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Inherited CARD9 deficiency: invasive disease caused by ascomycete fungi in previously healthy children and adults

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Abstract

Autosomal recessive CARD9 deficiency underlies life-threatening, invasive fungal infections in otherwise healthy individuals normally resistant to other infectious agents. In less than 10 years, 58 patients from 39 kindreds have been reported in 14 countries from 4 continents. The patients are homozygous ($n=49$; 31 kindreds) or compound heterozygous ($n=9$; 8 kindreds) for 22 different *CARD9* mutations. Six mutations are recurrent, probably due to founder effects. Paradoxically, none of the mutant alleles has been experimentally demonstrated to be loss-of-function. CARD9 is expressed principally in myeloid cells, downstream from C-type lectin receptors that can recognize fungal components. Patients with CARD9 deficiency present impaired cytokine and chemokine production by macrophages, dendritic cells and peripheral blood mononuclear cells (PBMCs), and defective killing of some fungi by neutrophils *in vitro*. Neutrophil recruitment to sites of infection is impaired *in vivo*. The proportion of Th17 cells is low in most, but not all patients tested. Up to 52 patients suffering from invasive fungal diseases (IFD) have been reported, with ages at onset of 3.5 to 52 years. Twenty of these patients also displayed superficial fungal infections. Six patients had only mucocutaneous candidiasis or superficial dermatophytosis at their last-follow-up visit, at the age of 19 to 50 years. Remarkably, for 50 of the 52 patients with IFD, a single fungus was involved; only two patients had IFDs due to two different fungi. IFD recurred in 44 of 45 patients who responded to treatment, and a different fungal infection occurred in the remaining patient. Ten patients died from IFD, between the ages of 12 and 39 years, whereas another patient died at the age of 91 years, from an unrelated cause. At the most recent scheduled follow-up visit, 81% of the patients were still alive, and aged from 6.5 to 75 years. Strikingly, all the causal fungi belonged to

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Conflicts of interest

We have no conflict of interest to declare.

the phylum Ascomycota: commensal *Candida*, and saprophytic *Trichophyton*, *Aspergillus*, *Phialophora*, *Exophiala*, *Corynespora*, *Aureobasidium* and *Ochroconis*. Human CARD9 is essential for protective systemic immunity to a subset of fungi from this phylum, but seems to be otherwise redundant. Previously healthy patients with unexplained invasive fungal infection, at any age, should be tested for inherited CARD9 deficiency.

Keywords

CARD9; primary immunodeficiency; invasive fungal disease; Ascomycota phylum; chronic mucocutaneous candidiasis; invasive candidiasis; deep dermatophytosis; phaeohyphomycosis; invasive aspergillosis; phagocytes; central nervous system

Introduction

Inherited CARD9 deficiency was first reported in 2009, as an autosomal recessive (AR) form of chronic mucocutaneous candidiasis (CMC) in an Iranian multiplex consanguineous family in which six of the seven affected individuals had CMC [1]. Since 2009, 16 additional CARD9-deficient patients (from 13 families) with CMC have been reported [2–11]. Inborn errors of interleukin (IL)-17 immunity were shown, from 2011 onward, to be the key genetic etiologies of CMC [12,13]. Accordingly, low *ex vivo* proportions of IL-17-producing T (Th17) cells and/or low levels of IL-17A and IL-17F production *in vitro* in response to polyclonal or fungal stimulation were found in 18 of the 27 CARD9-deficient patients tested [1,2,9,10,14–16]. Only six of these 18 patients had CMC at the time of study [1,9,10]. In addition to CMC, three of the seven patients reported in the original study had probably also had central nervous system (CNS) *Candida* infection, affecting the meninges and/or brain [1]. Support for this hypothesis was provided by the description of 14 additional patients (from 13 families) with the same clinical presentation [3,5–7,9–11,16,17]. However, although the CNS remains the main target organ of *Candida* infection in CARD9-deficient patients, *Candida* infections of other organs (eye: endophthalmitis, colon: colitis, bones: osteomyelitis) have been reported in seven patients (from seven families) [3,6–8,18,19]. Since the first description of CARD9 deficiency, the spectrum of invasive fungal diseases (IFD) in CARD9-deficient patients has progressively expanded, with the description of patients with extensive/deep dermatophytosis ($n=21$, from 12 families) [2,4,10,20,21], subcutaneous and invasive phaeohyphomycosis ($n=10$, from 10 families) [14,15,19,22,23], or extrapulmonary invasive aspergillosis (IA) ($n=2$, from 2 families) [8].

In addition to *C. albicans*, a growing number of other IFD-causing fungi have been identified in CARD9-deficient patients. All belong to the phylum Ascomycota (*Trichophyton*, *Phialophora*, *Exophiala*, *Corynespora*, *Aureobasidium*, *Ochroconis* and *Aspergillus*). Regardless of whether we consider the disease-causing fungus (e.g. *Corynespora*) or the clinical presentation (e.g. *Candida* CNS infection, isolated brain or intra-abdominal IA without lung damage), such IFDs are rare even among patients with classical primary immunodeficiencies (PIDs). These studies have thus shown that CARD9 deficiency is primarily a genetic etiology of IFD striking otherwise healthy individuals, rather than a genetic etiology of CMC. CARD9 deficiency is currently the only known PID involving a

specific increase in selective susceptibility to both superficial and invasive fungal diseases, and the only PID reported for some types of IFD (e.g. deep dermatophytosis). Phagocytes probably play a key role in invasive infections, as impaired cytokine and chemokine production by macrophages, peripheral blood mononuclear cells (PBMCs) or dendritic cells (DCs), defects of fungal killing by neutrophils, and impaired neutrophil recruitment to the site of infection have been found *in vitro* or *in vivo*, in both humans and mice. It has become clear that CARD9-mediated immunity is essential for systemic host defense against various fungi in humans, but the molecular and cellular bases of fungal diseases in CARD9-deficient patients remain poorly understood. We review here the molecular, cellular, and clinical features of human inherited CARD9 deficiency as well as the relevant features observed in CARD9-deficient inbred mice.

CARD9 expression in mice and humans

In 2000, Bertin *et al.* identified human CARD9 as a new caspase recruitment domain (CARD)-containing protein following a search of the Millenium Pharmaceuticals proprietary database for sequences encoding CARD motifs [24]. The gene encoding CARD9 is located on chromosome 9, at position q34.3, and has 13 exons (Figure 1). It encodes two main transcripts differing by 309 nucleotides, due to the alternative splicing of exons 12 and 13, based on the data in the Ensembl database. The first transcript is 2,123 base pairs (bp) long and is encoded by 13 exons (the first exon being a non-coding exon), and it yields a protein of 536 amino acids (isoform 1). The alternative transcript of 1,814 bp long (it lacks the last 67 nucleotides of exon 12 and the first 242 nucleotides of exon 13) is also encoded by 13 exons and yields a 492-amino-acid protein (isoform 2). Isoform 2 is smaller than isoform 1 and has a different Cterminus, with a different amino-acid sequence from residues 482 to 492, the stop-codon. All published functional studies have been performed on the first transcript, encoding isoform 1. In mice, the *Card9* gene is located on chromosome 2, at position 18.87cM. It also consists of 13 exons and encodes two transcripts. The first transcript is 1,947 bp long and is encoded by 12 exons. The second is 1,880 bp long and encoded by 13 exons (the first exon being a non-coding exon). Both transcripts yield the same 536-amino-acid protein based on the data in the Ensembl database. The human and mouse CARD9 proteins are cytosolic and contain one N-terminal CARD domain and one C-terminal coiled-coiled domain (CCD) [24]. Human and mouse CARD9 proteins are highly but not completely homologous (around 86%), which explains their functional similarities and perhaps also suggests that they might have functional differences [25]. The pattern of CARD9 expression across tissues seems to be similar in humans and mice. *CARD9* mRNA is detectable in the placenta, small intestine, skin, lungs and brain, whereas the CARD9 protein has been found in stomach and skin [1,7,24,26]. However, the highest levels of both mRNA and protein for CARD9 are found in the hematopoietic cells of the spleen, bone marrow, and blood, especially in myeloid cells such as macrophages and DCs.

CARD9 functions in mouse cells

In vitro studies on mice, mostly performed with bone marrow-derived DCs/macrophages (BMDCs/BMMs), have shown that CARD9 transduces signals downstream from C-type lectin receptors (CLRs), a family of pathogen-recognizing receptors (PRRs). These receptors

include Dectin-1 (CLEC7A) [27], Dectin-2 (CLEC6A) [28], Dectin-3 (CLEC4D) [29], and Mincle (CLEC4E) [30]. They are specific for β -glucans (Dectin-1), α -mannans (Dectin-2 and Dectin-3), and glycolipids (Mincle) of the fungal cell walls [31]. These receptors are activated by the phosphorylation, by Src family kinase (SFK), of immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic tail of the receptor (for Dectin-1), or of the associated signaling molecules, ITAM-containing common Fc receptor γ subunit (FcR γ) (for Dectin-2, Dectin-3 and Mincle). This activation induces a downstream activation cascade involving phosphorylation by different kinases. Phosphorylated ITAMs first recruit and activate spleen tyrosine kinase (SYK), which in turn phosphorylates protein kinase C (PKC)- δ , thereby engaging CARD9 in the canonical nuclear factor kappa light-chain enhancer of activated B cells (NF- κ B) and/or mitogen-activated protein kinase (MAPK) pathways through phosphorylation of the threonine 231 residue of CARD9, which is located in the CCD. The phosphorylated CARD9 recruits B-cell CLL/lymphoma 10 (BCL10) by CARD-CARD interaction. BCL10 immediately binds to the mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) paracaspase, leading to the formation of the CARD9-BCL10-MALT1 (CBM) complex [32–38]. This complex activates NF- κ B, the c-Jun N-terminal kinase (JNK), and p38 to stimulate the transcription of genes encoding pro-inflammatory cytokines and chemokines, such as IL-2, IL-10, IL-12, tumor necrosis factor (TNF)- α , pro-Th17 cytokines (IL-1 β , IL-6, IL-23), and CXCL1 or CXCL2 [26,32,39]. It has recently been shown that proteins of the Vav guanine nucleotide exchange factor (VAV) family (VAV1, 2, 3) can serve as alternative activators of CARD9, just like PKC- δ [40]. VAVs are activated upon binding to CLRs (Dectin-1, Dectin-2 and Mincle) via SYK-dependent activation. They, in turn, phosphorylate CARD9 complex, leading to activation of the NF- κ B pathway, but not the MAPK pathway, thereby inducing the production of pro-inflammatory cytokines (TNF, IL-2, IL-1 β and IL-10) [40]. However, the mechanisms leading to CARD9 phosphorylation by VAV and the role of the CBM are less well understood than CARD9 activation by PKC- δ . The Dectin-1/SYK signal can also engage CARD9 in the MAPK pathway independently of PKC- δ activation and CBM formation [41]. Upon β -glucan binding to Dectin-1, activated SYK phosphorylates Ras protein-specific guanine nucleotide-releasing factor 1 (Ras-GRF1), which in turn forms a complex with CARD9 that recruits and activates Harvey rat sarcoma viral oncoprotein (H-Ras), leading to extracellular signal-regulated kinase (ERK) activation and the production of cytokines (IL-1 β , IL-6 and TNF- α). Thus, in mice, binding of CLRs to fungal ligands engages CARD9 in cytokine and chemokine production through the activation of the NF- κ B and/or MAPK pathways, depending on SYK/PKC- δ or SYK/Ras-GRF1 activation.

CARD9: functions in human cells

Human CARD9 function has been studied mostly in overexpression systems and has been found to be very similar to that of the mouse protein. Indeed, human CARD9 transduces signals downstream from different CLRs (e.g. DECTIN1/CLEC7A, DECTIN2/CLEC6A, DECTIN3/CLEC4D and MINCLE/CLEC4E), from fungal components, through the SYK activation of ITAM, leading to activation of the NF- κ B and MAPK pathways and the production of pro-inflammatory cytokines (Figure 2) [29,34,42–45]. These receptors have been identified in humans and cloned during the last 10 years, and have been shown to

recognize the same fungal cell wall motifs as their mouse counterparts, which is not surprising given the high percentage identity between mouse and human CLR sequences [46–49]. Cytokine (e.g. IL-2, IL-10 or TNF- α) production was detected 24 hours after the stimulation of HEK 293 (human embryonic kidney) or RAW 264.7 (cell line derived from murine macrophages) cells transfected with human recombinant *DECTIN1/CLEC7A*, *DECTIN2/CLCEC6A* or *MINCLE/CLEC4E* alleles, with zymosan (principal β -glucan of the yeast cell wall [50]) or heat-killed *C. albicans* [43–45]. The human CBM complex proteins were identified by co-immunoprecipitation assays in which HEK cells were transfected with *BCL10*, *MALT1* and WT *CARD9* alleles and displayed complex formation [6]. Furthermore, in NF- κ B luciferase reporter assays, HEK cells transfected with *DECTIN1/CLEC7A*, *SYK*, *BCL10* and WT *CARD9* alleles displayed NF- κ B-dependent transcriptional activity after 24 hours of stimulation with *C. albicans* or *Exophiala dermatitidis* [3,22]. Finally, CARD9 activation by VAV protein has not yet been demonstrated in human cells. In primary cells (PBMCs, monocyte-derived macrophages (MDMs), monocyte-derived dendritic cells (MDDCs) and monocytes), CARD9-dependent signaling was assessed principally by measuring the production of pro-inflammatory cytokines and chemokines (e.g. IL-1 β , IL-6, TNF- α , CXCL1 and CXCL2) after 24 or 48 hours of stimulation with whole fungi or CLR agonists (see below for details of the cells tested) [1–3,5–8,10,14–17,22]. Dectin-1-induced SYK-dependent signaling was shown to activate the canonical (p65,c-Rel) and non-canonical (RelB) NF- κ B pathways in MDDCs after stimulation with curdlan (β -glucan from bacteria [51]) or *C. albicans* [52]. Moreover, the Dectin-1/Ras-GFR1/ERK pathway has been shown to be activated in human monocytes after stimulation with zymosan, leading to the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA (*CFS2*) and protein [6]. Thus, in overexpression system, the binding of human CLRs to fungal ligands or whole fungi induces cytokine production in a CARD9-dependent manner, with CBM formation, NF- κ B transcriptional activity and a high level of sequence identity between the human and mouse CLRs. In primary cells, Dectin-1/SYK signaling induces the activation of both canonical and non-canonical NF- κ B and MAPK pathways upon fungal stimulation, leading to the production of pro-inflammatory cytokines.

Human *CARD9* mutations

CARD9 deficiency has been reported in 58 patients from 39 kindreds. *CARD9* was sequenced in 51 of these patients (from 39 kindreds), leading to the identification of 22 mutations. CARD9 deficiency is strictly recessive, as none of the heterozygotes had any unusual infectious phenotype. The genotype of the other seven patients was, therefore, assumed to be the same as that of their affected siblings, according to a recessive mode of inheritance. Forty-nine individuals (84.5%) from 31 kindreds (79.5%) were found to carry the mutation in the homozygous state, and nine individuals (15.5%) from eight kindreds (20.5%) were found to carry the mutation in the compound heterozygous state (Figure 1, Table 1) [1,2,4–11,14–23]. The mutations identified were located in the 5' UTR, CARD, or CCD regions (Figure 1). Missense ($n=11$) [2–4,6–8,10,16–18,22], nonsense ($n=4$) [1–3,5,8,9,11,14,15,20,21] and synonymous ($n=1$) [18] mutations, small deletions ($n=3$, 2 in-frame and 1 frameshift) [19,22], small insertions ($n=2$, frameshift) [14,15,23] and a single-

nucleotide substitution in the 5' UTR region ($n=1$) [6] were identified (Figure 1). Six of the mutations were recurrent, suggesting a founder effect. The two homozygous nonsense mutations, Q289* ($n=18$, from 10 kindreds) and Q295* ($n=13$, from 6 kindreds), were the most frequent, being found in 53.4% of patients and 41% of kindreds. Q289* was reported in patients from North Africa (Algeria, Egypt, Morocco, Tunisia), and four unrelated Algerian patients harboring the same homozygous *CARD9* Q289* mutation shared a common haplotype around this mutation [2]. The Q295* mutation found in patients from the Middle East (Iran, Turkey, Pakistan) and in one patient of mixed European origin (no data about the country of origin) was not formally demonstrated to be due to a founder effect [1,3,5,8,9,11]. Three unrelated Chinese patients bearing the same homozygous insertion, D274fs*60, shared the same haplotype around the *CARD9* locus [15]. A founder effect is also probable for the R70W mutation, which was found in four Turkish patients from three kindreds [3,10]. Finally, a founder effect has been suggested for the Y91H and c.-529T>C mutations, which have been found in three and two unrelated French-Canadian kindreds, respectively [6,17].

Computational analysis of *CARD9* variants

The minor allele frequency (MAF) of mutant alleles underlying rare conditions is low (defined as <1%, with common alleles defined as having a MAF ≥ 1%) [53]. Only eight of the 22 mutant alleles identified in *CARD9*-deficient patients (Q289*, Q295*, R35Q, Y91H, R373P, E323del, L64fs*59 and D274fs*60) have been reported in public databases (gnomAD, ExAC, 1000Genom, dbSNP), with very low MAFs, ranging from 4.1×10^{-6} to 3.3×10^{-5} (gnomAD) (<http://gnomad.broadinstitute.org>), and these variants have been reported only in the heterozygous state (Table 2). The other variants were private. Candidate variants for further study can be selected by predicting their impact and the relevance of the mutated gene, with gene-level and variant-level methods. Gene-level methods include analyses of gene expression pattern and function, and calculation of the gene-damage index (GDI), a gene-level metric estimating the accumulation of damaging mutations in the general population [54]. The *CARD9* gene has a low GDI of 7.21 (which is less than 12.4 the standard GDI cutoff used in computational analysis when looking for PIDs-causing gene in general), reflecting a minimal mutational burden in the general population (<http://lab.rockefeller.edu/Casanova/GDI>) [54]. Variant-level methods can be used to determine whether a given variant is benign or deleterious. They include the combined annotation-dependent depletion (CADD), polymorphism phenotyping version 2 (PolyPhen-2), and sorting intolerant from tolerant (SIFT) scores [55–57]. The mutation significance cutoff (MSC) for a given gene is the lower limit for a given variant-level score (e.g. CADD) above which the variant is probably pathogenic [58]. Nineteen variants were predicted to be deleterious, with a high CADD score (above the MSC of 19, with 15 generally used as the standard cutoff) and/or a high PolyPhen-2/SIFT score (Table 2, Figure 3). Three private variants (R317R, A380P and c.-529T>C) were not predicted to be pathogenic by any of these scores and were found in the heterozygous state in the patients. The 5' UTR c.-529T>C allele was not predicted to have any functional impact *in silico* (e.g. splicing) but was suggested to be in linkage disequilibrium with the unidentified causal mutation in the three patients carrying the predicted pathogenic Y91H mutation in the other allele [6]. The

other two “benign” mutations, R317R (predicted to create a splicing site *in silico*) and A380P, were found in a compound heterozygous state within a single patient, the only patient in this series to have a predicted nonpathogenic genotype [18]. Thus, 19 of the 22 *CARD9* variants were predicted to be deleterious and disease-causing.

Experimental analysis of *CARD9* mRNAs

The impact of mutated *CARD9* alleles on mRNA levels was evaluated by real-time qPCR or classical RT-PCR in PBMCs, MDMs, MDCCs, and/or lymphoblastoid-derived cell lines (LCLs) from patients. Thirteen alleles were tested: *c.472C>T* (p.Q158*), *c.865C>T* (p.Q289*), *c.883C>T* (p.Q295*), *c.52C>T* (p.R18W), *c.104G>A* (p.R35Q), *c.208C>T* (p.R70W), *c.271T>C* (p.Y91H), *c.301C>T* (p.R101C), *c.184G>A* (p.G62fs*), *c.191–192insTGCT* (p.L64fs*59), *c.288C>T* (p.G96del96), *c.819–820insG* (p.D274fs*60) and, *c.–529T>C*. The Q295*, Y91H, L64fs*59, Q158* and D274fs*60 alleles gave rise to substantial amounts of mRNA in PBMCs, suggesting that their transcripts underwent little or no nonsense-mediated RNA decay [1,15,17]. Patients compound heterozygous for the *c.–529T>C/c.271T>C* variants had an allelic imbalance in PBMCs and LCLs, with only mRNA for the *c.271T>C* (p.Y91H) mutant allele detected [6]. The 5' UTR *c.–529C>T* substitution had no detectable impact, and this mutation was considered to be in probable linkage disequilibrium with the causal mutation rather than being a disease-causing allele itself. However, the second causal hit has not been identified in these patients, and may be an intronic mutation creating a splicing site. cDNA sequencing showed that *c.184G>A* (G62fs*) and *c.288C>T* (G96del36) created novel splice donor sites due to a 4 bp frameshift deletion and an in-frame deletion of 36 bp, respectively [19]. Both the *c.184G>A* and *c.288C>T* mRNAs were detected in LCLs. *CARD9 c.865C>T* (p.Q289*), *c.883C>T* (p.Q295*), *c.52C>T* (p.R18W), *c.104G>A* (p.R35Q), *c.208C>T* (p.R70W) and *c.301C>T* (p.R101C) mRNAs were also detected in the patients' MDMs, MDCCs and/or monocytes [2,3,22]. The *CARD9 c.3G>C* (p.M1I), *c.68C>A* (p.S23*), *c.170G>A* (p.R57H), *c.214G>A* (p.G72S), *c.302G>T* (p.R101L), *c.951G>A* (p.R317R), *c.1118G>C* (p.R373P), *c.1138G>C* (p.A380P) and *c.GAG967–969del* (p.E323del) mRNAs were not studied. These data indicate that the 13 *CARD9* mutant alleles are expressed at mRNA level in the cells from the patients tested, suggesting that none of these alleles is amorphic, at least in terms of mRNA production.

CARD9 protein production in the patients' cells

The impact of biallelic *CARD9* mutations on protein production was assessed by western blotting and/or flow cytometry, with monoclonal (mAb) or polyclonal (pAb) anti-CARD9 antibodies (Table 3) [1–3,5–8,14–17,19,22]. Endogenous CARD9 expression was assessed in the PMBCs, neutrophils, MDMs, and/or MDCCs of the patients. Western blotting detected no protein of the expected molecular weight (MW) (~65 kDa) in cells homozygous for Q289* (MDCCs), Q295* (PBMCs, monocytes, neutrophils), D274fs*60 (PBMCs, neutrophils), or M1I (PBMCs), or in cells compound heterozygous for G72S/R370P (neutrophils, monocytes), L64fs*59/Q158* (PBMCs), S23*/D274fs*60 (neutrophils), or L64fs*59/D274fs*60 (neutrophils) [1,2,5,8,14–16]. These mutant alleles are therefore probably loss-of-expression. By contrast, normal levels of CARD9 proteins (of normal MW)

were detected in cells homozygous for Y91H (PBMCs) and R57H (neutrophils, monocytes), or compound heterozygous for G62fs*/G96del36 (LCLs) [7,17,19]. Moreover, R18W and R57H protein levels in monocytes, MDMs or neutrophils, were found to be normal on flow cytometry, further suggesting that these mutations are not loss-of-expression [7,22]. However, the levels of the R101C and R70W proteins in MDCCs were found to be low on flow cytometry [2,3]. Finally, the expression of some *CARD9* alleles (Q289*, Q295*, R101C, R18W, R35Q, R70W, Y91H) was also assessed by western blotting with pAbs after transient overexpression in HEK cells [2,3,6,22]. The Q289* and Q295* *CARD9* alleles generated normal amounts of truncated protein (about 25 kDa). The R101C, R18W, R35Q, R70W and Y91H *CARD9* alleles produced proteins of normal MW (65 kDa) in amounts similar (R101C, R35Q, Y91H), higher (R18W) or lower (R70W) to those in HEK cells transfected with the WT *CARD9* allele. In overexpression conditions, none of the alleles tested was loss-of-expression. The impact of the E323del, R101L, A380P, and R317R *CARD9* alleles was not studied in cells from patients or in an overexpression system [4,18,22]. In conclusion, endogenous *CARD9* expression varies between the different genotypes, from the production of normal amounts of a protein of the expected MW to a complete absence of protein production.

Immunological status of patients with *CARD9* mutations

All 31 patients tested for peripheral neutrophils, T, B and natural killer (NK) cells had normal counts of these cells, and monocyte counts were normal in the 12 patients tested (Table 3) [1–5,7–11,14,17,19–22]. Extensive whole-blood immunophenotyping revealed no abnormalities other than low CD4⁺ and CD8⁺ T-cell counts in one patient [8] and a low CD4⁺ T-cell count in another [11]. T-cell proliferation in response to mitogen (phytohemagglutinin_PHA) was analyzed in nine patients, all of whom displayed normal proliferation, whereas proliferation in response to antigens, which was assessed in seven patients, (e.g. candidin, tetanus toxin, tuberculin) was normal in four patients, with a weak response to candidin and tuberculin in the other three [3–5,7–9,16,22]. The phagocyte oxidative burst was normal, as shown by the dihydrorhodamine assay (DHR), in the 15 patients tested [3,4,7,8,11,14,16,17,22]. Sixteen of the 25 patients tested had hypereosinophilia (64%), 12 of 19 had high serum IgE levels (63.2%), and eight of 17 patients tested had both (47%) [1–4,6–11,14,19,21]. The high serum IgE concentrations and hypereosinophilia are unexplained. No manifestations of atopy (e.g. allergies, eczema or dermatitis skin lesions) were reported in any of the patients with the exception of one patient with a history of food allergies, mild eczema and reactive airway atopy [8]. However, IgE allergen reactivity was not analyzed. Thus, hypereosinophilia and high serum IgE concentration in patients with IFD may be suggestive of inherited *CARD9* deficiency, in cases in which monoallelic dominant-negative *STAT3* mutations and biallelic loss-of-function *DOCK8* mutations have been excluded [59,60]. Thus, the immunological status of patients with *CARD9* deficiency is characterized by a normal leukocyte blood count, and about half the patients present hypereosinophilia and high serum IgE concentration.

Impaired mononuclear phagocyte responses to fungal stimulation

Cytokine production by PBMCs, monocytes, MDMs, and MDDCs after the stimulation of these cells with various fungal ligands (curdlan, zymosan, heat-killed *C. albicans*, *E. dermatitidis*, *E. spinifera*, *Phialophora verrucosa*, *Ochroconis musae*, *Corynespora cassiicola*, and/or *Aspergillus fumigatus*) was impaired in the 27 patients tested (Table 3) [1–3,5–8,10,14–17,22]. Indeed, impaired production of IL-1 β , IL-6, and TNF- α was observed in cells from homozygous (Q289*, Q295*, M1I, R18W, R57H, R70W, R101C and D274fs*60) and compound heterozygous (L64fs*59/Q158*, G72S/R373P, S23*/D274fs*60 and L64fs*59/D274fs*60) individuals tested after 24 or 48 hours of stimulation. This defect probably resulted at least in part from impaired NF- κ B pathway activation, as suggested by the abnormally low levels of NF- κ B p65 phosphorylation observed in monocytes from patients homozygous for D274fs*60 or compound heterozygous for S23*/D274fs*60 or L64fs*59/D274fs*60 after 30 minutes of stimulation with *E. spinifera* [14]. PBMCs from individuals homozygous for p.Y91H or compound heterozygous for c.-529T>C/c.271T>C (p.Y91H) mutations produced normal amounts of IL-1 β , IL-6 and TNF- α after 24 hours of stimulation with zymosan, whereas GM-CSF levels were very low [6,17]. Impaired GM-CSF production by PMBCs was also detected after 24 hours of stimulation with curdlan, *C. albicans*, and/or *A. fumigatus* in individuals homozygous for Q295*, M1I, R57H, or R70W [7,8,10]. Western blotting analyses suggested that ERK phosphorylation was impaired after stimulation with zymosan in monocytes from patients expressing CARD9 Y91H protein [6]. However, a recent report suggested normal ERK phosphorylation after zymosan in monocytes expressing CARD9 R57H protein [61]. In all CARD9-deficient patients tested, the levels of IL-1 β , IL-6 and/or TNF- α produced after 24 or 48 hours of stimulation with non-fungal ligands (e.g. heat-killed *Staphylococcus aureus* and/or lipopolysaccharides_LPS) were similar to those of healthy control cells. These data suggest that the 14 mutations tested are at least strongly hypomorphic, if not amorphic, in terms of cytokine production upon fungal stimulation. However, assays based on gene editing technology (e.g. CRISPR/Cas9 and rescue of mutant phenotype) were not performed, and it was not, therefore, possible to draw firm conclusions as to whether the mutants were completely (null) or partially (hypomorphic) loss-of-function.

Impaired functions of polymorphonuclear neutrophils

Neutrophil functions were studied in 10 patients (Table 3) [4,5,7,8,16,62,63]. It was initially suggested that the susceptibility to *Candida* CNS infections resulted from defective killing activity in CARD9-deficient neutrophils. Indeed, neutrophils from three patients with *Candida* meningitis were shown to have a selective defect impairing the *ex vivo* killing of unopsonized but not of opsonized *C. albicans* yeasts, resulting in an outgrowth of hyphae not observed with controls [5,7,16,63]. In line with these data, phagolysosomes with an abnormal ultrastructure were observed in these neutrophils on electron microscopy [16]. However, no neutrophil killing defect was observed with *C. albicans* hyphae (whether opsonized or unopsonized), the predominant form in infected tissues [7,64]. A defect of opsonized *C. albicans* spore-killing by neutrophils was observed in a standardized killing assay [65] for the only patient with deep dermatophytosis tested [4]. As observed for the *C. albicans* yeast killing defect, neutrophils from four patients with subcutaneous *P. verrucosa*

infection displayed a selective defect affecting the killing of unopsonized *P. verrucosa* conidia, but not of opsonized conidia [62]. However, the killing of opsonized and unopsonized *A. fumigatus* conidia and hyphae by neutrophils was not impaired in CARD9-deficient patients, suggesting that CARD9 may play different fungus-specific roles in neutrophil function [8]. Moreover, the observed killing defects were neutrophil-specific, as the *ex vivo* killing of *E. spinifera* conidia or *C. albicans* yeast by monocytes was normal in the patients with subcutaneous phaeohyphomycosis ($n=3$) or *Candida* meningitis ($n=1$) tested (by flow cytometry analysis of stained fungal particles [66]) [7,14]. Neutrophil recruitment was impaired in the affected organs [3,7–9,14,16]. Indeed, a lack of neutrophil accumulation was observed in the cerebral spinal fluid (CSF) of four patients with *Candida* CNS infections, whereas blood neutrophil counts were within the normal range [3,7,9,16]. By contrast, patients with *Candida* meningitis but no CARD9 deficiency presented neutrophil predominance in the CSF [7]. Consistent with this finding, histological analyses of skin, mesenteric lymph nodes (LN) or adrenal masses from patients with subcutaneous phaeohyphomycosis ($n=3$) or intra-abdominal aspergillosis ($n=2$) showed a lack of neutrophil infiltration [8,14]. These findings are suggestive of tissue-specific neutropenia in the absence of blood neutropenia. The impairment of neutrophil recruitment in the CNS of patients may result from a lack of CXC-chemokine induction at the site of infection, as suggested by the low levels of CXCL1 and CXCL2 in the CSF, whereas neutrophil-intrinsic chemotaxis functions were maintained *in vitro* [7]. A similar neutrophil recruitment defect was suggested in a patient with intra-abdominal aspergillosis and normal neutrophil-intrinsic chemotactic capacity [8]. Furthermore, *ex vivo* mRNA levels for *CXCL1*, *CXCL2*, and *CXCL8* were low in the PMBCs from three patients with subcutaneous phaeohyphomycosis after 24 hours of stimulation with heat-killed *C. albicans*, *E. spinifera*, *O. musae*, and/or *C. cassiicola* [14]. The cells with impaired chemokine production upon fungal recognition remain to be identified. Overall, these data suggest that CARD9 plays a crucial role in the recruitment of neutrophils to the site of infection, by governing CXC-chemokine induction upon fungal stimulation, and to a lesser extent in the selective killing of some fungi (*C. albicans* yeast and *P. verrucosa* conidia) with lower levels of opsonins at their surfaces.

Impairment of IL-17 immunity

T cell-dependent IL-17 immunity was evaluated in 27 patients by flow cytometry ($n=24$), ELISA ($n=15$), or both ($n=12$) (Table 3) [1–3,5,7–10,14–17,22]. The proportion of IL-17-producing T cells (Th17) among PBMCs was determined *ex vivo*, by flow cytometry with intracellular anti-IL-17A mAb or pAb staining, after 12 hours of stimulation with anti-CD2/CD3/CD28 antibody-coated beads or PMA (phorbol myristate acetate)/ionomycin ($n=16$), after 48 hours of stimulation with heat-killed *C. albicans* ($n=2$) or *P. verrucosa* ($n=3$), or after 6 days of stimulation with *C. albicans*, *E. spinifera*, *O. musae* and/or *C. cassiicola* ($n=3$). Almost two thirds of the patients tested (15/24; 62.5%) had low proportions of Th17 cells, as shown by *ex vivo* stimulation with PMA/ionomycin and/or antibody-coated beads (7/16; 43.8%), or with heat-killed fungi (8/8; 100%) [1,2,10,14,15]. By contrast, nine patients (56.2%) had proportions of Th17 cells *ex vivo* within the control range following stimulation with PMA/ionomycin and/or antibody-coated beads [3,5,7,8,17,22]. The production of IL-17A by whole-blood cells or PBMCs was evaluated by ELISA, after 24

hours of stimulation with PMA/ionomycin ($n=7$), 48 hours of stimulation with *C. albicans* ($n=2$) or *P. verrucosa* ($n=3$), or 6 days of stimulation with *C. albicans*, *E. spinifera*, *O. musae* and/or *C. cassicola* ($n=3$). IL-17A production was low in three of seven patients (42.9%) after stimulation with PMA/ionomycin, and in all patients after 48 hours or 6 days of stimulation with heat-killed fungi (8/8; 100%) [9,10,14–16]. However, IL-17A production was normal in four patients (57.1%) after stimulation with PMA/ionomycin [3,22]. Twelve patients were tested both for the proportion of Th17 cells *ex vivo* and the production of IL-17A. Eight of these patients (66.7%) had low proportions of Th17 cells and impaired IL-17A production after 48 hours or 6 days of fungal stimulation, whereas no defect was detected after 24 hours of stimulation with PMA/ionomycin in four (33.3%) patients [3,10,14,15,22]. Thus, IL-17-dependent immunity was impaired to some extent in 18 of the 27 patients tested (66.7%), but to a lesser extent following stimulation with PMA/ionomycin or antibody-coated beads (10/19, 52.6%) than after stimulation with whole fungi (8/8, 100%).

Other studies have shown that inborn errors of IL-17 immunity underlie CMC, but not IFD [67–69]. However, only one third of the 18 CARD9-deficient patients with low proportions of Th17 cells and/or low IL-17A production in this study had CMC (6/18; 33.3%). Four patients with CMC had impaired IL-17 immunity in response to PMA/ionomycin and/or antibody-coated beads and two others had impaired IL-17 immunity in response to *C. albicans* [1,9,10]. By contrast, six of nine patients with normal IL-17 production upon stimulation with PMA/ionomycin had CMC [3,5,7,8]. However, PMA/ionomycin is not a physiological form of stimulation, so there might still be a defect of IL-17 immunity after *C. albicans* stimulation. Furthermore, the *ex vivo* evaluation of blood proportions of Th17 cells does probably not reflect the IL-17 immunity in mucosa, that could be impaired in CARD9-deficient patients and explained their CMC despite a normal Th17 blood count. How the decreased proportions of blood Th17 cells translate into impaired IL-17 immunity in mucosae, which is crucial for anti-*Candida* immunity, is unclear. Unfortunately, the local (skin/mucosa) IL-17 immunity has not been tested in CARD9-deficient patients and might be impaired, despite normal blood Th17 cell proportions, as it has been shown in patients with AR IL-17RA, IL-17RC, ACT1 or AD IL-17F deficiency [70–72]. CARD9 probably influences Th17 cell differentiation, possibly by inducing the production of pro-Th17 cytokines (e.g. IL-6, IL-23) by myeloid cells upon fungal recognition. CARD9-deficient patients may, therefore, develop CMC when their proportions of Th17 cells and IL-17A and IL-17F production decrease. However, other explanations remain plausible. CARD9 deficiency may result in impaired Th17 cell recruitment to the skin and mucosae due to lower levels of Th17-attracting chemokine (e.g. CCL20) production, as reported for neutrophils. In addition, it remains possible that other IL-17-producing cells [73] are impaired or have impaired function in CARD9-deficient patients. Finally, IL-17 immunity may play an important role in susceptibility to subcutaneous phaeohyphomycosis, as the six patients whose responses to stimulation with whole fungi tested all had low proportions of Th17 cells and low levels of IL-17 production [14,15]. In conclusion, CARD9 affects IL-17 immunity, but its precise role, and that of Th17 cells in the development of IFD remain unclear. Further studies are required to improve our understanding of the links between the

CARD9 immunity mediated by myeloid cells, and IL-17 immunity mediated by lymphoid cells.

Demographic, epidemiologic and general characteristics of patients

Most of the CARD9-deficient patients (40/55; 72.7%; no data available for 3 patients) came from consanguineous kindreds (21/36; 58.3%; no data available for 3 patients). The sex ratio was balanced (M:F of 32:26). The families originated from 14 countries on four continents (Africa, America, Asia, and Europe), and a large proportion of the patients (20/55; 36.4%; no data available for 3 patients) and families (11/36; 30.5%; no data available for 3 patients) came from North Africa, and the Middle East (19/55; 34.5% and 10/36; 27.8%, respectively) [1–11,14–23] (Table 1). These patients and families originated from Algeria ($n=12$, 6 families), Morocco ($n=3$, 2 families), Tunisia ($n=4$, 2 families), Egypt ($n=1$, living in France), Iran ($n=9$, 3 families), Turkey ($n=9$, 6 families), Pakistan ($n=1$), Angola ($n=1$, living in France), China ($n=7$, 7 families), South Korea ($n=1$), Italy ($n=1$, living in Brazil), Canada ($n=4$, 3 families), Germany ($n=1$) and El Salvador ($n=1$, living in the US). In addition, one patient had a mixed European origin, another was of Afro-American origin, and no data are available for another patient. The first clinical manifestations of a superficial or invasive fungal disease occurred between birth and the age of 48 years (mean age: 15.2 years; median age: 9 years), and the underlying *CARD9* mutations were generally identified a few years later, at ages ranging from four to 64 years (mean age: 28.3 years; median age: 25.5 years; data available for 34 patients). The oldest patient still living was 75 years old at the time of publication. A few patients had comorbid conditions (10.3%): type 2 diabetes mellitus ($n=3$), ventricular septal defect ($n=1$), hypoparathyroidism ($n=1$), and spastic paraplegia ($n=1$) (Table 3) [1,2,8,10]. One of the patients with diabetes mellitus had a non-insulin-dependent form, and another had insulin-dependent diabetes. Both were diagnosed with diabetes before the onset of IFD (no data were available for the third patient with diabetes). Spastic paraplegia is a neurodegenerative condition caused by heterozygous *SPAST* mutations; infectious complications have never before been reported in patients with this condition [74].

Invasive fungal diseases: the key clinical manifestation

Six of the 58 patients reported (10.3%) had only superficial fungal infections (CMC or superficial dermatophytosis [1,9,10]) at their last follow-up visit (age range [19 – 50 years]) (Table 1). The other 52 (89.7%) patients had IFD, with a mean age at onset of 22.1 years (median age: 18 years; range [3.5 – 52.0 years]) (Table 1). Twenty patients had both superficial and invasive infections (34.5%), with IFD beginning years after the superficial disease (mean time interval: 8.4 years; median time interval: 5 years). However, this is probably an underestimation, due to the lack of information about age at onset of IFD in many reports. In all but two of the patients, invasive infections (of one or multiple organs) and relapses were due to a single fungus. One patient displayed IFD caused by two different fungi: he suffered from invasive intra-abdominal candidiasis at nine years of age and a probable cerebral IA at 18 years, with an intra-abdominal relapse seven years later [8]. The other patient had a single relapsing IFD due to two different fungi: endophthalmitis due to *Aureobasidium pullulans* in one eye, and then the same condition caused by *C. albicans* in

the contralateral eye 15 years later [19]. None of the patients had persistent fungemia (proven positive blood culture), but 32.8% of patients (19/58) displayed disseminated disease, as defined by reaching two or more organs [2,3,6–8,10,15,18,21,22]. The clinical penetrance of fungal disease linked to *CARD9* deficiency is complete in all the patients described to date, albeit only by the age of 52 years. However, no single fungal disease has been reported to be common to all patients, suggesting incomplete penetrance for each of the fungal diseases identified in these patients. However, all otherwise healthy patients with deep dermatophytosis studied to date have been found to carry biallelic *CARD9* mutations. The fungal infections in *CARD9*-deficient patients are caused by a small number of fungi, and none of the patients was reported to have an infection due to other common opportunistic fungi (e.g. *Cryptococcus*, *Histoplasma*, *Coccidioides*, *Paracoccidioides*, *Pneumocystis* [75–77]). Likewise, no increased susceptibility to severe or uncommon viral, bacterial/mycobacterial or parasitic infections have been reported to date. One Turkish girl, from a consanguineous kindred, was recently reported to have a private homozygous *CARD9* mutation (p.V261fs*) and bloody pancolitis due to *Prototheca zopfii*, a microalga containing β -glucans, that can colonize the human gastrointestinal tract [78]. The V261fs* mutation has a high CADD score (23.4) and was predicted *in silico* to create a premature stop codon (at position 362), but neither its function nor its expression were studied and it was, not, therefore, clearly demonstrated that this *CARD9* mutation was responsible for the patient's disease. Interestingly, inherited homozygous mutations of *BCL10* and *MALT1*, partners of *CARD9* in the CBM, lead to combined immunodeficiencies (CID) and severe combined immunodeficiencies (SCID). Patients with AR *BCL10* or *MALT1* deficiency are susceptible to a broad spectrum of infectious diseases, including viral and bacterial infections, CMC, but not IFD, suggesting that the susceptibility of *CARD9*-deficient patients to IFD is largely independent of *BCL10* and *MALT1* [79–81].

Disease-causing fungi in patients with *CARD9* mutations

Only fungi from the phylum Ascomycota have been identified in these patients to date: *Candida*, *Trichophyton*, *Exophiala*, *Phialophora*, *Corynespora*, *Ochroconis*, *Aureobasidium* and *Aspergillus* (Table 1). There are eight fungal phyla, three of which (Ascomycota, Basidiomycota and Zygomycota) can cause disease in humans, but Ascomycota contains most of the fungi causing human diseases (Figure 4) [82]. The different phyla are defined on the basis of their sexual reproduction characteristics. All ascomycetes produce a similar structure, the ascus, a sac-like structure that contains the ascospores during sexual reproduction [83]. The ultrastructural morphology of the ascus is used to classify fungi to the subdivisions of this phylum, which contains eight orders, five of which contain fungi responsible for disease in *CARD9*-deficient patients (Figure 4). About 64,000 ascomycetes are known, making Ascomycota the largest of the fungal phyla [83]. The cell walls of all fungi (from all phyla) contain chitin, glucan and glycoproteins [84]. Each fungus, regardless of the phylum in which it belongs, has a specific structure, which can contribute to its resistance to host defense mechanisms. Moreover, ascomycete morphology is diverse. These fungi can form yeasts, filaments (hyphae/pseudohyphae) and more complex forms. Some ascomycetes causing human diseases have not yet been described in *CARD9*-patients. This may be because *CARD9*-deficient patients described to date have not been sufficiently

exposed to the agents of endemic mycoses, such as histoplasmosis, (para)-coccidioidomycosis, or blastomycosis, even if they lived in endemic zones. CARD9-deficient patients infected with these fungi may be identified in the future. The only exception could be pneumocystis pneumonia (due to *Pneumocystis jirovecii*), as usually such infections occurred very early in life in patients with inborn errors of T cell immunity, such as SCID, HLA-II-deficient, or CD40/CD40L-deficient patients [85]. In conclusion, CARD9-deficient patients are susceptible to a narrow spectrum of fungi from the phylum Ascomycota, suggesting a specific role of CARD9 in anti-ascomycete immunity. As suggested in a previous study, susceptibility to a narrow spectrum of pathogens reflects a *high degree of redundancy* of the gene in host defense, whereas susceptibility to a large spectrum of pathogens reflects a *low level of redundancy* of the gene [86]. *CARD9* is the perfect example of a *highly redundant gene*: patients are susceptible exclusively to a narrow spectrum of fungi. In CARD9 deficiency, penetrance is complete for fungal infection, but incomplete for each type of fungus. These data suggest that although human CARD9 is essential for host defense against ascomycetes, in the sense that each patient is vulnerable to at least one ascomycete, it is also largely redundant, in the sense that all patients are resistant to most ascomycetes.

Ascomycetes causing disease in patients with CARD9 mutations

Candida spp. are dimorphic fungi that can grow as yeast or filamentous forms [87]. *C. albicans*, the most frequent of the *Candida* spp., is a commensal yeast colonizing the digestive tract, urogenital mucosae and skin of healthy individuals [87]. However, when immunity is compromised, *C. albicans* can cause mucocutaneous candidiasis (CMC) or invasive infections [70,88,89]. Hyphae seem to play an important role in tissue invasion [87]. Other *Candida* spp. of clinical importance in terms of human disease include *C. glabrata* and *C. dubliniensis*, which have also been reported in CARD9-deficient patients [3,16], and *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (not yet reported in CARD9-deficient patients) [90]. *Trichophyton* spp. are filamentous fungi from the dermatophyte group. They are classified into geophilic, zoophilic, and anthropophilic groups according to their usual habitat, in soils, animals, and humans, respectively. The most frequent mode of contamination is the inter-human transmission of anthropophilic dermatophytic spores [91,92]. *T. rubrum* and *T. violaceum* are the major anthropophilic pathogenic species worldwide, and both have been reported in CARD9-deficient patients. The transmission to humans of zoophilic dermatophytes from infected animals (e.g. dogs or cats), mostly *T. mentagrophytes* (found in CARD9-deficient patients), or *Microsporum canis*, occurs more frequently than the sporadic transmission of geophilic dermatophytes, of which *M. gypseum* is the main human pathogen. Dermatophytes are keratinophilic fungi responsible for common superficial dermatophytosis in both temperate and tropical countries. This condition affects 20–25% of the general population worldwide [91,92], and severe invasive infections have been reported in patients with acquired immunodeficiencies [93]. Dematiaceous fungi form a group of pigmented filamentous ascomycete molds (growing as multicellular filaments known as hyphae) containing more than 100 subtropical or tropical species with brown melanin in their cell walls [94]. The principal pathogens of relevance in human disease include *Exophiala* and *Phialophora*, both of which have been reported in CARD9-deficient patients

[14,15,22], and *Alternaria*, *Curvularia* and *Cladophialophora*. More rarely *Aureobasidium*, *Corynespora* or *Ochroconis* may cause human disease [94–97]; all three have been reported in CARD9-deficient patients [14,19,23]. They commonly cause a range of diseases, including phaeohyphomycosis, a general term defining a broad spectrum of infections from chronic subcutaneous/cutaneous and corneal infections to invasive infections; chromoblastomycosis, a localized subcutaneous infection displaying no dissemination; and mycetoma, a cutaneous infection characterized by ulcerative masses potentially involving bones. Subcutaneous inoculation commonly occurs after traumatic lesions and causes local disease. By contrast, disseminated infections are rare and occur after the inhalation of hyphae or conidia, in patients with acquired immunodeficiencies [94,98–100]. Chronic sinusitis (which may extend to the orbit and brain) and allergic sinusitis are frequent manifestations in the general population worldwide [98]. *Aspergillus* spp. live in organic debris and soils. Conidia are frequently inhaled but rarely cause disease, which is characterized by three clinical manifestations. Allergic bronchopulmonary aspergillosis and chronic pulmonary aspergillosis occur in apparently “immunocompetent” individuals or patients with “mild” risk factors, respectively, and have never been reported in CARD9-deficient patients [101]. Invasive aspergillosis (IA) is a severe pulmonary or, more rarely, disseminated infection occurring mostly in immunodeficient patients with neutrophil defects [102,103]. About 90% of aspergillosis cases in humans are due to *Aspergillus fumigatus*, but other species, such as *A. flavus*, *A. niger*, *A. terreus* and *A. nidulans*, may also cause disease [101,102]. However, *A. fumigatus* is the only pathogen from this genus to have been reported in CARD9-deficient patients to date [8].

Invasive *Candida* infections

Candida infections occurred in 29 patients (50%) (Tables 1 and 4); 22/29 (75.9%) patients had CMC, 21/29 (72.4%) had invasive infections, and 14/29 (48.3%) had both superficial and invasive candidiasis. CMC was the only clinical manifestations in four patients (4/29; 13.8%) [1,10]. The onset of CMC was variable, occurring at a mean age of 12.9 years (median age: 8 years; range [birth – 42 years]). Of the 21 patients with invasive candidiasis, 17 (17/21; 80.9%) had a probable or proven CNS infection occurring with ($n=12$) or without ($n=5$) CMC. CNS infections affect the meninges, cerebral parenchyma, and basal ganglia, resulting in clinical meningo-encephalitis (5/17; 29.4%), brain abscesses or single/multiple masses mimicking metastasis (5/17; 29.4%) or a combination of these manifestations (7/17; 41.2%) [1,3,5–7,9–11,16,17]. Analysis of CSF samples from patients have revealed pleocytosis with mostly mononuclear cells (lymphocytes and/or monocytes) and eosinophils but no neutrophils, hyperproteinorrhachia and hypoglycorrachia [3,7,9,16]. Three of these patients also suffered from severe colitis [3], multifocal vertebral osteomyelitis [7], or endophthalmitis with cervical spine osteomyelitis [6]. One adolescent was reported to have fungal masses in the brain, which brain biopsy revealed to contain conidia and non-septate hyphae compatible with *Candida* spp. or *Aspergillus* spp., but no culture was performed [11]. However, this fungal CNS infection was considered to be a *Candida* infection rather than an *Aspergillus* infection as the patient also suffered from proven oral CMC. Four patients were reported to have invasive *Candida* infections without CNS involvement: relapsing endophthalmitis [19], endophthalmitis with hip osteomyelitis [18], severe colitis

[3], and intra-abdominal candidiasis (liver and mesenteric LNs) [8]. The onset of invasive disease varied, occurring at a mean age of 21.9 years (median age: 17.5 years; range [3.5 – 58.0 years]). The CARD9-deficient patients with CMC and/or invasive *Candida* infections came from nine countries worldwide (Algeria, Morocco, Iran, Turkey, Pakistan, Canada, Italy, El Salvador and South Korea) and one patient was of mixed European origin. Unsurprisingly, of all *Candida* infections (superficial and invasive), *C. albicans* was the most frequently involved, being found in 72.4% ($n=21$) of the patients; *C. dubliniensis* and *C. glabrata* were each found in one patient [3,16] (Table 1). About 40 cases of “idiopathic” *Candida* CNS infection and one case of *Candida* endophthalmitis have been reported worldwide [3,104]. Some of the affected individuals may suffer from CARD9 deficiency. But all reported cases of other invasive *Candida* infections were associated with acquired immunosuppressive risk factors [3,105–108]. In summary, CARD9 deficiency confers a predisposition to both superficial and invasive *Candida* infections, and particularly to *Candida* infections of the CNS, even though *Candida* spp. are not typically considered a neurotropic pathogen.

Invasive dermatophytosis

Dermatophytosis was reported in 29 patients (50%) (Tables 1 and 4); 8/29 (27.6%) patients had superficial infections and 21/29 (72.4%) patients had extensive or deep infection. Superficial dermatophytosis, also known as ringworm or “tinea”, is a benign localized disease of the stratum corneum of skin (“tinea corporis”), scalp (“tinea capitis”) or nails. Isolated “tinea corporis” was reported in two patients [1,9], whereas, in six other patients, this condition was associated with CMC ($n=2$) [1], invasive *Candida* infections ($n=3$) [6,9] or intra-abdominal aspergillosis ($n=1$) [8]. The onset of superficial disease occurred at ages of 3 to 42 years (mean age: 17.7 years; median age: 8 years). Extensive/deep dermatophytosis is rare and has been reported worldwide in about 65 immunocompromised patients, mostly after solid organ transplantation (SOT) [93,109–112]. The first description of “dermatophytic-disease” in otherwise healthy individuals was published in 1959 and characterized by the progression of a superficial infection to the deep dermis and hypodermis, resulting in skin ulcerations and abscesses associated with the destruction of soft tissues and highly mutilating lesions [113]. Internal organs (e.g. LNs, brain, liver, digestive tract, and bone) may be involved and damaged [93,114,115]. Up to 63 cases of deep dermatophytosis have been reported in otherwise healthy individuals, mostly originating from and living in North Africa ($n=45$) [2]. In 2013, 17 such patients from eight consanguineous kindreds were reported to display deep dermatophytosis and CARD9 deficiency [2]. All these patients came from North Africa: Algeria ($n=11$), Morocco ($n=2$) and Tunisia ($n=4$). Four additional patients have since been described, from Turkey ($n=1$) [10], Egypt ($n=1$, living in France) [20], Italy ($n=1$, living in Brazil) [4], and Algeria ($n=1$) [21]. All these patients had skin lesions characterized by nodules, painful infiltrated plaques, severe pruritic rash with scraping lesions and ulcerations. LNs were involved in 11 patients, in some of whom brain ($n=2$), bone ($n=1$) and perineum ($n=1$) involvement were also noted [2,10,21]. These clinical presentations are not significantly different from those previously described in patients with acquired immunodeficiencies [93]. In addition to invasive disease, four patients suffered from oral CMC [2,4,10]. The onset of invasive disease varied,

occurring at a mean age of 24.1 years (median age: 19 years; range [12 – 52 years]). *T. violaceum* (31.0%; *n*=9) and *T. rubrum* (27.6%; *n*=8) were the two most common dermatophytes found in these patients, consistent with the distribution of *Trichophyton* in the general population (Table 1). Overall, CARD9 deficiency confers a predisposition to superficial and deep dermatophytosis, and should especially be considered in patients from North Africa and Middle-East.

Invasive phaeohyphomycosis

Phaeohyphomycosis has been reported in 10 patients (10/58; 17.2%) (Tables 1 and 4) [14,15,19,22,23]. The first report of phaeohyphomycosis related to CARD9 deficiency described four unrelated Chinese patients with persistent red plaques and ulcerative nodules on the face, scalp and/or ears due to *P. verrucosa* [15]. Three additional unrelated Chinese patients with subcutaneous facial infections due to *C. cassiicola* (generally a plant pathogen), *E. spinifera* or *O. musae* (new combined name of *Scolecobasidium musae* and *Ochroconis mirabilis* [97]), respectively, have been reported [14,23]. Facial lesions were described, with chronic, red, extensive, infiltrative, purulent, painful plaques and papules associated with post-auricular LNs in one patient [23] and progressing to the trunk and limbs in another [14]. The clinical characteristics of the skin lesions of CARD9-deficient patients are similar to those previously reported in both immunocompetent patients and patients with acquired immunodeficiencies (e.g. on immunosuppressive drugs, SOT, human immunodeficiency virus (HIV) infection). Indeed, the lesions were typically described as a rash that darkened, forming painless nodules or masses progressing to cystic abscesses and ulcerations [100]. Two additional patients with invasive *Exophiala* infection have been described [22]. A liver and biliary tract infection due to *E. dermatitidis* (the most common *Exophiala* spp. causing human disease, also known as *Wangiella dermatitidis* [116]), followed by a severe brain relapse (complicated pachimeningitis) after 25 months was described in a patient originating from Angola. An Iranian patient had a subcutaneous (nodules with LN involvement) and bone infection due to *E. spinifera*. This patient died from a lung relapse five years later (Dr Davood Mansouri, personal communication). Finally, a German patient had endophthalmitis (left eye) due to *A. pullulans* leading to a complete loss-of-vision, with a relapse 15 years later in the form of endophthalmitis (right eye) due to *C. albicans* [19]. Overall, the mean age at disease onset for CARD9-deficient patients with phaeohyphomycosis (subcutaneous and invasive) was 24.6 years (median age: 19 years; range [5 – 48 years]). In published cases, CNS involvement, characterized by single or multiple brain masses, is the most common clinical presentation of disseminated infections in both immunocompetent patients and patients with acquired immunodeficiencies, including, in particular, those with cancers or undergoing SOT (about 100 reported cases, reviewed in [117]). Nevertheless, CNS involvement in immunocompetent patients was mostly secondary to sinusitis. Other organs (e.g. lungs, heart, bones, joints) are rarely involved and such involvement is mostly reported in patients with acquired immunodeficiency (e.g. SOT or HIV infection) [98,100,117]. About 32 cases of “idiopathic” phaeohyphomycosis caused by fungi found in CARD9-deficient patients have been reported in the literature: two subcutaneous infections due to *P. verrucosa* in Asia [118], 23 invasive infections due to *E. dermatitidis* and three due to *E. spinifera* in patients from around the

world [22], and four skin and invasive infections due to *A. pullulans* in Norway, China and Nepal [119–121]. Some of these patients probably have CARD9 deficiency. Subcutaneous *C. cassiicola* infections have been reported in four patients with risk factors (trauma or diabetes mellitus) in Africa and Asia [122–125]. No other case of *O. musae* infection has been reported. Thus, CARD9 deficiency confers high susceptibility to subcutaneous infections with dematiaceous fungi, particularly in China, where phaeohyphomycetes are endemic. To date, no case of sinusitis has ever been reported in CARD9-deficient patients and only one patient has been shown to have CNS involvement. These common manifestations of phaeohyphomycosis in the general population thus appear to be rare in patients with CARD9 deficiency.

Invasive aspergillosis

One study reported two CARD9-deficient patients with extrapulmonary IA (2/58; 3.4%) (Tables 1 and 4) [8], a life-threatening infection mostly observed in patients with acquired immunodeficiencies, particularly those with neutrophil defects (number or function). Its incidence is about 5 to 25% in patients with acute leukemia (AL) and about 5 to 10% in patients who have undergone allogeneic hematopoietic stem cell transplantation (HSCT) [102]. The main clinical manifestations of IA are acute pulmonary infections and acute invasive rhinosinusitis. Disseminated extrapulmonary IA is rare. It mostly involves the brain, but also the bones, eyes, skin, LNs, liver, digestive tract, kidneys and urinary tract, and generally causes the formation of masses in the affected organs, with or without concomitant fungemia [101,102,126]. The first CARD9-deficient patient reported was of European origin and had strongly suspected brain IA, characterized by cerebral masses in the thalamus and capsula interna, disseminating a few years later to the liver and mesenteric LNs. Biopsies revealed the presence of septate hyphae compatible with *Aspergillus* spp. Nine years earlier, he had had invasive intra-abdominal candidiasis (liver and mesenteric LNs). The second patient, of Afro-American origin, was reported to have proven intra-abdominal aspergillosis due to *A. fumigatus* described clinically as necrotizing granulomatous masses encompassing celiac, mesenteric and renal vessels [8]. Neither of these patients had pulmonary IA or sinusitis, the most common clinical forms of this infection. In these two patients, disease onset occurred at eight and 18 years, respectively (mean and median age: 13 years). More than 300 cases of extrapulmonary IA in the absence of identified risk factors have been reported (reviewed in [13]), and some of these patients may have CARD9 deficiency. In conclusion, CARD9 deficiency confers a predisposition to uncommon and severe extrapulmonary IA in apparently healthy individuals worldwide. Nevertheless, the description of new cases would improve the clinical, microbiological and geographic description of IA in CARD9 deficiency.

Immunological features of Card9-deficient mice

CARD9 immunity has been studied in a *Card9* knockout (*Card9*^{-/-}) mouse model. *Card9*^{-/-} mice were born alive with no anatomical abnormalities and they grew normally [32,33]. The development of neutrophils and monocytes was not impaired in *Card9*^{-/-} mice [7,33,127]. No differences in basal immunoglobulin levels (total IgA, IgM, IgG and subtypes of IgG) were observed between *Card9*^{-/-} and WT mice [32,33]. Unfortunately, IgE levels and

eosinophil counts were not evaluated. Extensive immunophenotyping of T and B cells revealed that each of the cell subsets developed normally, and T-cell proliferation after stimulation with anti-CD3/CD28 antibody-coated beads or PMA/ionomycin was similar in *Card9*^{-/-} and WT mice [32,33]. Functional studies *in vitro* showed that pro-inflammatory cytokine production was impaired in *Card9*^{-/-} mice, consistent with human data. Indeed, *Card9*-deficient BMDCs and BMMs displayed a strong dose-dependent impairment of IL-1 β , IL-2, IL-6 and TNF- α production after 48 hours of stimulation with zymosan, heat-killed *C. albicans*, *C. tropicalis*, *P. verrucosa* and/or *E. spinifera* [32,41,66,127]. Cytokine production in response to *A. fumigatus* stimulation was not assessed. Post-infection pro-inflammatory cytokine (IL-1 β , IL-6 and TNF- α) levels *in vivo* were determined in footpad homogenates from mice infected with *P. verrucosa* or *E. spinifera*, three days and one week after infection. These levels were markedly lower in *Card9*^{-/-} mice than in their WT counterparts [14,66]. Thus, mice with CARD9 deficiency are viable and display no particular impairment of myeloid or lymphoid cell development. Functionally, pro-inflammatory cytokine production by myeloid cells from *Card9*^{-/-} mice in response to fungal stimulation (agonists and whole fungi) is strongly impaired, both *in vitro* and *in vivo* (Table 5). However, no specific cellular phenotype of *Card9*^{-/-} mice has been identified, due to the absence of studies in mice with conditional knockouts of *Card9*^{-/-} in particular cell subtypes. Such studies would provide strong evidence concerning the type of immune (hematopoietic and non-hematopoietic) cells involved in CARD9-immunity, their contribution to clinical phenotype and their individual role in anti-fungal immunity.

Candida infections in Card9-deficient mice

Susceptibility to superficial and invasive *Candida* infections has been assessed in *Card9*^{-/-} mice, in a model of oropharyngeal candidiasis (OPC) and a model of intravenous infection, respectively. The role of CARD9 in immunity to OPC was studied in a rechallenge model of local infection. *Card9*^{-/-} mice were challenged sublingually with *C. albicans*, then rechallenged after six weeks; they developed OPC during the second infection [128]. The proportion of Th17 cells and IL-17A production measured in cervical LNs were both significantly impaired in *Card9*^{-/-} mice relative to the WT 24 hours after rechallenge with heat-killed *C. albicans* [128]. These data suggest that susceptibility to OPC is dependent on CARD9, through IL-17 adaptive immunity. Invasive *Candida* infection was mimicked by the intravenous injection, into *Card9*^{-/-} mice, of live *C. albicans* or *C. tropicalis* yeasts. The mice failed to control systemic infections, with 100% of mortality recorded after 5 or 10 days, respectively [32,127]. Uncontrolled disseminated infections, characterized by a high fungal load in internal organs (e.g. brain, kidneys, liver), were observed three and five days post-infection, respectively [7,32,127]. The development of *C. albicans* infection of the CNS in *Card9*^{-/-} mice was explained by brain-specific neutropenia due to a lack of CNS CXC-chemokine induction [7]. CXC-chemokine induction, as assessed by determining chemokine (e.g. CXCL1 or CXCL2) levels, was proportional to fungal load in affected organs 72 hours post-infection in WT mice and was associated with massive neutrophil recruitment. *Card9*^{-/-} mice displayed a defect of CXC-chemokine induction (low chemokine levels measured), associated with an impairment of neutrophil recruitment specific to the CNS and not observed in other organs (e.g. kidneys) 72 hours post-infection (or even at 24

hours). The CNS neutropenia observed in *Card9*^{-/-} mice was not associated with peripheral neutropenia or bone-marrow insufficiency. The intrinsic trafficking capacity of neutrophils for mobilization was similar between *Card9*^{-/-} and WT mice. This defect was also specific to *C. albicans*, as CNS neutrophil recruitment was not impaired in either *Card9*^{-/-} or WT mice 48 hours after the intravenous injection of *S. aureus*, whereas mice displayed concomitant bacterial dissemination to the brain [7]. This suggests that brain neutrophil recruitment during bacterial infection is CARD9-independent and that CARD9 deficiency would not result in a predisposition to bacterial infections of the brain, consistent with the human data. Brain IL-17 (mRNA and protein) levels were similar between *Card9*^{-/-} and WT mice, suggesting that IL17 immunity is not involved in the development of CNS *Candida* infection. The capacity of neutrophils to kill *C. tropicalis* evaluated *ex vivo* was similar in WT and *Card9*^{-/-} mice, whereas neutrophil recruitment was not studied [127]. IL-17 immunity did not seem to be impaired, as *Il17* mRNA levels in the kidneys were similar in WT and *Card9*^{-/-} mice. Thus, consistent with human data, these findings suggest that mouse CARD9 plays a key role in brain neutrophil recruitment during *C. albicans* infection, by inducing CXC-chemokine production.

Phaeohyphomycosis in Card9-deficient mice

Phaeohyphomycosis was modeled by the subcutaneous injection of live *P. verrucosa* or *E. spinifera* conidia into the footpads of *Card9*^{-/-} mice [14,66]. Chronic infections with large ulcerative and abscessed lesions occurred in *Card9*^{-/-} mice, whereas WT mice had small self-healing cutaneous lesions eight weeks after infection. Moreover, dissemination of the subcutaneous infection to internal organs (e.g. brain, kidneys, LNs, lungs, liver and spleen) was observed in 100% of *Card9*^{-/-} mice and was responsible for their death 12 weeks after infection, whereas no dissemination was observed in WT mice, all of which were alive after 12 weeks. Neutrophil recruitment was strongly impaired in *Card9*^{-/-} mice infected with *E. spinifera*, as shown by comparison with the WT [14]. Indeed, neutrophils were absent from the skin biopsy specimens of *Card9*^{-/-} mice, and the percentage of neutrophils was significantly lower than that in the WT in single-cell analyses of footpad homogenates three days after infection. Accordingly, low levels of *Cxcl1* and *Cxcl2* chemokine mRNA were measured in footpad homogenates three days after infection [14]. Moreover, BMDM phagocytosis and the killing of *P. verrucosa* were assessed in *in vitro* assays, which showed conidial uptake and the increase in ROS production to be similar in *Card9*^{-/-} and WT mice [66]. However, CARD9-deficient mice displayed a strong impairment of BMDM killing. Impaired phosphorylation of p65 (NF- κ B) and p38 (MAPK) was observed in response to stimulation for 30 or 60 minutes with heat-killed *E. spinifera*, and this resulted in impaired pro-inflammatory cytokine and chemokine production [14]. IL-17a production levels in footpad homogenates one week post-infection were significantly lower in *Card9*^{-/-} mice infected with *P. verrucosa* or *E. spinifera* than in the WT [14,66]. Similarly, in an *in vitro* T cell differentiation assay (allogeneic naïve T cells co-cultured with BMDCs), the proportion of Th17 cells after 24 hours of stimulation with *P. verrucosa* was lower in *Card9*^{-/-} mice [66]. As in humans, IL-17 immunity may play a key role in the development of subcutaneous phaeohyphomycosis. The defect of Th17 differentiation in *Card9*^{-/-} mice may also be due to defective pro-Th17 cytokine production by myeloid cells in response to fungal

stimulation. Mouse subcutaneous phaeohyphomycosis reproduces the clinical and histological phenotype of CARD9-deficient patients. Consistent with human data, Card9 plays a key role in neutrophil recruitment in mice, by regulating chemokine production upon *E. spinifera* stimulation.

***Aspergillus* infection in Card9-deficient mice**

Susceptibility to *A. fumigatus* was studied in a pulmonary infection model based on the intratracheal injection of large numbers (7×10^7) of conidia. *Card9*^{-/-} mice developed exclusively pulmonary infections, with no extrapulmonary manifestations, after 12–24 hours, contrasting with findings for humans [129,130]. A lack of neutrophil recruitment was observed in the lungs of *Card9*^{-/-} mice, consistent with observations in mesenteric LNs of CARD9-deficient patients with intra-abdominal aspergillosis [130]. As in mice infected with *Candida* or *Exophila*, a defect of CXC-chemokine production, as shown by lung chemokine mRNA levels, was observed in *Card9*^{-/-} mice relative to the WT. However, in this model, CARD9 was required for neutrophil recruitment to the lung only at later (more than 24 hours) stages of infection, whereas this process was MyD88-dependent at earlier stages. These data again suggest that neutrophil recruitment to the site of infection is CARD9-dependent. Contrary to findings for humans, *Card9*^{-/-} mice had much weaker neutrophil-killing activity against *A. fumigatus* *in vitro* than WT mice [129]. The isolated lung infection may be due to the number of conidia inhaled by the *Card9*^{-/-} mice: too many for the clearance of the fungus from the lungs by macrophages and/or epithelial cells resulting in the development of lung infection immediately after inoculation with conidia. It also seems likely that pulmonary IA will be reported in future CARD9-deficient patients. In conclusion, mouse models of superficial and invasive infections with fungi causing disease in humans mimic human infections, with the exception of IA, as *Aspergillus* caused only lung disease in *Card9*^{-/-} mice (Table 5). *Card9*^{-/-} mice have not been tested for superficial and deep dermatophytosis. Such models would help to identify the critical immune cells involved in immunity to fungal infections and to decipher the role of CARD9 in pathogenesis.

Other fungal infections in Card9-deficient mice

Card9^{-/-} mice are susceptible to additional ascomycetes not yet identified in human CARD9-deficient patients, such as *Coccidioides posadasii*, but also to basidiomycetes such as *Cryptococcus neoformans*. In a model of coccidioidomycosis, the subcutaneous injection of *C. posadasii* spores into *Card9*^{-/-} mice led to the development of massive necrotizing subcutaneous abscesses and disseminated infections in the lungs and spleen 16 days after challenge. The mice failed to control infection, with 100% of mortality achieved after 40 days [131]. The authors suggested that the susceptibility of *Card9*^{-/-} mice to infection was due to an impairment of IFN- γ production by skin CD4⁺ T cells. A defect of recruitment of neutrophils, T- and B cells was identified by comparing the skin of deficient and WT mice. However, chemokine production was not studied. *Card9*^{-/-} mice were shown to be susceptible to *C. neoformans* in a model of pulmonary infection based on the intratracheal injection of live yeasts [132]. Infected *Card9*^{-/-} mice displayed massive yeast multiplication and had a high lung fungal load without granulomatous responses in the alveolar spaces 14 days post-infection. The susceptibility of deficient mice to *C. neoformans* was explained by

impaired IFN- γ production by NK and T (CD44^{bright} subset) cells, which were poorly recruited to the lungs. Like the defect of neutrophil recruitment observed during fungal infection, the lack of NK- and T-cell recruitment was shown to result from defective chemokine (CXCL4, CXCL9, CXCL10) production by the lung DCs. However, no cases of coccidioidomycosis or cryptococcosis have been observed in CARD9-deficient patients, probably because these infections are experimental, as opposed to natural infections, and they do not reflect host defense *in natura* [133,134]. However, it would not be surprising if new patients with infections due to *Coccidioides* or other ascomycetes were reported in the future. By contrast, we would not expect to see *Cryptococcus* in new CARD9-deficient patients, as this fungus belongs to phylum Basidiomycota.

Bacterial and viral infections in Card9-deficient mice

Card9^{-/-} mice are susceptible not only to fungal diseases, but also to intracellular bacterial infections. In a model of pulmonary tuberculosis, mice were infected with a virulent *Mycobacterium tuberculosis* strain by aerosol inoculation [135]. All infected *Card9*^{-/-} mice died from pyogranulomatous pneumonia on day 34 post-challenge and lung histology showed a higher mycobacterial load, with the massive recruitment of neutrophils, contrasting with the lack of neutrophil recruitment observed during fungal infections. The internalization and killing of *M. tuberculosis* by BMDMs *in vitro* after IFN- α stimulation was similar in *Card9*^{-/-} and WT mice [135]. *Card9*^{-/-} mice challenged with live *Listeria monocytogenes* in an intravenous infection model displayed impaired bacterial clearance, with a high bacterial load in the liver and spleen after two days of infection [26,136]. BMDMs from *Card9*^{-/-} mice had impaired *L. monocytogenes*-killing capacity *in vitro* relative to BMDMs from WT mice [136]. In both models, *in vitro* BMDCs or *in vivo* macrophages from *Card9*^{-/-} mice displayed highly impaired cytokine production (IL-1 β , IL-6, TNF- α , and IL-12) after 24 hours of stimulation with *M. tuberculosis*-derived molecules (e.g. peptidoglycan) [135] or three hours after infection with live *L. monocytogenes* [26,136]. Neutrophil recruitment and CXC-chemokine induction were not studied. Interestingly, in a model of severe influenza pneumonia caused by intratracheal inoculation with a lethal dose of virus, *Card9*^{-/-} mice were found to be protected [137]. In conclusion, mouse CARD9 plays a key role in cytokine production by myeloid cells in response to both bacterial and fungal stimulation. However, like the greater susceptibility to non-ascomycete fungi observed in *Card9*^{-/-} mice, but not in CARD9-deficient patients, intracellular bacterial infections would not be expected in CARD9-deficient patients. Indeed, the impairment of CARD9-dependent neutrophil recruitment through the defect of CXC-chemokine induction seems to play a crucial role in fungal infections whereas this recruitment is not impaired during bacterial infections, suggesting that there are CARD9-independent mechanisms of host defense against intra-cellular bacteria, resulting in protection from these infections in CARD9 deficiency. There are, thus, differences in infection spectrum between mouse and human CARD9 deficiencies (Table 5). Indeed, human CARD9 deficiency underlies a narrow spectrum of fungal infections, restricted to a single fungal phylum, and therefore reflects a *high degree of redundancy* of human *CARD9* [86]. By contrast, in mice, *Card9* deficiency leads to a larger spectrum of both fungal (from different phyla) and bacterial infections, suggesting a *lower level of redundancy* for mouse

Card9. Mouse studies remain useful for studies of the pathogenesis of human diseases, but it should always be borne in mind that mouse models are artificial. Caution is therefore required when extrapolating data from mouse models to humans.

Diagnosis of inherited CARD9 deficiency

The clinical penetrance of IFD in CARD9-deficient patients is globally complete by the age of 52 years. The number of fungi identified as disease-causing in these patients is growing. The main acquired risk factors for IFD are HIV infection, hematological malignancies, HSCT, SOT, immunosuppressive therapies and chemotherapy [138–141]. All children and adults with unexplained IFD should be screened for *CARD9* mutations by gene sequencing. Only a few other PIDs have been reported to underlie IFD, typically in the context of other infectious diseases due to viruses (e.g. SCID, AD GATA2 deficiency), bacteria (e.g. SCID, AD-hyper IgE syndrome [AD-HIES]), mycobacteria (e.g. SCID, AR IL-12R β 1 deficiency) and/or parasites (e.g. SCID, X-linked hyper-IgM syndrome [XL-HIGM]) (Table 6, reviewed in [142]). Therefore, depending on the fungal disease, other genes, in addition to *CARD9*, should be screened. Invasive *Candida* infections are rarely reported but mostly occur in patients with HIGM, and exceptional cases have been described in other PIDs associated with syndromic IFD (see Table 6) [143–147]. The main genetic etiology of IA is CGD, but cases have also been reported in patients with AD/AR- severe congenital neutropenia (SCN), AD GATA2 deficiency, AD STAT1 gain-of-function, or AD-HIES [143–145,148,149]. By contrast, inherited CARD9 deficiency is currently the only PID associated with invasive dermatophytosis or phaeohyphomycosis (with the exception of rare cases reported in CGD-patients). Endemic mycoses or cryptococcosis have never been reported in CARD9-mutated patients, but may occur in some patients with inborn errors of the IL-12/IFN- γ axis (e.g. *IL12RB1*, *IFNGR1*) [150]. *P. jirovecii* infections typically occur in patients with inherited T-cell defects, such as SCID (more than 30 genetic etiologies), HLA-II deficiency, or defects disrupting CD40-CD40L interaction (e.g. AD/AR anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) or HIGM), which are generally diagnosed in the first few months of life [146,151,152]. If no mutation in any of the known candidate genes is found by gene sequencing, analysis by next-generation sequencing (NGS), including whole-exome sequencing (WES) or whole-genome sequencing (WGS), should be performed to identify new variants of known PID-related genes or new PID genes [53]. Furthermore, as CARD9 deficiency is a recessive defect, genetic analyses should be performed on other members of the patient's family, and genetic counseling should be proposed before conception.

Treatment of fungal diseases in CARD9-deficient patients

CMC is managed by life-long treatment with topic azole agents as first-line treatment. Systemic agents (e.g. azole agents, echinocandins) are used for extensive or uncontrolled disease and for cases not responding to topical agents [153]. Invasive *Candida* infections must be treated intravenously (e.g. with echinocandins, fluconazole or liposomal amphotericin B (AmB) with flucytosine) for 1–2 weeks and then with oral fluconazole consolidation therapy [154]. Treatment duration depends on the organs affected and should be adapted according to the clinical, radiological and biological responses of the patient (e.g. sterilization of the CSF in cases of CNS candidiasis). Surgery should be performed for intra-

abdominal masses and endophthalmitis. Superficial dermatophytosis is treated with topical agents as a first-line treatment (e.g. terbinafine, ciclopirox olamine) [155,156]. Treatment duration ranges from six weeks to one year, depending on the molecule used and the type of lesion. Onychomycosis and scalp lesions are treated with systemic antifungal agents as a first-line treatment (mostly azole agents) for six to 12 months. Extensive/deep dermatophytosis is treated with systemic agents (e.g. terbinafine, griseofulvin, azole agents) for several months and, if the disease becomes mutilating, surgery may be performed to control progression [155,156]. However, no guidelines have been published and there are no standardized recommendations for treating phaeohyphomycosis. However, oral azole therapy (mostly with itraconazole) for several months is considered a treatment of choice for subcutaneous infection. In cases of extensive skin lesions, local excisions may be performed [98,157,158]. The most appropriate treatment for disseminated phaeohyphomycosis is unclear, but combinations of systemic antifungal therapies (e.g. voriconazole or posaconazole plus echinocandin) may be effective [158]. Brain abscesses should be completely removed by surgery if possible. In the latest update of the guidelines for IA treatment, systemic voriconazole is recommended as the first-line treatment, for a minimum of 6–12 weeks, depending on site of infection. Systemic liposomal AmB or isavuconazole are possible alternative treatments [159].

Immunotherapeutic approaches in patients with **CARD9** mutations

In terms of clinical outcome, alternative/adjuvant treatments may be required to cure patients or to control the fungal disease. Defects of GM-CSF production by PBMCs and monocytes in response to stimulation with zymosan or *C. albicans* were observed in five patients with *Candida* infections of the CNS [6,7,17]. The impaired GM-CSF production by myeloid cells was initially related to the impaired ERK phosphorylation downstream HRAS/Ras-GFR1/ERK pathway after stimulation with zymosan in patients homozygous for Y91H or compound heterozygous for *c.-529T>C/c.271T>C* (p.Y91H) alleles [6]. Based on these data, three CARD9-deficient patients with relapsing *Candida* CNS infection were successfully treated with GM-CSF (*n*=2) [6,17] or G-CSF (*n*=1) [9] immunotherapy as an adjuvant to standard oral azole therapy. Complete clinical recovery was reported with CSF sterilization after six days of subcutaneous GM-CSF (500 µg/d), or three weeks of subcutaneous G-CSF (450 µg twice/week). After 18 months of subcutaneous GM-CSF (250 µg/d) with oral voriconazole, or three months of subcutaneous G-CSF (450 µg twice/week) with oral fluconazole, adjuvant treatments were stopped, and no relapse occurred during one year of follow-up. By contrast, a fourth CARD9-deficient patient, with relapsing *Candida* CNS infection, treated with subcutaneous GM-CSF (200 µg/d) and oral fluconazole for 15 months showed an ineffective response, resulting clinically in an enlargement of the fourth ventricle, for which discharge was required [61]. The clinical worsening was characterized by a massive eosinophil infiltration in the CSF and the brain, associated to persistent fungal pathogen in brain biopsies and in CSF. GM-CSF was stopped and the patient showed clinical and biological improvement under high-dose of oral fluconazole alone, and no relapse occurred during 2.5 years of follow-up. This patient homozygous for the R57H allele was found with a functional HRAS/Ras-GFR1/ERK pathway, like one patient compound heterozygous for *c.-529T>C/c.271T>C* (p.Y91H) alleles, who was not treated by GM-CSF.

The non-response to GM-CSF observed in this fourth patient may be associated to other CARD9-independent immune defects, but also to fungal virulence or chronicity of infection. Further studies are required to understand the variability to GM-CSF immunotherapy between CARD9-deficient patients. Strong conclusions about the GM-CSF-efficacy and CARD9-related pathway are difficult to draw because of the very small number of CARD9-deficient patients treated with GM-CSF. However, in patients with relapsing *Candida* infection of the CNS, subcutaneous GM-CSF/G-CSF could be considered as an adjuvant immunological treatment for use with classical azole agents during the consolidation therapy. However, clinicians must use adjuvant GM-CSF therapy with caution because of a possible risk of disease exacerbation and complications related to eosinophil-driven immunopathology of GM-CSF. The potential therapeutic role of GM-CSF was assessed in *Card9*^{-/-} mice infected intravenously with *C. albicans*, a model that mimics the susceptibility to *Candida* CNS infection observed in CARD9-deficient patients [61]. Infected mice were intraperitoneally injected with GM-CSF (5µg) immediately following infection or 24 hours after infection. *Card9*^{-/-} mice showed fungal burden in CNS and kidneys significantly higher than in their WT counterparts, which were not reduced after GM-CSF treatment as compared to untreated mice, 72 hours after infection. Thus the use of GM-CSF did not appear efficient in a mouse model of *Candida* CNS infection. The HRAS/Ras-GFR1/ERK pathway has not been studied in *Card9*^{-/-} mice. Adjuvant treatment with GM-CSF or G-CSF could be extended to invasive fungal infections other than relapsing *Candida* CNS infections, in other models of infection in *Card9*^{-/-} mice (e.g. subcutaneous or invasive phaeohyphomycosis, IA and deep dermatophytosis). The impact of GM-CSF or G-CSF on fungal load, survival, and outcome should be evaluated in mice. Improvements of our understanding of the CARD9-dependent antifungal immunity should contribute to the development of new immunological therapies for fungal diseases occurring in a context of CARD9 deficiency.

Hematopoietic stem cell transplantation in patients with **CARD9** mutations

Only one patient with CARD9 deficiency and intra-abdominal aspergillosis refractory to multiple antifungal therapies and local debulking surgery underwent HSCT, at the age of 12 years. The patient died from a fatal diffuse alveolar hemorrhage after two consecutive HSCTs [8]. The potential utility of HSCT in CARD9-deficient patients therefore remains unclear. Indeed, patients seem to have a single IFD throughout their lifetime, but of the 45 patients treated with multiple and/or long-term therapies with or without adjuvant treatment, the outcome was eventually satisfactory in 29/43 (67.4%), 8/43 (18.6%) still have active fungal disease and 6/43 (13.9%) died (no data available for two patients). In some PIDs conferring a predisposition to syndromic IFD, such as CID, CGD, or AD GATA2 deficiency, HSCT is performed as a last resort, with complete clinical recovery if engraftment is successful and no adverse effects occur (e.g. rejection, secondary infections) [160–162]. In CGD or AD GATA2 deficiency, patients have a myeloid cell defect, as in patients with AR CARD9 deficiency. In a recent prospective trial of CGD patients undergoing HSCT, myeloid engraftment rates exceeded 70% [163]. It has also been shown that, in patients undergoing HSCT for lymphoid or myeloid malignancies, monocyte recovery occurs after 30 days, and is then maintained and associated with high median survival [164]. In GATA2-deficient

patients, HSCT is also performed to avoid transformation into AL [165,166]. Nevertheless, HSCT is a heavy treatment associated with high mortality rates, mostly due to disseminated infections, such as IFD, and rejection problems [167]. The decision to perform HSCT is based on the optimal benefit/risk ratio for each patient. We suggest that CARD9-deficient patients with fungal disease refractory to standard/adjunct therapies should be considered for allogeneic HSCT, with caution, due to the lack of available data. The success of HSCT in CARD9-deficient patients would provide formal proof of the involvement of hematopoietic cells in CARD9 deficiency.

Outcome of CARD9-deficient patients

Overall, 45 of the 58 patients were treated. All received multiple and/or long-term antifungal therapies to control the fungal disease [1–11,14–23] (Table 3). A satisfactory outcome was obtained in six patients (6/44; 13.6%, no data concerning one patient), three of whom received secondary prophylaxis after initial treatment [3,5,15]. However, 38 patients relapsed immediately upon treatment cessation (38/43; 88.4%, no data available for one patient). The fungal disease was eventually successfully controlled in 23 of these patients (with adjunct immunological therapy in three patients), but not in 14 patients, eight of whom still had active disease and six of whom died. Indeed, ten of 58 patients (17.2%) died from active disease at a mean age of 23.1 years (median age: 19 years; range [12 – 39 years]). Six patients died from active disease despite treatment (deep dermatophytosis ($n=3$), *Candida* CNS infection ($n=1$), IA ($n=1$), invasive *Exophiala* infection ($n=1$), [2,8,11] and personal communication from Dr Davood Mansouri); one died from deep dermatophytosis without treatment [2], and three died from *Candida* CNS infections (no treatment data available) [1]. One patient died from old age, at 91 years of age [2]. CMC seems to be controlled by chronic oral treatment with antifungal agents in affected patients [1–5,7–11]. *Candida* CNS infections were treated in 13 patients (according to guidelines in 6 patients [3,9,10,16]), eight of whom experienced relapses (including 3 patients treated according to guidelines) [3,5–7,9–11,16,17] (no data available on outcome for one patient [6]). Four of these patients received adjunct GM-CSF/G-CSF treatment, which controlled the infection perfectly [6,9,17] in three patients but was ineffective in the fourth one [61] and, three others also needed brain surgery [3,6,17]. *Candida* endophthalmitis ($n=2$) [18,19], osteomyelitis ($n=3$) [6,7,18], colitis ($n=1$) and intra-abdominal infection ($n=1$) [3,8] were successfully treated with systemic long-term antifungal agents and the patients with hip osteomyelitis needed a hip replacement [18]. Outcome is unknown for one treated patient with *Candida* meningitis, endophthalmitis and osteomyelitis [6]. Seventeen patients with extensive/deep dermatophytosis were treated with multiple systemic antifungal agents for nine–12 months, but all relapsed when treatments were stopped [2,4,10,20,21]. Life-long therapy has controlled the disease in 11 patients [2,10,21], one still have active disease, and three died despite treatment [2]. Second-line oral posaconazole therapy yielded complete clinical remission in two patients after eight and 12 months, respectively [4,20]. Surgery was required in three patients [2,10]. The seven patients with subcutaneous phaeohyphomycosis were treated with combinations of antifungal agents followed by long-term oral azole therapy. The improvement of skin lesions was poor in six of these patients, but one patient was successfully treated with a combination of itraconazole and the excision of skin nodules

[14,15,22,23]. The two patients with invasive *Exophiala* infections were treated with multiple antifungal treatments and invasive therapeutic interventions [22]. One improved, but the other died from a pulmonary relapse. Cerebral IA was treated with systemic agents in addition to the surgical excision of a brain mass, and the intra-abdominal IA relapse was successfully treated with oral itraconazole in this patient [8]. The other patient with intra-abdominal IA did not respond to multiple treatments and died from complications of HSCT [8]. Overall survival and mortality were estimated at 86.7% (39/45) and 13.3% (6/45), respectively, for treated patients. At their last follow-up, 81% (47/58) of *CARD9*-deficient patients (treated or not) were alive (Figure 5).

Primary and secondary prophylaxis in patients with *CARD9* mutations

More than 85% of patients with *CARD9* mutations suffered relapses of fungal disease when treatment was stopped, indicating that longer courses of treatment or life-long treatment are required to prevent recurrence. Long-term therapies, mostly with azole agents (or griseofulvin in deep dermatophytosis) were used in 16 patients with invasive *Candida* infection [3,5–7,9–11,16–19,61], 17 patients with deep dermatophytosis [2,4,10,20,21] and nine patients with phaeohyphomycosis [14,15,22,23], and control of the fungal disease was achieved in 13, 13, and 2 patients, respectively. Secondary prophylaxis (more than one year of antifungal treatment) was given to seven patients (Table 3); oral itraconazole used for over 20 years prevented relapses of invasive candidiasis and aspergillosis in one patient [8]. No relapse of *Candida* osteomyelitis or endophthalmitis occurred in a patient treated with oral fluconazole after 2.5 years of follow-up [18]. No relapse of *Candida* meningitis has occurred on oral fluconazole treatment over follow-up periods of one to two and a half years in four patients [3,5,9,61]. Finally, one patient with subcutaneous phaeohyphomycosis was treated with oral itraconazole for more than one year with no relapse [15]. Consistent with these data, each *CARD9*-mutated patient should be given secondary prophylaxis with oral azole agents after the first episode of IFD, which should be treated over a long period (at least 9–12 months) without changing the molecule used if no recurrence occurs on treatment. We do recommend drug-based prophylaxis with oral fluconazole (100–200mg/d), a known safely anti-fungal drug, for siblings of patients found to have *CARD9* mutations. This should avoid life-threatening *Candida* CNS infections which can occur insidiously, and which have been found in about 30% of *CARD9*-deficient patients. The use of oral fluconazole should also prevent the occurrence of CMC. However, we do not recommend drug-based prophylaxis for other IFDs (e.g. invasive aspergillosis, deep dermatophytosis or phaeohyphomycosis) with other azole agents such as voriconazole or posaconazole because such treatments place considerable constraints on the individual (e.g. drug monitoring in the blood), can cause resistance problems, in addition to be costly. However, patients should be monitored for the occurrence of IFD and we recommend careful checking for the occurrence of CMC by rigorous screening of the skin and oral cavity even if they receive primary drug prophylaxis.

Conclusion

Inherited *CARD9* deficiency is a genetic etiology of superficial and invasive fungal diseases caused by various fungi from phylum Ascomycota. The defect is AR, but it is not known

whether it is complete or partial, due to a lack of robust assays for testing the function of individual alleles experimentally. Clinical penetrance is globally complete by the time patients reach their early 50s, but it is incomplete in younger individuals and, if fungal infections are considered individually, it is incomplete for the type of infection [168,169]. Fungal diseases can occur at any age, from early childhood to late adulthood. The adult onset seen in several CARD9-deficient patients is an uncommon feature of inborn errors of immunity, and must encourage clinicians to evoke CARD9 deficiency in differential diagnosis of adults presenting with unexplained IFD. This variability of disease onset may be due to host or environmental factors. Most patients are prone to a single IFD, a striking observation that remains unexplained. It may reflect the chronic activation of antifungal immunity against the invading fungus, providing protection against other fungi. The reasons for the observed selective vulnerability to fungi from a single phylum also remain unclear. Most of these disease-causing fungi are almost ubiquitous. The narrow spectrum of fungi causing infection in these patients may be explained by shared structural characteristics and their connection with CARD9. The asci, which are the fungal sacs containing the spores produced by sexual reproduction, and which are seen at least under specific *in vitro* conditions, are specific to ascomycetes. They might play a role in the initial stages of infection in CARD9-deficient hosts. Other ascomycete infections may subsequently be identified in CARD9-deficient patients. In this context, any patient, even adults, with unexplained IFD due to an ascomycete fungus should be tested for *CARD9* mutations. More generally, the possibility of CARD9 deficiency should be considered in any patient with unexplained IFD. WES in patients with IFD without *CARD9* mutations will probably reveal new genetic defects that may clarify the pathogenesis of fungal infections in CARD9-deficient patients. In parallel, the study of CARD9 deficiency at the cellular level, although difficult, should be pursued. Myeloid cells are thought but not proven to play a key role in susceptibility to IFD, by mechanisms that also remain to be deciphered. The use of conditional *Card9*^{-/-} mutant mice may help to identify the cells involved in CARD9-dependent antifungal immunity. The defect of Th17 cells seen in most, but not all patients, should also be studied, as the underlying mechanism is unclear. When these patients present CMC, years before IFD, it may be due to a primary defect of Th17 cells. However, most CARD9-deficient patients do not suffer from CMC and many have no apparent Th17 cell deficit. Furthermore, the link between CARD9 and Th17 is unclear. Overall, studies of the molecular and cellular bases of CMC and IFD in humans with inherited CARD9 deficiency should shed light on the mechanisms of antifungal immunity in humans.

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KEY POINTS

- Inherited CARD9 deficiency (OMIM #212050) is an AR PID due to mutations that may be present in a homozygous or compound heterozygous state.
- CARD9 is expressed principally in myeloid cells and transduces signals downstream from CLR activation by fungal ligands.
- Endogenous mutant CARD9 levels differ between alleles (from full-length normal protein to an absence of normal protein).
- The functional impacts of *CARD9* mutations involve impaired cytokine production in response to fungal ligands, the impairment of neutrophil killing and/or recruitment to infection sites, and defects of Th17 immunity.
- The key clinical manifestations in patients are fungal infections, including CMC, invasive (in the CNS in particular) *Candida* infections, extensive/deep dermatophytosis, subcutaneous and invasive phaeohyphomycosis and extrapulmonary aspergillosis.
- The clinical penetrance of CARD9 deficiency is complete, but penetrance is incomplete for each of the fungi concerned.
- Age at onset is highly heterogeneous, ranging from childhood to adulthood for the same fungal disease.
- All patients with unexplained IFD should be tested for *CARD9* mutations. Familial screening and genetic counseling should be proposed.
- The treatment of patients with *CARD9* mutations is empirical and based on antifungal therapies and the surgical removal of fungal masses. Patients with persistent/relapsing *Candida* infections of the CNS could be considered for adjuvant GM-CSF/G-CSF therapy. The potential value of HSCT for CARD9-deficient patients remains unclear.

Figure 1.
Structure of human CARD9 protein and schematic representation of the 22 reported mutations in patients with CARD9 deficiency

Figure 2.
Human CARD9 signaling pathway

Figure 3.
Analysis in silico of CARD9 variants

Figure 4.
Phylogenetic classification of fungi causing diseases in humans

Figure 5.
Kaplan-Meier survival curve for CARD9-deficient patients

Table 1.

and genetic features of patients with biallelic *CARD9* mutations

Patients	Age at onset, y	Age at last follow-up, y	Sex	Country of origin	Familial status	Fungal disease		Fungus	Patient status	CARD9 status: cDNA → protein	References
						Superficial	Invasive				
P1	3	19	M	Iran	CSG	CMC (oral)	-	<i>C. albicans</i>	Alive	c.883C>T → p.Q295*	[1]
P2	Early childhood	19	M	Iran	CSG	CMC (oral)	Meningitis with hydrocephalus (no brain masses) (18y)	<i>C. albicans</i>	Dead (19y)	NA	[1]
P3	42	50	F	Iran	CSG	CMC (vaginal) Tinea corporis	-	<i>C. albicans</i> <i>Trichophyton spp.</i>	Alive	c.883C>T → p.Q295*	[1]
P4	Early childhood	NA	F	Iran	CSG	CMC (oral, vaginal) Tinea corporis	-	<i>C. albicans</i> <i>Trichophyton spp.</i>	Alive	c.883C>T → p.Q295*	[1]
P5	Childhood	NA	M	Iran	CSG	Tinea corporis	-	NA	Alive	c.883C>T → p.Q295*	[1]
P6	Childhood	15	F	Iran	CSG	CMC (skin)	Brain abscess (13y)	<i>C. albicans</i>	Dead (15y)	NA [§]	[1]
P7	Early childhood	15	F	Iran	CSG	CMC (oral)	Meningoencephalitis, brain abscess (13y)	<i>C. albicans</i>	Dead (15y)	NA [§]	[1]
P8	7	13	F	South Korea	Adopted	-	Meningoencephalitis (relapsing, no brain masses)	<i>C. dubliniensis</i>	Alive	c.214G>A → p.G72S c.1118G>C → p.R373P	[16]
P9	6	75	M	Algeria	CSG	Tinea capitis/corporis	Deep dermatophytosis (skin, scalp, nails, lymph nodes, 52y)	<i>T. violaceum</i>	Alive	c.865C>T → p.Q289*	[2]
P10	2	29	M	Algeria	CSG	Tinea capitis/corporis	Deep dermatophytosis (skin, scalp, nails, lymph nodes, brain, 25y)	<i>T. violaceum</i>	Dead (29y)	NA	[2]
P11	9	40	F	Algeria	CSG	Tinea capitis/corporis	Deep dermatophytosis (skin, scalp, nails, lymph nodes, 12y)	<i>T. rubrum</i>	Alive	c.865C>T → p.Q289*	[2]
P12	8	56	M	Algeria	CSG	Tinea capitis/corporis CMC (oral)	Extensive dermatophytosis (skin, scalp, nails)	<i>T. violaceum</i> <i>Candida spp.</i>	Alive	c.865C>T → p.Q289*	[2]
P13	8	34	M	Algeria	CSG	Tinea capitis/corporis CMC (oral)	Deep dermatophytosis (skin, scalp, nails, lymph nodes, 15y)	<i>T. violaceum</i> <i>C. albicans</i>	Dead (34y)	NA	[2]
P14	8	41	F	Algeria	CSG	Onychomycosis	Extensive dermatophytosis (nails)	<i>T. violaceum</i>	Alive	c.865C>T → p.Q289*	[2]
P15	19	43	M	Algeria	CSG	Tinea capitis/corporis	Deep dermatophytosis (skin, scalp, nails, lymph nodes, 19y)	NA [§]	Alive	c.865C>T → p.Q289*	[2]
P16	21	40	M	Algeria	CSG	Tinea capitis/corporis	Deep dermatophytosis (skin, scalp, perineum, lymph nodes, 21 y)	NA [§]	Alive	c.865C>T → p.Q289*	[2]
P17	NA	28	M	Algeria	CSG	Tinea corporis	Deep dermatophytosis (skin ulcerations and pseudo-tumor)	NA [§]	Dead (28y)	NA	[2]
P18	Childhood	39	M	Algeria	CSG	Tinea capitis/corporis	Deep dermatophytosis (skin granuloma, scalp, nails, 27y)	<i>T. violaceum</i>	Dead (39y)	c.865C>T → p.Q289*	[2]

J Clin Immunol. Author manuscript; available in PMC 2019 August 22.

Patients	Age at onset ^y	Age at last follow-up ^y	Sex	Country of origin	Familial status	Fungal disease		Fungus	Patient status	CARD9 status: cDNA → protein	References
						Superficial	Invasive				
P19	Childhood	37	F	Algeria	CSG	Onychomycosis	Extensive dermatophytosis (nails)	NA	Alive	c.865C>T → p.Q289*	[2]
P20	Childhood	40	M	Morocco	CSG	Tinea corporis	Deep dermatophytosis (skin, nails, bone, lymph nodes, 35y)	<i>T. rubrum</i>	Alive	c.301C>T → p.R101C	[2]
P21	Childhood	49	F	Morocco	CSG	Tinea capitis Onychomycosis	Extensive dermatophytosis (nails)	NA	Alive	c.301C>T → p.R101C	[2]
P22	6	91	M	Tunisia	CSG	Tinea capitis/corporis Onychomycosis	Extensive dermatophytosis (nails)	NA	Dead ^d (91y)	c.865C>T → p.Q289*	[2]
P23	12	44	M	Tunisia	CSG	Tinea corporis	Deep dermatophytosis (skin granuloma, nails, 16y)	<i>T. rubrum</i>	Alive	c.865C>T → p.Q289*	[2]
P24	5	52	F	Tunisia	CSG	Tinea capitis/corporis	Deep dermatophytosis (skin, scalp, nails, lymph nodes, 12y)	<i>T. rubrum, T. violaceum</i>	Alive	c.865C>T → p.Q289*	[2]
P25	6	62	M	Tunisia	No CSG	Tinea capitis/corporis	Deep dermatophytosis (skin, scalp, nails, lymph nodes, 40y)	<i>T. rubrum, T. violaceum</i>	Alive	c.865C>T → p.Q289*	[2]
P26	30	41	M	Canada (French)	No CSG	-	Meningoencephalitis, brain abscess (relapsing)	<i>C. albicans</i>	Alive	c.271T>C → p.Y91H	[17]
P27	13	21	M	China	No CSG	-	Subcutaneous phaeohyphomycosis (skin ulcerations and nodules)	<i>P. verrucosa</i>	Alive	c.191-192insTGCT → p.L64fs*59 c.472C>T → p.Q158*	[15]
P28	6	17	M	China	No CSG	-	Subcutaneous phaeohyphomycosis (skin ulcerations)	<i>P. verrucosa</i>	Alive	c.819-820insG → p.D274fs*60	[15]
P29	20	43	F	China	No CSG	-	Subcutaneous phaeohyphomycosis (skin ulcerations and nodules)	<i>P. verrucosa</i>	Alive	c.819-820insG → p.D274fs*60	[15]
P30	48	64	M	China	No CSG	-	Subcutaneous phaeohyphomycosis (skin ulcerations, scalp, eyes)	<i>P. verrucosa</i>	Alive	c.819-820insG → p.D274fs*60	[15]
P31	5	8	F	Angola (living in France)	No CSG	-	Invasive phaeohyphomycosis (liver and biliary tract, 7y; brain with hydrocephaly and cranial hypertension, 9y)	<i>E. dermatitidis</i>	Alive	c.52C>T → p.R18W	[22]
P32	18	26	F	Iran	CSG	-	Subcutaneous (nodules, lymph nodes) and invasive (bones, 19y, lungs, 24y) phaeohyphomycosis	<i>E. spinifera</i>	Dead (>26y) ^f	c. GAG967-969del → p.E323del	[22]
P33	36	42	F	Turkey	CSG	CMC (vulvo-vaginal)	Meningitis, brain abscess (39y)	<i>C. albicans</i>	Alive	c.208C>T → p.R70W	[3]
P34	5	8	F	Turkey	CSG	CMC (oral, nails)	Meningitis, brain and medullar masses (7y) (relapsing)	<i>C. albicans</i>	Alive	c.208C>T → p.R70W	[3]

Patients	Age at onset _y	Age at last follow-up _y	Sex	Country of origin	Familial status	Fungal disease		Fungus	Patient status	CARD9 status: cDNA → protein	References
						Superficial	Invasive				
P35	17	28	M	Iran	CSG	-	Brain abscess, fungal sinusual infection with orbital and intracranial extension (17y), digestive tract (colitis, 22y)	<i>C. glabrata</i>	Alive	c.104G>A → p.R35Q	[3]
P36	34	37	F	Morocco	CSG	CMC (oral)	Meningitis, multiple brain masses (37y)	<i>C. albicans</i>	Alive	c.865C>T → p.Q289*	[3]
P37	26	34	M	Pakistan	CSG	CMC (oral, esophagus)	Digestive tract (colitis, 29y)	<i>C. albicans</i>	Alive	c.883C>T → p.Q295*	[3]
P38	13	40	M	Egypt (living in France)	No CSG	Tinea corporis	Extensive dermatophytosis (skin, nails, 18y)	<i>T. rubrum</i>	Alive	c.865C>T → p.Q289*	[20]
P39	3	24	M	Italy (living in Brazil)	No CSG	Tinea corporis CMC (oral)	Deep dermatophytosis (skin ulcerations, nails, alopecia) (12y)	<i>T. mentagrophytes</i> <i>Candida spp.</i>	Alive	c.302G>T → p.R101L	[4]
P40	1,5	6,5	F	Turkey	CSG	CMC (oral)	Meningitis (no brain masses) (3,5y)	<i>C. albicans</i>	Alive	c.883T → p.Q295*	[5]
P41	35	38	F	China	No CSG	-	Subcutaneous phaeoerythromycosis (ulcerations, necrotizing lesions on face)	<i>Corynespora cassiicola</i>	Alive	c.191-192insTGCT → p.L64fs*59 c.819-820insG → p.D274fs*60	[23] [14]
P42	NA	38	M	Canada (French)	No CSG	-	Meningitis, multiple cystic masses (relapsing)	<i>C. albicans</i>	Alive	c.271T>C → p.Y91H c.-529T>C → ?	[6]
P43	NA	39	F (twins)	Canada (French)	CSG	Tinea corporis	Endophthalmitis, brain abscess, osteomyelitis (vertebral) (39y)	<i>C. albicans</i> <i>Trichophyton spp.</i>	Alive	c.271T>C → p.Y91H c.-529T>C → ?	[6]
P44	Childhood	NA	F (twins)	Canada (French)	CSG	Tinea corporis Possible CMC (oral)	Lesions in basal ganglia bilaterally (CNS)	<i>Trichophyton spp.</i> <i>Candida spp.</i>	Alive	c.271T>C → p.Y91H c.-529T>C → ?	[6]
P45	Birth	13,5	F	El Salvador (living in U.S)	CSG	CMC (oral)	Meningoencephalitis (relapsing), brain abscess, osteomyelitis (cervical spine) (8y)	<i>C. albicans</i>	Alive	c.170G>A → p.R57H	[7]
P46	Childhood	40 or +	M	Mixed Europe	CSG	CMC (oral)	Intra-abdominal candidiasis (liver and mesenteric lymph nodes, 9y). Probable cerebral abscess aspergillosis (18y); probable liver and mesenteric lymph nodes aspergillosis (25y)	<i>Candida spp.</i> <i>Aspergillus spp.</i>	Alive	c.883C>T → p.Q295*	[8]
P47	Childhood	12	M	Afro-american	NA	Tinea corporis	Intra-abdominal aspergillosis (granuloma) (8y)	<i>Trichophyton spp.</i> <i>A. fumigatus</i>	Dead (12y)	c.3G>C → p.M11	[8]
P48	3	25	M (twins)	Turkey	CSG	CMC (oral) Tinea corporis	Meningoencephalitis (relapsing, no brain masses, 25y)	<i>C. albicans</i> <i>Trichophyton spp.</i>	Alive	c.883C>T → p.Q295*	[9]
P49	NA	NA	M (twins)	Turkey	CSG	Tinea corporis	-	<i>Trichophyton spp.</i>	Alive	c.883C>T → p.Q295*	[9]

Patients	Age at onset _y	Age at last follow-up _y	Sex	Country of origin	Familial status	Fungal disease		Fungus	Patient status	CARD9 status: cDNA → protein	References
						Superficial	Invasive				
P50	25	31	F	NA	NA	-	Endophthalmitis (right eye, 25y) and osteomyelitis (hip, 27y)	<i>C. albicans</i>	Alive	<i>c.1138G>C</i> → p.A380P <i>c.951G>A</i> → p.R317R	[18]
P51	8	55	M	Turkey	CSG	CMC (oral) Tinea capitis/corporis	Deep dermatophytosis (skin, scalp, nails, lymph nodes 41y)	<i>Candida spp.</i> <i>T. violaceum</i> , <i>T. rubrum</i> , <i>T. verrucosum</i>	Alive	<i>c.208C>T</i> → p.R70W	[10]
P52	8	NA	M	Turkey	CSG	CMC (skin)	-	<i>Candida spp.</i>	Alive	<i>c.208C>T</i> → p.R70W	[10]
P53	5	NA	M	Turkey	CSG	CMC (skin, nails)	Encephalitis with carotid artery aneurysm and thrombosis (5,3y)	<i>C. albicans</i>	Alive	Refused to be tested	[10]
P54	10	47	F	Algeria	CSG	Tinea corporis	Deep dermatophytosis (lymph nodes 17y; brain abscess 47y)	<i>T. rubrum</i>	Alive	<i>c.865C>T</i> → p.Q289*	[21]
P55	43	59	F	Germany	No CSG	-	Endophthalmitis (relapsing) (left eye, 43y; right eye, 58y)	<i>A. pullulans</i> (43y) <i>C. albicans</i> (58y)	Alive	<i>c.184G>A</i> → p.G62fs* <i>c.288T>C</i> → p.G96del36	[19]
P56	13	18	F	China	No CSG	-	Subcutaneous phaeoerythromycosis (rash and papules on face, trunk, limbs)	<i>E. spinifera</i>	Alive	<i>c.C68A</i> → p.S23* <i>c.819-820insG</i> → p.D274fs*60	[14]
P57	45	53	F	China	No CSG	-	Subcutaneous phaeoerythromycosis (rash and nodules on face)	<i>O. musae</i>	Alive	<i>c.819-820insG</i> → p.D274fs*60	[14]
P58	7	17	F	Turkey	No CSG (same village)	CMC (oral)	Brain masses (relapsing, 17y). Complications: right hemiparesis and hemiplegia, generalized tonic-clonic convulsions, left blurred vision. Para-aortic lymph nodes (< 1cm)	<i>C. albicans</i> (CMC) <i>Candida spp.</i> or <i>Aspergillus spp.</i> in brain biopsies	Dead (17y)	<i>c.883C>T</i> → p.Q295*	[11]

J Clin Immunol. Author manuscript; available in PMC 2019 August 22.

post-mortem
phae on biopsies
old age

M Mansouri, personal communication.

Available; y: years; M: male; F: female; CSG: consanguineous; CMC: chronic mucocutaneous candidiasis

Table 2.

Computational analysis of *CARD9* variants in *CARD9* deficiency

<i>CARD9</i> alleles <i>cDNA</i> (protein)	CADD score	Allele frequency ^{\$}	ClinVar database	PolyPhen-2/SIFT (score)
<i>c.3G>C</i> (p.M11)	26.7	Not reported	Not reported	Not calculable
<i>c.52C>T</i> (p.R18W)	32.0	Not reported	Not reported	Probably damaging (1) / Deleterious (0)
<i>c.68C>A</i> (p.S23*)	35.0	Not reported	Not reported	Not calculable
<i>c.104G>A</i> (p.R35Q)	33.0	3.3×10^{-5} (gnomAD)	Not reported	Probably damaging (1) / Deleterious (0)
<i>c.170G>A</i> (p.R57H)	29.9	Not reported	Not reported	Probably damaging (1) / Deleterious (0)
<i>c.184G>A</i> (p.G62fs*)	23.5	Not reported	Not reported	Probably damaging (1) / Deleterious (0)
<i>c.191-192insTGCT</i> (p.L64fs*59)	24.0	5.3×10^{-6} (gnomAD)	Not reported	Not calculable
<i>c.208C>T</i> (p.R70W)	25.3	Not reported	Not reported	Probably damaging (1) / Deleterious (0)
<i>c.214G>A</i> (p.G72S)	27.3	NA (dbSNP)	Pathogenic	Probably damaging (1) / Deleterious (0)
<i>c.271T>C</i> (p.Y91H)	25.0	2.4×10^{-5} (gnomAD)	Not reported	Probably damaging (1) / Deleterious (0)
<i>c.288C>T</i> (p.G96del36)	6.9	Not reported	Not reported	Probably damaging (1) / Deleterious (0)
<i>c.301C>T</i> (p.R101C)	24.2	NA (dbSNP)	Pathogenic	Probably damaging (1) / Deleterious (0)
<i>c.302G>T</i> (p.R101L)	24.9	Not reported	Not reported	Probably damaging (1) / Deleterious (0)
<i>c.472C>T</i> (p.Q158*)	35.0	Not reported	Not reported	Not calculable
<i>c.819-820insG</i> (p.D274fs*60)	22.7	3.3×10^{-5} (gnomAD)	Not reported	Not calculable
<i>c.865C>T</i> (p.Q289*)	35.0	8.4×10^{-6} (gnomAD)	Pathogenic	Not calculable
<i>c.883C>T</i> (p.Q295*)	36.0	1.3×10^{-5} (gnomAD)	Pathogenic	Not calculable
<i>c.951G>A</i> (p.R317R)	7.3	NA (dbSNP)	Not reported	Not calculable
<i>c.GAG967-969del</i> (p.E323del)	21.4	4.1×10^{-6} (gnomAD)	Not reported	Not calculable
<i>c.1118G>C</i> (p.R373P)	23.3	2.3×10^{-5} (gnomAD)	Pathogenic	Possibly damaging (0.6) / Deleterious (0.1)
<i>c.1138G>C</i> (p.A380P)	6.9	Not reported	Not reported	Benign (0.1) / Tolerated (0.2)
<i>c.-529T>C</i>	4.1	Not reported	Not reported	Not calculable

^{\$}Public databases: gnomAD, 1000Genom (<http://internationalgenom.org>) and dbSNP (<https://www.ncbi.nlm.nih.gov/SNP>). CADD: combined annotation-dependent depletion; MSC: mutation significance cutoff.

CADD: combined annotation-dependent depletion; MSC: mutation significance cutoff.

Table 3.

and immunological features and outcome of patients with biallelic *CARD9* mutations

Patients	CARD9 protein	Protein expression	Immunological phenotype					Defect of neutrophils	Treatments	Comorbidities - Other clinical manifestations	References
			T CD4 ⁺ , T CD8 ⁺ , B, NK, PNN, monocytes count	IgE (IU/ml) - Eo (/mm ³)	Cytokine production defect ^c	Blood Th17 cells – IL-17A production					
P1	p.Q995*	Absent ^a	Normal	NA – 8088	TNF-α	Low ^f – NA	NA	Long-term p.o. ketaconazole	Aphthous lesions	[1]	
P2	p.Q995*	NA	NA	NA	NA	NA	NA	NA	NA	[1]	
P3	p.Q995*	Absent ^a	Normal	NA – 1082	TNF-α	Low ^f – NA	NA	NA	Diabetes mellitus 2, nephrolithiasis	[1]	
P4	p.Q995*	Absent ^a	Normal	NA – 617	TNF-α	Low ^f – NA	NA	NA	NA	[1]	
P5	p.Q995*	Absent ^a	Normal	NA – 472	TNF-α	Low ^f – NA	NA	NA	NA	[1]	
P6	p.Q995*	Absent ^a	NA	NA	NA	NA	NA	NA	Ventricular septal defect	[1]	
P7	p.Q995*	NA	NA	NA	NA	NA	NA	NA	NA	[1]	
P8	p.G725R/R373P	Absent ^a	NA	NA	IL-1 β, IL-6	NA – Low ^j	PNN killing (unopsonized <i>C. albicans</i>) [£] CNS neutropenia	i.v. AmB then p.o. fluconazole (6 months) [§] Relapse: i.v. 5-flucytosine + fluconazole	Diabetes mellitus 2 NID (50y)	[16] [63] [£]	
P9	p.Q289*	NA	NA	NA–1700	NA	NA	NA	Long-term p.o. itraconazole	Diabetes mellitus 2 ID (35y)	[2]	
P10	p.Q289*	NA	NA	NA – 2700	NA	NA	NA	Ineffective long term p.o. itraconazole	NA	[2]	
P11	p.Q289*	NA	Normal (monocytes NA)	NA	NA	NA	NA	Long-term p.o. itraconazole	Diabetes mellitus 2 ID (35y)	[2]	
P12	p.Q289*	NA	Normal (monocytes NA)	1300 – NA	NA	NA	NA	Long-term p.o. griseofulvin	NA	[2]	
P13	NA	NA	NA	1300 – 2600	NA	NA	NA	Ineffective long term p.o. griseofulvin	NA	[2]	
P14	p.Q289*	NA	NA	NA	NA	NA	NA	Long-term p.o. griseofulvin	NA	[2]	
P15	p.Q289*	NA	NA	NA	NA	NA	NA	Long-term p.o. griseofulvin + p.o. fluconazole	NA	[2]	
P16	p.Q289*	NA	NA	NA	NA	NA	NA	Multiple antifungal therapy + perineal surgery (with colostomy) then long-term p.o itraconazole +	NA	[2]	

Patients	CARD9 protein	Protein expression	Immunological phenotype				Defect of neutrophils	Treatments	Comorbidities - Other clinical manifestations	References
			T CD4 ⁺ , T CD8 ⁺ , B, NK, PNN, monocytes count	IgE (IU/ml) - Eo (mm ³)	Cytokine production defect ^c	Blood Th17 cells - IL-17A production				
P17	NA	NA	NA	NA	NA	NA	NA	terbinafine		
P18	p.Q289*	NA	NA	NA - 550	NA	NA	NA	Ineffective long term p.o. griseofulvin		[2]
P19	p.Q289*	NA	NA	NA	NA	NA	NA	NA		[2]
P20	p.R401C	Diminished^a Normal^b (size - amount)	Normal (monocytes NA)	1741 - 1500	IL-6	Low ^f - NA	NA	Multiple antifungal therapies + IFN-γ + Foot amputation + failed long-term p.o voriconazole.		[2]
P21	p.R401C	NA	NA	NA	NA	NA	NA	NA		[2]
P22	p.Q289*	NA	NA	NA	NA	NA	NA	NA		[2]
P23	p.Q289*	Absent^{a,b}	NA	NA	IL-6	Low ^f - NA	NA	p.o. fluconazole then long-term p.o. itraconazole		[2]
P24	p.Q289*	NA	NA	NA	NA	NA	NA	Long-term p.o. griseofulvin + fluconazole + ketoconazole		[2]
P25	p.Q289*	Absent^{a,b}	NA	5119 - 2700	IL-6	Low ^f - NA	NA	Multiple antifungal therapies then long-term p.o. voriconazole		[2]
P26	p.Y21H	Normal^a (size - amount)	Normal	NA	GM-CSF IL-1 β , IL-6, TNF- α Normal	Normal ^f - NA	NA	Brain surgery ; i.v. fluconazole + AmB Relapse: s.c. GM-CSF + p.o. voriconazole (18 months)		[17]
P27	p.L64fs*59/p.Q158*	Absent^a	NA	NA	IL-1β, IL-6, TNF-α, IL-23p19	Low ^g - Low ^g - Low ^j (IL-22 ^e)	PNN killing (unopsonized <i>P. verrucosus</i>) ^f	i.v. AmB then escaping p.o. itraconazole		[15] [62] ^f
P28	p.D274fs*60	Absent^a	NA	NA	NA	Low ^g - Low ^j (IL-22 ^e)	PNN killing (unopsonized <i>P. verrucosus</i>) ^f	i.v. AmB then long-term p.o. itraconazole (for 2 y, with recurrence)		[15] [62] ^f
P29	p.D274fs*60	Absent^a	NA	NA	IL-1 β , IL-6, TNF- α , IL-23p19	Low ^g - Low ^g (IL-22 ^e)	PNN killing (unopsonized <i>P. verrucosus</i>) ^f	Skin nodes surgery + p.o. itraconazole Prophylaxis: p.o. itraconazole (>1y)		[15] [62] ^f

J. Clin. Immunol. Author manuscript; available in PMC 2019 August 22.

Patients	CARD9 protein	Protein expression	Immunological phenotype				Defect of neutrophils	Treatments	Comorbidities - Other clinical manifestations	References
			T CD4 ⁺ , T CD8 ⁺ , B, NK, PNN, monocytes count	IgE (IU/ml) - Eo (mm ³)	Cytokine production defect ^c	Blood Th17 cells - IL-17A production				
P30	p.D274fs*60	NA	NA	NA	NA	NA	PNN killing (unopsonized <i>P. verrucosa</i>) [£]	p.o. itraconazole + p.o. terbinafin (6 months, mild response)		[15] [62] [£]
P31	p.R35W	Normal ^a Normal size; high amount ^b	Normal (monocytes NA)	NA	IL-6, TNF-α	Normal ^f – Normal ⁱ	NA	Irrigation of biliary tract + local i.v. AmB; then p.o. voriconazole (22 months). Brain relapse: VP shunt + intra-thecal i.v. AmB + i.v. voriconazole + p.o. terbinafine		[22]
P32	p.E33del	NA	Normal (monocytes NA)	NA	NA	NA	NA	Combination of azole agents (5y) (fluconazole, itraconazole, voriconazole). Lung relapse: NA		[22]
P33	p.R10W	Diminished^a Normal size; low amount ^b	Normal (monocytes NA)	NA	IL-6, TNF-α	Normal ^f – Normal ⁱ	CNS neutropenia	VP shunt; i.v. AmB+5-flucytosine then p.o. fluconazole [§] . Prophylaxis: p.o. fluconazole (>2y)		[3]
P34	p.R10W	Diminished^a Normal size; low amount ^b	Normal (monocytes NA)	NA	IL-6, TNF-α	Normal ^f – Normal ⁱ	NA	i.v. AmB then p.o. fluconazole [§] (twice) then long-term p.o. fluconazole (>6 months)		[3]
P35	p.R35Q	Normal ^b (size – amount)	Normal (monocytes NA)	1700 – 1500	NA	NA	NA	Sinus surgery + p.o. fluconazole then long-term p.o. itraconazole (5y) and relapse		[3]
P36	p.Q289*	Normal amount of truncated protein ^b	Normal (monocytes NA)	NA	NA	NA	NA	i.v. AmB + flucytosine then long-term p.o. fluconazole (>10 months) ^{§§}		[3]
P37	p.Q295*	Normal amount of truncated protein ^b	Normal (monocytes NA)	4979 – NA	IL-6, TNF-α	Normal ^f – Normal ⁱ	NA	i.v. AmB then p.o. posaconazole		[3]
P38	p.Q289*	NA	Normal (monocytes NA)	NA	NA	NA	NA	Multiple antifungal therapies: improvement with p.o. posaconazole (8 months)		[20]

Patients	CARD9 protein	Protein expression	Immunological phenotype					Defect of neutrophils	Treatments	Comorbidities - Other clinical manifestations	References
			T CD4 ⁺ , T CD8 ⁺ , B, NK, PNN, monocytes count	IgE (IU/ml) - Eo (/mm ³)	Cytokine production defect ^c	Blood Th17 cells - IL-17A production					
P39	p.R101 L	NA	Normal (monocytes NA)	>2000 – 1368	NA	NA	NA	PNN killing (opsonized <i>C. albicans</i>)	Multiple antifungal therapies: improvement with p.o. posaconazole (12 months)		[4]
P40	p.Q295* <i>J Clin Immunol.</i> Author manuscript; available in PMC 2015 August 22.	Absent^a	Normal (monocytes NA)	High [#] – High [#]	IL-1β, IL-6	Normal ^f – NA		PNN killing (unopsonized <i>C. albicans</i>) [£]	Triple p.o. antifungal therapy then p.o. voriconazole alone (4 months). Prophylaxis: p.o. fluconazole (>2y)	<i>Ascaris</i> spp. infection [#]	[5] [63] [£]
P41	p.L64fs*59fs p.D274fs*60	Absent^a	Normal	Normal	IL-1β, IL-6, TNF-α	Low ^h – Low ^k (Th22 ^g – IL-22 ^g)		Skin neutropenia	i.v. AmB + p.o. terbinafine		[23] [14]
P42	p.Y91H/Le-529T>C	Normal^b (size – amount)	NA	NA	GM-CSF (+ERK activation) ^d IL-1β, IL-6, TNF-α Normal	NA	NA	NA	Brain surgery + p.o. fluconazole Relapse: s.c. GM-CSF + p.o. fluconazole (18 months)		[6]
P43	p.Y91 H/Le-529T>C	Normal^b (size – amount)	NA	NA	GM-CSF (+ERK activation) ^d IL-1β, IL-6, TNF-α Normal	NA	NA	NA	Fluconazole (duration: NA)		[6]
P44	p.Y91 H/Le-529T>C	Normal^b (size – amount)	NA	NA	GM-CSF (+ERK activation) ^d IL-1β, IL-6, TNF-α Normal	NA	NA	NA	NA		[6]
P45	p.R57H	Normal^a	Normal	Normal	IL-1β, IL-6, TNF-α, GM-CSF, IFN-γ (+ normal ERK activation) ^d	Normal ^f – NA		PNN killing (unopsonized <i>C. albicans</i>) CNS neutropenia	i.v. AmB + 5-flucytosine + voriconazole (1 month) then i.v. 5-flucytosine + voriconazole then p.o. fluconazole (6 months) Relapse: s.c. GM-CSF (15 months) + p.o. fluconazole Disease's progression: p.o. fluconazole (high dose) + STOP GM-CSF + VP shunt Prophylaxis: p.o. fluconazole (high dose; >2.5y)		[7] [61]

Patients	CARD9 protein	Protein expression	Immunological phenotype					Defect of neutrophils	Treatments	Comorbidities - Other clinical manifestations	References
			T CD4 ⁺ , T CD8 ⁺ , B, NK, PNN, monocytes count	IgE (IU/ml) - Eo (/mm ³)	Cytokine production defect ^c	Blood Th17 cells – IL-17A production					
P46	p.Q295*	Absent ^a	T CD4 ⁺ 300/mm ³ T CD8 ⁺ 136/mm ³ Normal B, NK, PNN, monocytes	NA	IL-1β, IL-6, TNF-α, GM-CSF, IFN-γ	Normal ^f – NA	Mesenteric lymph nodes neutropenia Normal chemotactic capacity No PNN killing defect	Intra-abdominal candidiasis: i.v. AmB Relapse: Cerebral aspergillosis: i.v. AmB + cerebral surgery. Intra-abdominal aspergillosis: p.o itraconazole. Prophylaxis: p.o itraconazole (>20y)	AD spastic paraplegia (heterozygous <i>SPAST</i> mutation)	[8]	
P47	p.Q11	Absent ^a	Normal	828 – Elevated	IL-1β, IL-6, TNF-α, GM-CSF, IFN-γ	Normal ^f – NA	Suprarenal mass neutropenia Normal chemotactic capacity No PNN killing defect	Intra-abdominal debulking surgery + combination of anti-fungal therapies. Two failed HSCT: failure to engraft then hepatic veno-occlusive disease (and dead)	Mild eczema, food allergies, reactive airway disease	[8]	
P48	p.Q295*	NA	Normal (monocytes NA)	Normal	NA	NA – Low ^j	CNS neutropenia	i.v. fluconazole + i.v. AmB then p.o. voriconazole [§] Relapse: i.v. fluconazole + i.v. AmB then i.v. capsosfungin then s.c. G-CSF (3 months) + p.o fluconazole Prophylaxis: p.o. fluconazole (>1y)		[9]	
P49	p.Q295*	NA	NA	NA	NA	NA – Low ^j	NA	NA	Meningitis (8y) (viral, bacterial, fungal?)	[9]	
P50	p.A380P/p.R317R	NA	NA	NA	NA	NA	NA	Endophthalmitis: eyes surgery + p.o. voriconazole Relapse: Osteomyelitis: hip replacement + p.o. fluconazole. Prophylaxis: p.o. fluconazole (>2,5y)		[18]	
P51	p.R70W	NA	Normal (monocytes NA)	1820 – High	IL-6, GM-CSF	Low ^g – Low ^g (IL-22) [§]	NA	Multiple antifungal therapies + chest nodule resection		[10]	
P52	p.R70W	NA	High T CD4+ (monocytes NA)	843 – Normal	IL-6, GM-CSF	Low ^g – Low ^g (IL-22) [§]	NA	NA		[10]	
P53	NA	NA	Normal (monocytes NA)	161 – High	NA	NA	NA	i.v. AmB + 5-flucytosine then p.o. fluconazole [§]	Hypoparathyroidism	[10]	

Patients	CARD9 protein	Protein expression	Immunological phenotype				Defect of neutrophils	Treatments	Comorbidities - Other clinical manifestations	References
			T CD4 ⁺ , T CD8 ⁺ , B, NK, PNN, monocytes count	IgE (IU/ml) - Eo (/mm ³)	Cytokine production defect ^c	Blood Th17 cells - IL-17A production				
P54	p.Q289*	NA	Normal (monocytes NA)	Normal	NA	NA	NA	(duration: NA) p.o. griseofulvin (several courses of treatment) then long-term p.o. itraconazole		[21]
P55	p.G62fs*/p.G96del36	Normal^d	Normal	Normal	NA	NA	NA	Aureobasidium infection: NA Relapse: Candida infection: Intra-vitreally + i.v. voriconazole then p.o. voriconazole (9 months)		[19]
P56	p.S23*/p.D274fs*60	Absent^d	Normal	Normal	IL-1β, IL-6, TNF-α (+p65 phosphorylation) ^e	Low ^h - Low ^k (Th22 [§] - IL-22 [§])	Skin neutropenia	p.o. posaconazole (several months) Relapse: p.o. terbinafine + i.v. itraconazole (ineffective)		[14]
P57	p.D274fs*60	Absent^d	Normal	Normal	IL-1β, IL-6, TNF-α (+p65 phosphorylation) ^e	Low ^h - Low ^k (Th22 [§] - IL-22 [§])	Skin neutropenia	p.o. itraconazole (3 months) then p.o. itraconazole and terbinafine (4 months) then i.v. AmB (several weeks, little improvement)		[14]
P58	p.Q295*	NA	T CD4 ⁺ 186/mm ³ T CD8 ⁺ 222/mm ³ Normal B, NK, PNN (monocytes NA)	1810 - Normal	NA	NA	NA	p.o. voriconazole (3 months) + phenytoin (convulsions) Relapse: i.v. AmB + p.o. voriconazole	Warts (hands), labial herpetic lesions, upper respiratory tract infection (childhood)	[11]

were assessed by western blotting and/or flow cytometry with mAb or pAb in patients' cells

expression system

amount of protein are specified in each case of CARD9 protein detection on western blots. Cytokine production was assessed by ELISA after 24 or 48 hours of myeloid cell (e.g. PBMCs, DCs) stimulation with fungal agonists (e.g. zymosan, curdlan) or whole fungi (e.g. heat-killed *C. albicans*, *P. verrucosa*, *E. phialophora*, *E. spinifera*, *C. corynespora*, *O. musae*, *A. fumigatus*)

) phosphorylation was impaired in monocytes from patient compound heterozygous for c.529T>C/c.271T>C (p. Y91H) CARD9 mutations and normal in monocytes from patient or R57H CARD9 allele after 15 minutes of stimulation with zymosan, as shown by immunoblots of cell lysates with a mAb

subunit (NF-κB) phosphorylation was found in the patients' monocytes after 30 minutes of stimulation with *E. spinifera*, in flow cytometry analysis with a mAb

n of IL-17A+ CD4+ T cells in PBMCs was assessed ex vivo by flow cytometry after 12 hours of stimulation with anti-CD2/CD3/CD28 antibody-coated beads or PMA/ionomycin

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^g after 48 hours of stimulation with heat-killed *C. albicans* or *P. verrucosa*

^h or after six days of stimulation with heat-killed *C. albicans*, *P. verrucosa*, *E. spinifera*, *C. cassicola* and/or *O. musae*

ⁱ IL-17A production by whole-blood cells or PBMCs was assessed by ELISA after 24 hours of stimulation with PMA/ionomycin or anti-CD2/CD3/CD28 antibody-coated beads

^j 48 hours of stimulation with *C. albicans* or *P. verrucosa*

^k or six days of stimulation with heat-killed *C. albicans*, *P. verrucosa*, *E. spinifera*, *C. cassicola* and/or *O. musae*

[§] The proportion of IL-22+ IL-17- CD4+ T cells and IL-22 production by PBMCs were assessed in the same experiment, by flow cytometry or ELISA with the anti-IL-22 mAb, and they were found to be low

[#] High eosinophil count and high serum IgE levels in a context of parasitic infection (*Ascaris* spp.).

^f Neutrophil killing assays for the patients were also reported in separate papers.

[§] Treatment according to guidelines.

NA: not available; WT: wild-type; PNN: polynuclear neutrophils; NK: natural killer cells; Eo: eosinophils; CNS: central nervous system; p.o.: per os; s.c.: subcutaneous; i.v.: intravenous; AmB: amphotericin B; VP: ventriculo-peritoneal; HSCT: hematopoietic stem cell transplantation; AD: autosomal dominant; NID: non-insulin-dependent; ID: insulin-dependent.

Table 4.

Main characteristics of fungal diseases in CARD9 deficiency

Fungal Diseases	Number of cases (58)	Median age at onset (y)	Mean age at onset (y)	Range of age at onset (y)	Outcome Alive - Dead
CMC	22 (37.9%)	8.0	12.9	[birth - 42]	17 - 5 [#]
Superficial dermatophytosis	8 (13.8%)	8.0	17.7	[3 - 42]	7 - 1 [#]
Invasive <i>Candida</i> infections	21 (36.2%)	17.5	21.9	[3.5 - 58 [*]]	17 - 4
Extensive/deep dermatophytosis	21 (36.2%)	19.0	24.1	[12 - 52]	16 - 5 [£]
Phaeohyphomycosis	10 (17.2%)	19.0	24.6	[5 - 48]	9 - 1
Invasive extrapulmonary aspergillosis	2 (3.4%)	13.0	13.0	[8 - 18]	1 - 1

* Fungal disease onset occurred at the age of 43 years, but *Candida* endophthalmitis started at the age of 58 years.

[#] Patients died from the associated invasive disease, not from the superficial infection.

[£] One patient died from old age.

CMC: chronic mucocutaneous candidiasis; y: years.

Table 5.

Comparison between human and mouse CARD9

Gene position	Human CARD9		Mouse CARD9	
	Chr 9q34.3		Chr 2 18.87cM	
mRNA production	Hematopoietic cells (spleen, bone marrow, blood) [#] ; myeloid cells (macrophages – DCs) Placenta – small intestine – skin – brain – lungs		Hematopoietic cells (spleen, bone marrow, blood) [#] ; myeloid cells (macrophages – DCs) Placenta – small intestine – skin – brain – lungs	
Protein	Hematopoietic cells (spleen, bone marrow, blood) [#] ; myeloid cells (macrophages – DCs) Stomach – Skin		Hematopoietic cells (spleen, bone marrow, blood) [#] ; myeloid cells (macrophages – DCs) Stomach – Skin	
Defect of cytokine production[*] upon fungal stimulation <i>in vitro</i>	IL-1β, IL-6, TNF-α, GM-CSF		IL-1β, IL-2, IL-6, TNF-α	
Defect of neutrophil killing <i>in vitro</i>	Unopsonized <i>C. albicans</i> yeast Unopsonized <i>P. verrucosa</i> conidia No defect to (un)-opsonized <i>A. fumigatus</i> hyphae and conidia		<i>P. verrucosa</i> conidia <i>A. fumigatus</i> hyphae and conidia No defect to <i>C. tropicalis</i> yeast	
Defect of neutrophil recruitment <i>in vivo</i>	CNS – intra-abdominal LN – suprarenal mass – skin		CNS – lungs – skin	
Defect of CXC-chemokines induction	CXCL1, CXCL2, CXCL5, IL-8 (mRNA and protein)		CXCL1, CXCL2, CXCL5 (mRNA and protein)	
Defect of IL-17 immunity	Low or normal <i>ex vivo</i> proportions of Th17 cells and IL-17A/F production <i>in vitro</i> upon polyclonal (PMA/ionomycin or coated beads) or fungal stimulation (<i>C. albicans</i> , <i>P. verrucosa</i> , <i>E. spinifera</i> , <i>O. musae</i> , and/or <i>C. corynespora</i>)		Low <i>ex vivo</i> proportions of Th17 cells and IL-17A/F production <i>in vitro</i> upon fungal stimulation (<i>C. albicans</i> , <i>P. verrucosa</i> and/or <i>E. spinifera</i>)	
Blood cell counts	Normal T CD4+, T CD8+, B, NK, PNN, monocyte count Hyper IgE – hyper eosinophilia		Normal T CD4+, T CD8+, B, NK, PNN count IgE, eosinophils, monocytes count NA	
Susceptibility to fungal diseases (associated fungi)	CMC (<i>C. albicans</i>) Invasive candidiasis (<i>C. albicans</i> , <i>C. glabrata</i> , <i>C. dubliniensis</i>) Phaeohyphomycosis (subcutaneous – invasive) (<i>E. dermatitidis</i> , <i>E. spinifera</i> , <i>P. verrucosa</i> , <i>C. cassicola</i> , <i>A. pullulans</i> , <i>O. musae</i>) Extrapulmonary invasive aspergillosis (<i>A. fumigatus</i>) Deep dermatophytosis (<i>T. rubrum</i> , <i>T. violaceum</i> , <i>T. mentagrophytes</i>)		CMC (<i>C. albicans</i>) Invasive candidiasis (<i>C. albicans</i> , <i>C. tropicalis</i>) Phaeohyphomycosis (subcutaneous – invasive) (<i>P. verrucosa</i> , <i>E. spinifera</i>) Pulmonary invasive aspergillosis (<i>A. fumigatus</i>) Pulmonary cryptococcosis (<i>Cryptococcus neoformans</i>) Cutaneous coccidioidomycosis (<i>Coccidioides posadasii</i>)	
Susceptibility to non-fungal diseases (associated pathogens)	Not reported		Pulmonary tuberculosis (<i>M. tuberculosis</i>) Invasive listeriosis (<i>L. monocytogenes</i>)	

[#] Peak of CARD9 mRNA and protein levels.^{*} 24 hours of stimulation with fungal agonist or whole fungus.

DCs: dendritic cells; CNS: central nervous system; LN: lymph nodes; NK: natural killer cells; PNN: polynuclear neutrophils; CMC: chronic mucocutaneous candidiasis.

Table 6.

Selected primary immunodeficiencies underlying invasive fungal diseases

PID	Candidiasis	Dermatophytosis	Phaeohyphomycosis	Aspergillosis	Histoplasmosis	Pneumocystosis	Paracoccidioidomycosis/ coccidiomycosis	Cryptococcosis	Other
CARD9 deficiency (AR)	+	+	+	+	-	-	-/-	-	-
GATA2 deficiency (AD-MonoMAC)	-	-	-	+	+	-	-/-	\$ ⁺	+
CYBB deficiency (XL-CGD)	+	-	\$ ⁺	+	-	-	-/-	-	# ⁺
CYBA/NCF1/NCF2 /NCF4 deficiency (AR-CGD)	+	-	\$ ⁺	+	-	-	-/-	-	# ⁺
STAT1 GOF (AD-CMC)	+	-	-	\$ ⁺	\$ ⁺	\$ ⁺	\$ ⁺ /-	\$ ⁺	# ⁺
STAT3 LOF (AD-HIES)	-	-	-	+	-	-	\$ ⁺ /-	\$ ⁺	-
DOCK8 deficiency (AR-HIES)	+	-	-	-	-	\$ ⁺	-	\$ ⁺	-
CD40L deficiency (XL-HIGM)	+	-	-	-	+	+	+/-	+	-
IL-12RB1 deficiency (AR-MSMD)	-	-	-	-	\$ ⁺	-	\$ ⁺ /+	\$ ⁺	-
IFN-γR1 deficiency (AD/AR-MSMD)	-	-	-	-	\$ ⁺	-	\$ ⁺ /-	-	-
NEMO deficiency (XL-EDA-ID)	-	-	-	-	-	+	-	-	-
IκBα deficiency (AR-EDA-ID)	-	-	-	-	-	+	-	-	-
ELA2 deficiency (AD-SCN)	\$ ⁺	-	-	+	-	-	-	-	-
HAX1 deficiency (AR-SCN)	\$ ⁺	-	-	+	-	-	-	-	-
CD18 deficiency (AR-LAD 1)	\$ ⁺	-	-	+	-	-	-	-	# ⁺
SCID [*]	+	-	-	+	-	+	-	-	-

#Fungi belonging to phyla other than Ascomycota.

* More than 30 genetic defects lead to SCID

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\$
not frequently reported.

PID: primary immunodeficiency (mode of inheritance: AR: autosomal recessive; AD: autosomal dominant; XL: X-linked); IFD: invasive fungal disease; CGD: chronic granulomatous disease; MSMD: Mendelian susceptibility to mycobacterial disease; CMC: chronic mucocutaneous candidiasis; EDA-ID: anhidrotic ectodermal dysplasia with immunodeficiency; SCN: severe congenital neutropenia; LAD1: leucocyte adhesion deficiency type 1; SCID: severe combined immunodeficiency; HIES: hyper IgE syndrome; HIGM: hyper IgM syndrome.