as a potential genetic cause of deep and severe phaeohyphomycosis caused by *C. cassiicola*. Also we presented a patient with disseminated Histoplasmosis with autosomal recessive *NCF4* variant. Finally, we also obtain advance and preliminary result with candidate genes in patients with Juvenile PCM.

CARD9 is an intracellular adaptor protein in the signaling pathway downstream from dectin-1, dectin-2 and macrophage- inducible C-type lectin primarily expressed in myeloid-lineage cells. Its function involves the pathways of ITAM-tyrosine kinase, Toll-like receptors (TLR) and NOD2 signaling pathways dependent or independent of NF-kappa-B transcription factor (181, 182). This protein plays a critical role in the host defense against fungal pathogens both in mice (183-185) and humans (186, 187). CARD9 knockout (CARD9 KO) mice are profoundly susceptible to *Phialophora verrucosa* infection compared to wild-type mice. The inability of these mice to control *P. verrucosa* infection was associated with lack of Th17 differentiation and reduction of TNFα, interleukin IL-1β, IL-6, and IL-17A levels in footpad homogenates (188). Other researchers have showed that CARD9 KO mice are also highly susceptible to *Candida albicans, Mycobacterium tuberculosis, Cryptococcus neoformans* and *Listeria monocytogenes* (183, 189-191).

In humans, CARD9 deficiency is an autosomal recessive primary immunodeficiency caused by biallelic mutations in *CARD9* gene. Clinical phenotype associated are heterogeneous conferring persistent and severe fungal infection at different anatomical sites mainly present on the skin, mucosal surfaces, subcutaneous tissue, central nervous system and systemic

mycosis (158, 159, 192-194). Approximately sixteen human CARD9 mutations have now been identified and characterized in more than 50 patients around the world (84-86, 95, 155, 159, 162, 186, 187, 192, 193, 195-197). In general, these patients present with abroad spectrum of isolated fungal infections, including *Candida spp* (chronic mucocutaneous candidiasis, colitis, meningoencephalitis, vertebral osteomyelitis), *Aspergillus fumigatus* (extrapulmonary infection) as well as deep dermatophytosis caused by *Trycophyton violaceus*, *Trycophyton rubrum*, *Trycophyton mentagrophytes*; invasive aspergillosis and dematiaceous fungus causing phaeohyphomycosis (*Phialophora verrucosum, Exophiala spp* and *Corynespora cassiicola)*. The identified defects are nonsense and missense mutations affecting both domains of the CARD9 protein (CARD and Coiled-coil), have an autosomal recessive allele segregation (homozygous or compound heterozygous) and the majority are associated with a reduced number of circulating Th17 cells (159).

To our knowledge have been documented five confirmed *C. cassiicola* phaeohyphomycosis reported in humans, all of them adults (**table 3**) (87, 152-154, 198). The first report was a description of a foot mycetoma in a farmer from Eritrea (198). The second case was a 76-years-old male farmer from Japan without previously history of diabetes mellitus and ocular trauma from soil or plants, who developed keratomycosis by *C cassiicola* (154)**.** Third, described a 69-year-old Chinese female farmer with diabetes mellitus who developed subcutaneous infection in both hands (152). The 4th patient, was a previously healthy, 57-years-old Chinese farmer with a 2-month history of indurated plaques, nodules, erosions and ulcers on both legs caused by *C. cassiicola* (153). The last patient, was a 37-year-old Chinese woman, who presented, with a facial subcutaneous phaeohyphomycosis that was genetically associated with the CARD9 mutation c.191–192InsTGCT (p.L64fsX59)(87).

In contrast, our patient represents the first description of a young Colombian girl with juvenile phaeohyphomycosis due to *Corynespora cassiicola*, causing a severe and invading facial infection, that affected soft tissue and bone structures, associated with a *CARD9* compound heterozygous mutation for a frameshift mutation with premature stop codon (p.D7fs10X), and a nonsense mutation (p.Q289X)

In our case, the patient presented severe and invasive phaeohyphomycosis with a perforation of the nasal bridge, hard palate and cheek due to *Corynespora cassiicola* infection associated with *CARD9* compound heterozygous mutation affecting the expression of the CARD9 protein in PBMCs. Likewise, there are reports in the literature related with CARD9 mutations and dematiaceous fungi causing phaeohyphomycosis or systemic fungal diseases. The first report included two patients, a 8-year-old girl from a nonconsanguineous Angolan kindred, who developed disseminated *E. dermatitidis* disease and a 26 year-old woman from an Iranian consanguineous kindred, who developed disseminated *E. spinifera* disease. Patients was homozygous for loss-of-function mutations (R18W and E323del) affecting both the CARD and coiled-coil domain of CARD9 respectively (85). In 2014 Wang et al reported 4 Chinese patients with subcutaneous phaeohyphomycosis caused by *Phialophora verrucosa* with CARD9 mutations and Th17 cell deficiencies. Patients come from apparently non-consanguineous families. All patients presented persistent red plaques and nodules on their faces, with ulcerations and crusting. Three mutations of CARD9 were detected: one patient was found to be carrier of a compound heterozygous mutation (p.L64fsX59 and p.Q158X) affecting the CARD and coiled-coil domains, and three patients presented the homozygous frameshift mutation (p.D274fsX60) affecting coiled-coil domain. These CARD9 mutations impaired proinflamatory cytokines production (IL-6, TNF-alpha, IL-1beta, and IL-23p19) in

innate immune cells and the Th17 cells (CD4+/IL-17+) cells were strikingly decreased (155). Similarly, Yang et al in 2016 reported the first case of phaeohyphomycosis caused by *C. cassiicola* in a Chinese woman who at the age of 35 presented nail-sized erythematous plaques on both sides of the nose and face while the a CARD9 mutation (p.L64fsX59) affecting the CARD domain was identified (87).

Altogether, our data strongly suggest that the CARD9 mutation identified in our patient, is affecting the expression of CARD9 protein and consequently impaired immune response, therefore explaining the predisposition to *C. cassiicola* infection in this patient (184).

Our second patient has a form of autosomal recessive defect in the $p40^{phox}$ subunit of the NADPH oxidase. The patient presented at 2 years of age with disseminated *Histoplasma capsulatum* infection, oral candidiasis, respiratory and gastrointestinal *Klebsiella pneumonie* infection. Neutrophil NADPH oxidase assays showed a considerable defect in intracellular production of oxidants in response to fMLF, *Candida albicans* and *Histoplasma capsulatum*, while the PMA response was normal. NADPH oxidase assays in EBV B cells also showed a defect in production of oxidants in response to PMA. Genetic analysis identified a homozygous transition c.C172T in exon 3 of the *NCF4* gene that predicts a missense mutation p.R58C in the PtdIns(3)P-binding PX domain of $p40^{phox}$. The R58C mutation possible compromises PtdIns(3)P binding, when considering prior studies on the importance of R58 and the PX domain in human cells (31, 199-201) and mouse models (202-205).
In neutrophils isolated from $p40^{phox-/-}$ mice, the expression of $p67^{phox}$ is reduced by ~55% and oxidase responses to TNFα, immunoglobulin G latex beads, *Staphylococcus aureus*, formyl-methionyl-leucyl-phenylalanine, and zymosan were significantly reduced (206). The

defect in ROS production by p40$^{phox-/-}$ neutrophils in response to *S. aureus* resulted in a severe, CGD-like defect in the killing of this organism both *in vitro* and *in vivo* (206). Also, in mice carrying a heterozygous (p40$^{phoxR58A/-}$) or homozygous (*p40*$^{phoxR58A/R58}$) mutation in the residue Arg58 of the PX domain (which prevents high-affinity binding to PtdIns(3)P), neutrophils exhibit significantly reduced intracellular oxidase responses after phagocytosis of *Staphylococcus aureus* and *E. coli,* and were significantly compromised in their ability to kill *S. aureus in vivo* (204, 207). Recently, it was demonstrated an impaired *p40*$^{phoxR58A/R58}$ macrophages NADPH oxidase activity in response to particles and soluble ligands, including IgG-opsonized particles and a TLR2 agonist. Compared with wild-type (WT) mice, p40$^{phoxR58A/R58A}$ mice had elevated numbers of newly recruited neutrophils and monocytes in the peritoneal inflamed elicited by zymosan, monosodium urate (MSU) crystals, or sodium periodate, and as well as delayed resolution of inflammation (202). All these data in mice demonstrate the physiological importance of the interaction dependent on PtdIns3P-binding to p40^{phox} in both neutrophils and macrophages. Also, shows the importance of the residue Arg58 of the PX domain, which is conserved in humans and mice and surprisingly, was the same residue mutated in our patient described here with the R58C mutation.

In contrast to the other *phox* subunits, p40^{phox} is not necessary for a high-level of NADPH enzyme activity on the plasma membrane of phagocytes or in cell-free systems, but instead plays a specialized role in phagosomes via PtdIns(3)P signal (30). This role was first elucidated in studies using genetically engineered COS^{phox} cells (31, 199) and mouse models described above. In 2009, a CGD patient was described with autosomal recessive mutations in *NCF4*, who had selective and profound defects in neutrophil phagosome but not plasma membrane oxidant production (200). This patient was a compound heterozygote for

mutations leading to a premature stop codon (g.3957_3966dup; p.K52RfsX79) and a missense mutation predicting a R105Q substitution in the PX domain that ablated PtdIns(3)P binding (17, 30). The patient presented at 3 years of age with severe Crohn's disease-like inflammatory bowel disease, which also develops in 10 – 20% of CGD patients. His neutrophils show a normal extracellular release of superoxide elicited by phorbol ester (PMA) or formyl-methionyl-leucyl-phenylalanine (fMLF, better known as a fMLP), whereas, neutrophils showed a substantial defect in intracellular superoxide production during phagocytosis (17). Although no other cases of $p40^{phox}$ deficiency have been reported up to date, suggesting that plasma membrane and/or residual phagosomal ROS are sufficient for microbial killing, that study underlines the importance of $p40^{phox}$ in the differential regulation of NADPH oxidase (17, 30).

The patient described here, presented at 2 years of age with disseminated *Histoplasma capsulatum* infection, oral candidiasis, and respiratory and gastrointestinal *Klebsiella pneumonie* infection. Although bacterial *Klebsiella spp* and fungal *Candida albicans* infections are usually present in CGD patients (48-50), dimorphic fungi infections such as histoplasmosis have not been reported in CGD (48, 51). Moreover, a disseminated histoplasmosis is often associated with compromised host defenses due to acquired immunodeficiency syndromes such as HIV infection, leukemias, lymphomas and patients under immunosuppression after transplantation (101, 111-114). There are also some inborn errors of immunity-related genes that result in susceptibility to *Histoplasma* infection such us *IFNGR1*, *IL12RB1*, *GATA2*, *STAT1* and *STAT3* (69, 119-122, 208).

In patient's neutrophils, NADPH oxidase assays showed a considerable defect in intracellular production of oxidants in response to fungi *Candida albicans* and *Histoplasma capsulatum*, and fMLF, all these findings associated with his defect in the $p40^{phox}$ PX domain. To our

knowledge, this is the first reported case of autosomal recessive CGD involving *NCF4* in a child who initially presented with disseminated histoplasmosis. It is important to highlight that although PMA-stimulated neutrophil oxidant production detected by DHR was not different when compared to control values; an impaired production after fMLF stimulus was observed. These results suggest that when screening patients with possible NADPH oxidase defects, the DHR assay may be performed using different stimulus, such as PMA and fMLF.

Of particular importance for the NADPH oxidase is phosphatidylinositol-3-phosphate PtdIns(3)P, which is found on Rab5-positive endosomes and phagosomes. PtdIns(3)P is generated by the action of Class III PI3 kinase (209, 210). The $p40^{phox}$ subunit plays a specialized role in the regulation of intracellular NADPH oxidase activity through its PX domain, which binds with high affinity to the PtdIns(3)P (32, 211-213). In studies made by Bravo et al using a X-ray crystal structure, they describe that the fold of the $p40^{phox}$ PX has a N-terminal three- stranded, meander topology β sheet packed against a helical subdomain beginning at residue 59 that consists of four α helices, a 3_{10} helix, and a type II polyproline helix (62). Arg58, a residue within the conserved β3/α2 loop that connects the β sheet to the helical subdomain, forms the most extensive interactions with the 3-phosphate of the bound PtdIns(3)P. Oxygens of the 3-phosphate form hydrogen bonds with NH2 and NE in the side chain of Arg58. When it is mutated to Gln, PtdIns(3)P binding *in vitro* is lost and eliminates the distinct punctate pattern characteristic of the $p40^{phox}$ PX domain endosomal localization *in vivo* (62).

Our patient presented unaffected NADPH complex protein expression, however the $p67^{phox}$ expression was reduced. Similar results were reported for $p40^{phox}$-null murine neutrophil

(204) and another group of p40*phox* deficient patients not reported (personal communication Jacinta Bustamante). Supporting this idea, in p67*phox*-deficient CGD patients, the p40*phox* expression is reduced, suggesting that p40*phox* and p67*phox* mutually regulate each other's steady-state expression (207).

Recent focus on p40*phox*, NADPH oxidase complex, and the N*CF4* gene has provided support for their association with numerous diseases, including inflammatory bowel disease, autoimmune disorders, infections, defective innate immunity and autophagy
(17, 202, 214-216). We have strong evidence to support the pathogenicity of the variant identified in our patient since we have identified a decrease in the expression of the p40*phox* protein in the neutrophils and from the functional point of view there is a defect in neutrophils and in the EBV-B cell lines. This *NCF4* patient is a child with invasive disseminated fungal disease, however in general the clinical manifestations of the other patients under analysis are essentially auto inflammatory, very few made severe infections (data unpublished, personal communication Jacinta Bustamante).

Although healthy carriers of the homozygous mutation, the young brothers and sister may benefit from future medical follow up to monitor future possible development of autoimmune or infectious diseases. There is still very limited information in terms of the penetrance and expressivity of the phenotype conferred by the mutation. There may also be other epidemiologic and sociodemographic factors contributing to disease expression. Nevertheless, the similarities or differences in clinical manifestations with *NCF4* affected mutations remains to be defined.

This report provides the molecular characterization of a second patient with NCF4 deficiency. With these results we hypothesized that our patient phenotype is possibly due to

the homozygous variation in exon 3 (c.172C>T; p.R58C) of the *NCF4* gene, which affects the NADPH oxidase activity, and confers susceptibility to intracellular microorganism as the yeast *H. capsulatum.*

We also studied a cohort of three patients (P3, P4 and P5), that presented a common clinical phenotype of Juvenile PCM. Since the genetic susceptibility to Juvenile PCM has not been cleared stablished, few studies have identified specific genetic defects in association with the phenotype (*IL12RB1/IL23R, CD40L, GATA2,* STAT4) (139-142). Here we aimed to use NGS tools to determine the genetic susceptibility to Juvenile PCM in a Colombian cohort of patients. Two of these three patients (P3 and P5) came from consanguineous parents giving rise to the possibility of autosomal recessive inheritance underlying a genetic susceptibility to this microorganism. Regarding to these 3 patients, we presented a preliminary WES analysis and according with the bioinformatics analysis tools we found possible candidate genes causing PCM, based on their low frequency, biological function and high damaging prediction. Some of these genes have been previously associated with fungal diseases such as *IL17RA (81, 217)*. However, we have not yet done any functional assay to probe their pathogenicity. Future studies are required for a better understanding of the pathophysiology of this disease and the implication of these genes in the immune response to PCM.

In conclusion, the present study provides the molecular characterization of genetic variants identified in 2 patients with infections by *H. capsulatum* and *C. cassiicola*. Our results strongly suggest the $p40^{phox}$ protein as an important player during oxidative responses mediated by specific stimuli. The phenotype of our patient is marked by disseminated

histoplasmosis and bacterial infections in GIT, making this CGD-subtype a relatively distinct entity.

All the above results highlight the importance of both genes (*CARD9 and NCF4*) in the immune response against these microorganisms. Regarding patients with Juvenile PCM further studies will help us clarify their role in the immune response against infection in humans.

This work represents the first report of genetic susceptibility to fungal infections diseases in Colombian patients thought high throughput performance next generation sequencing technologies. Implementation of WES showed to have advantages for identify known or unknown genetic causes of possible primary immunodeciencies (PIDs) in coding regions at low cost, however WES present limitations related with sequencing depth which are affected by poor or incomplete exome capture, non covering of complete splicing sites and finally WES cannot detect non-coding or structural variants like big deletions or insertions (218).

This information can be useful to employ more specific drug treatment and appropriate management of patients with immunity disorders. It may also promote the development of novel therapeutic strategies that are targeted focused and oriented to counteract the underlying gene defect (gene therapy). More importantly, the accumulated knowledge provided by our study will help improve the life quality of these patients as well as others with similar immunodeficiency phenotypes and patients with genetic defects in general.

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"And, when you want something, all the universe conspires in helping you to achieve it."

Be persistence¡ ― Paulo Coelho, The Alchemist

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ADDITIONAL ACTIVITIES

INTERSHIP

I did an internship as a visiting student in the Laboratory of Human Genetic of Infectious Diseases (HGID) under the tutory of Jacinta Bustamante. It was between September 26th and December 7th, 2016.

ORAL PRESENTATIONS

1. Arango-Franco CA, Góngora DE, Orrego JC, Alvarez JA, Fernández DP, Rojas JL, Velasquez MM, Gonzalez Loaiza D, Montoya CJ, Moncada-Vélez M, Rocha YC, Vélez GJ, López JA, Patiño PJ, Trujillo CM, Franco JL and Arias AA (2015). Molecular characterization of Primary Immunodeficiency Diseases from the group of Primary Immunodeficiencies in Colombia, 2015: an update. Front Immunol. Conference Abstract: IMMUNOCOLOMBIA 2015 - 11th Congress of the Latin American Association of Immunology - 10o. Congreso de la Asociación Colombiana de Alergia, Asma e Inmunología. doi: 10.3389/conf.fimmu.2015.05.00353

2. Fernández Echeverri D, González Loaiza D, Moncada-Vélez M, Orrego J, Rojas J, Arango-Franco CA, Gonzalez N, Pérez Vélez C, Sierra J, Wilches A, Arango C, Restrepo A, Trujillo M, Garcés C, Isaza-Correa J, Gongora D, Casanova J, Arias AA, Bustamante J and Franco JL (2015). Mendelian Susceptibility to Mycobacterial Diseases (MSMD) in Colombian Patients. Front. Immunol. Conference Abstract: IMMUNOCOLOMBIA2015 - 11th Congress of the Latin American Association of Immunology - 10o. Congreso de la Asociación Colombiana de Alergia, Asma e Inmunología. doi: 10.3389/conf.fimmu.2015.05.00109

3. Carlos Andrés Arango-Franco, Marcela Moncada-Vélez, Sara Daniela Osorio, Lorena Castro, Lina Vanessa Gómez, Ana María Muñoz, Verónica Molina, Delsy Yurledy del Río Cobaleda, Ana Cristina Ruiz Suárez, Claudia Patricia Beltrán, Rosalba Vivas, Indira Berrio, Andrea Restrepo, Mónica Trujillo, Carlos Garcés, Juan

Fernando Alzate, Felipe Cabarcas, Julio Cesar Orrego, Jean Laurent Casanova, Jacinta Bustamante, Anne Puel, Andrés Augusto Arias, José Luis Franco. Defecto de CARD9 asociado a infección por *Corynespora cassiicola* en un paciente colombiano. IX Jornadas de Investigación y Extensión de la Escuela de Microbiología "Conociendo la Investigación y la Extensión hecha en casa" 28 y 29 de Abril de 2016, presentación en la modalidad Oral. Medellín – Colombia.

4. **Carlos A. Arango-Franco,** Marcela Moncada-Vélez, Sara Daniela Osorio, Lorena Castro, Lina Vanessa Gómez, Ana María Muñoz, Verónica Molina, Delsy Yurledy del Río Cobaleda, Ana Cristina Ruiz, Claudia Patricia Beltrán, Rosalba Vivas, Indira Berrio , Cristian Mogollón, Andrea Restrepo, Mónica Trujillo, Carlos Garcés, Juan Fernando Alzate, Felipe Cabarcas, Julio Cesar Orrego, Jean Laurent Casanova, Jacinta Bustamante, Anne Puel, Andrés Augusto Arias, José Luis Franco. Invasive *Corynespora Cassiicola* infection associated with inherited CARD9 Deficiency in a Colombian Patient. Latin American Society for Primary immunodeficiencies Summer School from February 23th – 26, 2017. Cancun – Mexico.

POSTER PRESENTATIONS

1. Carlos Andrés Arango-Franco, Diego Gongora, Julio Cesar Orrego, Jesús Armando Alvarez, Diana Patricia Fernandez, Jesica Lineth Rojas, Margarita Velásquez, Daniel González, Juan Álvaro López, Carlos Julio Montoya, Pablo Javier Patiño, Marcela Moncada-Velez, Gabriel Jaime Vélez, Yermis Carolina Rocha, Bodo Grimbacher, Silvia Danielian, Sergio Rosenzweig, Jolan E. Walter, Fabio Candotti, Jean Laurent Casanova, Jacinta Bustamante, Claudia Milena Trujillo-Vargas, Andrés Augusto Arias, José Luis Franco. Molecular characterization of genes mutated in patients with Primary Immunodeficiency Diseases (PIDs) at the group of Primary Immunodeficiencies in Colombia, 2015: an update. First International Meeting LASID FAIC SAI, November 18th – 21st 2015, Buenos Aires-Argentina, Poster presentation. J Clin Immunol (2015) 35 (Suppl 1):S1–S57 DOI 10.1007/s10875-015-

0199-4

2. Marcela Moncada-Velez, Andrés Augusto Arias, Carlos Andres Arango-Franco, Sara Daniela Osorio, Lorena Castro, Miyuki Tsumura, Shiho Nishimura, Sonoko Sakata, Satoshi Okada, Juan Fernando Alzate, Felipe Cabarcas, Julio Cesar Orrego, Jean-Laurent Casanova, Jacinta Bustamante, José Luis Franco. Hallazgo de tres mutaciones nuevas que confieren ganancia de función en STAT1 y predisposición a infecciones por *Candida spp* y *Mycobacterium tuberculosis*. IX Jornadas de Investigación y Extensión de la Escuela de Microbiología "Conociendo la Investigación y la Extensión hecha en casa" 28 and 29 April 2016, poster presentation. Medellín – Colombia

3. Carlos A. Arango-Franco, Marcela Moncada-Vélez, Sara Daniela Osorio, Lorena Castro, Lina Vanessa Gómez, Ana María Muñoz, Verónica Molina, Delsy Yurledy del Río Cobaleda, Ana Cristina Ruiz, Claudia Patricia Beltrán, Rosalba Vivas, Indira Berrio , Cristian Mogollón, Andrea Restrepo, Mónica Trujillo, Carlos Garcés, Juan Fernando Alzate, Felipe Cabarcas, Julio Cesar Orrego, Jean Laurent Casanova, Jacinta Bustamante, Anne Puel, Andrés Augusto Arias, José Luis Franco. Invasive *Corynespora Cassiicola* infection associated with inherited CARD9 Deficiency in a Colombian Patient. "17th Biennial Meeting of the EUROPEAN SOCIETY FOR IMMUNODEFICIENCIES" 21 and 24 september 2016. Barcelona - Spain

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