Genetic Susceptibility to Opportunistic Fungal Infections

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Genetic Susceptibility to Opportunistic Fungal Infections

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Genetic Susceptibility to Opportunistic Fungal Infections

Doctoral Thesis

To obtain the degree of doctor from Radboud University Nijmegen on the authority of the Rector Magnificus prof. Th. L. M. Engelen according to the decision of the Council of Deans to be defended in public on Thursday January 15, 2015 at 16.30 hours

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CHAPTER 1

General Introduction
and Outline of the Thesis
General Introduction

*Candida albicans* is a dimorphic yeast that often resides in the gastrointestinal and genital tract of the human host in a commensal interaction [1, 2]. However, in clinical situations in which the host defense decreases, *C. albicans* can become an opportunistic pathogen and can cause infections of the mucosa and, through blood dissemination, it can invade other organs. Mucosal *Candida* infections are mainly described at the level of the mouth, pharynx and esophagus (oropharyngeal candidiasis – OPC) or at the level of the vagina (vulvovaginal candidiasis – VVC). These infections are often seen in pregnant women, patients with diabetes or HIV, or those receiving immunosuppressive therapy or antibiotics, and they determine a significant decrease in the life quality, although they are very rarely fatal [3, 4]. In contrast, disseminated *Candida* infections are a major complication of neutropenia or immunosuppressive treatment, major surgery or lengthy Intensive Care Unit (ICU) stay [5-7]. The systemic *Candida* infections are characterized by a high mortality reaching 30 to 40% [8]. Although other *Candida* species can be related to disseminated candidiasis, the most common strain associated with human pathologies is *Candida albicans* [5, 6].

Although several risk factors for fungal infections have been previously described, as noted above, many patients develop mucosal infections such as recurrent VVC (RVVC) without any risk profile [9]. Moreover, only a small minority of patients at risk develops a systemic fungal infection [10]. These facts strongly argue that host factors such as the genetic make-up is likely to play a crucial role in determining which patients will develop *Candida* infections. This thesis will focus on the study of the genetic variation in host defense genes that may determine an increased susceptibility to infections caused by *Candida* spp.

Host defense against *Candida albicans* is initiated by the recognition of the yeast by the innate immune system. Recognition is enabled by a large diversity of receptors known as pattern recognition receptors (PRRs), that detect conserved molecular structures of pathogens called pathogen-associated molecular patterns (PAMPs). Detection of PAMPs depends on different families of PRRs, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like helicases, all acting in a coordinated manner to initiate downstream signaling in innate immune cells, thereby inducing production of proinflammatory cytokines and activation of T-cell responses [11].

Two classes of PRRs have been described to be the most important receptors for *C. albicans* recognition: Toll-like receptors (TLR) and lectin-like receptors (LR) [12]. TLR4 and mannose receptor (MR) detect cell wall mannans [13], while TLR2/TLR1 and TLR2/
TLR6 heterodimers detect phospholipomannans [14]. In addition, TLR2 acts in synergy with the archetypical β-glucan receptor dectin-1 for the production of proinflammatory cytokines [15-17]. Dectin-1 can also induce TLR-independent signals for the production of IL-17, IL-6 and IL-10 through a Syk/CARD9-dependent pathway [18]. The dectin-1 receptor is expressed mainly by cells of the myeloid lineage (monocytes/macrophages, dendritic cells, and neutrophils) [19]. Moreover, the ability of the dectin-1 receptor to synergize with TLR2 and TLR4 promotes pro-inflammatory cytokine production and induction of Th1 and Th17 mediated host defense against C. albicans [15]. A scheme of Candida recognition by the PRRs can be found in Figure 1.

**Figure 1** Recognition of the *Candida albicans* cell wall components by the innate immune system. The Pattern recognition receptors (PRRs) are present in cells of the myeloid lineage (dendritic cells, neutrophils and monocytes/macrophages) and recognize conserved pathogen-associated molecular patterns (PAMPs). The signal is transduced by different pathways, activating NF-κB and the expression of pro-inflammatory cytokines.
A large part of the genetic variation in the human genome is represented by single nucleotide polymorphisms (SNPs). Previously, a Dutch family was described with members suffering from onycomycosis and recurrent vulvovaginal candidiasis, in which genetic studies have demonstrated that the presence of an early stop polymorphism (Y238X rs16910526) in the \textit{DECTIN-1} gene led to a partial or total deficiency in $\beta$–glucan recognition. This defect led to an impaired recognition of \textit{C. albicans} and decreased cytokine production by immune cells, especially for cytokines involved in the T-helper (Th) 17 pathway [20], and was also associated with a 3-fold higher susceptibility to mucosal colonization and infections in patients undergoing stem cells transplantation [21]. The support for the hypothesis of an important role of dectin-1 recognition for anti-\textit{Candida} defense was provided by the description of another family with a defective \textit{CARD9} gene [22].

In \textit{Chapter 2}, the effect of genetic variation in Dectin-1 and \textit{CARD9} on the prevalence of candidemia will be explored. The genotype of the rs16910526 Y238X SNP in \textit{DECTIN-1} and the S12N rs4077515 SNP in \textit{CARD9} will be analyzed and compared with both functional and clinical data.

Inflammation is an essential part of the immune response against infection, and cysteine protease enzymes of the caspase family have been shown to have a crucial role in inflammatory processes [23]. One important member of the caspase family is caspase-12. Caspase-12 is an inflammatory caspase, in which a loss-of-function genetic variant has been fixed in some populations by still undefined evolutionary pressures [24]. This loss-of-function is due to the presence of a T/C single nucleotide polymorphism on nucleotide position 125 in the \textit{CASPASE-12} gene [25]. Although the ancestral variant is still present in African populations, of which 20–30% express the active variant of caspase-12, it is absent in Asian and Caucasian populations [25]. Functional studies have suggested that functional caspase-12 is a negative regulator of caspase-1 activation, which might result in lower cytokine production in response to recognition through PRRs. Thus, based on the proposed inhibitory effect on caspase-1 and, consequently, lower IL-1\textbeta and IL-18 production, functional caspase-12 may have an effect on the immune homeostasis, which as a consequence can result in an increased susceptibility to severe sepsis and/or worsen the clinical outcome of sepsis patients [25]. In \textit{Chapter 3} the effect of the \textit{CASPASE-12} genotype on the susceptibility to \textit{Candida} sepsis in African-American patients will be evaluated.

Another important process for host defense is autophagy. Autophagy is an evolutionary conserved housekeeping molecular process important for cellular development, maintenance, energy turnover and antigen presentation. The autophagy machinery leads to the sequestration of components of the cytoplasm into double membrane
vesicles that ultimately fuse with the lysosome for degradation of the autophagosomal content [26]. Previous studies have demonstrated that genetic variation in autophagy genes affects the modulation of inflammation by autophagy [27]. Related to the autophagy process, a genetic variant in ATG16L1 (Thr300Ala, rs2241880) has been related with predisposition to Crohn’s disease, with the ATG16L1*300A conferring a higher risk [28–30]. Similarly, genetic polymorphisms in the IRGM (Immunity-related GTPase family M protein) gene are also associated with susceptibility to Crohn’s disease [31]. Interestingly, it has been shown that one of these IRGM SNPs, rs13361189 SNP, is in perfect linkage disequilibrium with a 20 kb insertion/deletion polymorphism situated upstream of the IRGM promoter region. Consequently, this genetic variant modulates the expression of the IRGM gene [32].

Moreover, recent studies have shown that this ATG16L1 polymorphism not only influences autophagy [33], but also modulates proinflammatory cytokine responses, especially IL-1β [34], and thus interferes with the host defense against microorganisms such as mycobacteria [35]. We have therefore hypothesized that genetic variation in autophagy genes may influence inflammation and host defense during fungal infections, in particular candidiasis. In Chapter 4, studies assessing the relation of the polymorphisms in ATG16L1 (rs2241880) and IRGM (rs13361189 and rs4958847) with predisposition to oropharyngeal candidiasis (OPC) and candidemia will be described.

Downstream signals transduced from the PRRs lead to the production of the inactive pro-cytokine form of IL-1β. Pro-IL-1β requires to be cleaved by the cysteine protease caspase-1 in order to be activated and secreted. In turn, activation of caspase-1 is under control of a protein complex called the inflammasome [39]. The inflammasome consists of a NLR protein [40], pro-caspase-1 and apoptosis-associated speck-like protein (ASC) [41]. After the interaction of the protein complex with a specific ligand, caspase-1 is activated and cleaves pro-IL-1β into the active form that is secreted from the cell through the pannexin-1 channel. A schematic representation of the inflammasome complex is shown in Figure 2. Th17-mediated immune responses against C. albicans, and thus antifungal mucosal host defense, relies strongly on IL-1β production [38].

In addition to systemic candidiasis and RVVC, oropharyngeal candidiasis (OPC) is an opportunistic infection that characterizes HIV infection. In addition to OPC, other common opportunistic infections in HIV positive patients are Mycobacterium avium complex (MAC) disease, Pneumocystis carinii pneumonia (PCP), cytomegalovirus (CMV) retinitis, extrapulmonary cryptococcosis, toxoplasmic encephalitis, tuberculosis, chronic herpes simplex, disseminated histoplasmosis and chronic cryptosporidiosis [42]. However, not all HIV patients with low CD4+ counts will eventually develop opportunistic infections. We hypothesize that genetic variation in the Dectin-1/CARD9 pathway
may affect the predisposition to opportunistic infections in immunocompromised HIV positive patients. The effect of the genotype in DECTIN-1 and CARD9 on the susceptibility of OPC and other opportunistic infections in HIV positive patients will be assessed in Chapter 5.

Not only susceptibility to systemic candidiasis may be influenced by genetic polymorphisms, but predisposition to mucosal fungal infections as well. In addition to the dectin-1 early stop codon, the mutant allele of the cytosine to thymine variation in the promoter region of the IL-4 gene (C589T) was related to a higher susceptibility to RVVC [36]. Moreover, a link between susceptibility to RVVC and the Gly54Asp SNP in the MBL gene, coding for Mannose binding lectin (MBL), was described. In this study it was demonstrated that the mutant allele was associated with reduced
vaginal MBL concentrations [37]. Finally, the relation between the genotype for a variable number tandem repeat polymorphism (VNTR) in intron 4 of the gene coding for the NACHT-LRR-PYD-containing protein 3 (NLRP3) with the incidence of RVVC and vulvar vestibulitis syndrome (VVS) was demonstrated [38]. In Chapter 6 I will study the effect of genetic variation in genes related to PRRs in susceptibility to RVVC. The dectin-1 (Y238X, rs16910526), CARD9 (S12N, rs4077515), mannose receptor (rs1926736, Ser396Gly), TLR1 (rs5743611, Arg80Thr), TLR2 (rs5743704, Pro631His) and TLR4 (rs4986790, Asp299Gly, rs4986791, Thr399Ile) genetic variants will be assessed for their role in RVVC susceptibility. Furthermore, the functional effects of these genetic variants for cytokine production will be investigated.

In order to present an up-to-date overview of the knowledge regarding genetic susceptibility to RVVC, in Chapter 7 I will provide a bibliographic review concerning the relation between genetic variations and susceptibility to this important mucosal Candida infection.

Finally, an overall summary of the findings presented in this thesis will be presented in Chapter 8. In this same chapter I will also propose future directions to follow for the increase in our understanding of the role of genetic variation in the host genome and Candida infections.
References


CHAPTER 2

Genetic Variation in the Dectin-1/CARD9 Recognition Pathway and Susceptibility to Candidemia
Abstract

**Background.** Candidemia is an important cause of morbidity and mortality in critically ill patients or patients undergoing invasive treatments. Dectin-1 is the main β-glucan receptor, and patients with a complete deficiency of either dectin-1 or its adaptor molecule CARD9 display persistent mucosal infections with *Candida albicans*. The role of genetic variation of *DECTIN-1* and *CARD9* genes on the susceptibility to candidemia is unknown.

**Methods.** We assessed whether genetic variation in the genes encoding dectin-1 and CARD9 influence the susceptibility to candidemia and/or the clinical course of the infection in a large cohort of American and Dutch candidemia patients (n = 331) and noninfected matched controls (n = 351). Furthermore, functional studies have been performed to assess the effect of the *DECTIN-1* and *CARD9* genetic variants on cytokine production *in vitro* and *in vivo* in the infected patients.

**Results.** No significant association between the single-nucleotide polymorphisms *DECTIN-1* Y238X and *CARD9* S12N and the prevalence of candidemia was found, despite the association of the *DECTIN-1* 238X allele with impaired *in vitro* and *in vivo* cytokine production.

**Conclusions.** Whereas the dectin-1/CARD9 signaling pathway is nonredundant in mucosal immunity to *C. albicans*, a partial deficiency of β-glucan recognition has a minor impact on susceptibility to candidemia.
Introduction

In developed countries, candidemia is one of the most prevalent bloodstream infections in hospital settings and is associated with significant morbidity and mortality. Candidemia is a systemic infection with *Candida* spp, predominantly *Candida albicans*, that occurs mostly in patients that are immunocompromised due to neutropenia, the use of broad-spectrum antibiotics, or invasive surgery, or by total parenteral nutrition [1, 2]. The first line of defense against fungal infections is the innate immune system that initiates recognition and initial elimination of pathogens, and subsequently induces the activation of adaptive immunity. Pattern recognition receptors (PRRs) are expressed by innate immune cells, and they specifically recognize moieties expressed on the surface of the microbial pathogens, designated as PAMPs (pathogen-associated molecular patterns) [3].

The recognition of PAMPs relies on several families of PRRs, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I helicases, all acting in concert to activate downstream signaling in innate immune cells, thereby inducing production of proinflammatory cytokines and activation of T-cell responses [4]. In the case of fungal pathogens, it is especially TLRs and CLRs that mediate recognition [3]. Among the PRRs recognizing *C. albicans*, the archetypal CLR dectin-1 detects β-glucans [5-8], which comprise 40% of the total cell wall of *C. albicans*. Upon β-glucan recognition, intracellular signaling is induced involving the adaptor proteins spleen tyrosine kinase (Syk) and CARD9 (caspase recruitment domain family member 9), as well as Bcl-10 and Malt1, ultimately activating the transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) [7, 9]. The dectin-1 receptor is expressed mainly by cells of the myeloid lineage (monocytes/macrophages, dendritic cells, and neutrophils) [7, 10].

Previously, we described a Dutch family with members suffering from onycomycosis and recurrent vulvovaginal candidiasis, in which genetic studies have demonstrated that the presence of an early stop mutation in the *DECTIN-1* gene led to a partial or total deficiency in β-glucan recognition [11]. This genetic defect resulted in impaired recognition of *C. albicans* and decreased cytokine production by immune cells, especially for cytokines involved in the T-helper (Th) 17 pathway [11]. The support for the hypothesis of an important role of dectin-1 recognition for mucosal anti-*Candida* defense was provided by the description of another family with a defective *CARD9* gene. Interestingly, family members with *CARD9* deficiency exhibited a similar clinical phenotype with predominantly recurrent mucocutaneous *Candida* infections. Non-functional CARD9 was also associated with defects in Th17 responses [12]. Of note, patients with nonfunctional dectin-1 appeared not to be predisposed to invasive
Candida infection, whereas CARD9-deficient patients were severely prone to develop invasive Candida infections, especially in the brain [11, 12].

These studies implicate the dectin-1/CARD9 pathway as non-redundant in mucosal host defense against C. albicans. However, the role of the dectin-1/CARD9 innate pathway for the host defense against systemic Candida infections has not been directly investigated, although it would be expected to be less important. In the present study, a cohort of patients with candidemia has been studied for the potential association of susceptibility to candidemia with genetic variation in the DECTIN-1 and CARD9 genes. The early stop codon mutation in the DECTIN-1 gene that was previously characterized in the Dutch family described above is a polymorphism with a general allele frequency of 6%–8% in Caucasian populations and 4% in African populations. This polymorphism, which clearly affects dectin-1 function, could therefore be studied as a population-wide potential determinant in susceptibility to candidemia. In contrast, the mutation in the CARD9 gene causing CARD9 deficiency is a very rare mutation, which until now was only found in 1 family [12]. Alternatively, a common genetic variation in the coding region of the CARD9 gene has been detected with a single-nucleotide polymorphism (SNP) at amino acid position 12. This nonsynonymous SNP leads to an amino acid substitution from a serine to an asparagine residue. Because of its potential to influence CARD9 function and high minor allele frequency in both Caucasian (53%) and African (25%) populations, this SNP was also selected in this genetic study [13]. Therefore, the aim of this study was to determine whether these DECTIN-1 and CARD9 SNPs influence susceptibility to candidemia.

Materials and Methods

Patients

Patients were enrolled after informed consent (or waiver as approved by the institutional review board) at the Duke University Hospital (DUMC, Durham, North Carolina) and Radboud University Nijmegen Medical Center (RUNMC, Nijmegen, the Netherlands). The study was approved by the institutional review boards at each study center, and enrollment occurred between January 2003 and January 2009. The clinical characteristics of the patients, both infected and non-infected, are presented in Table 1.

To be included in the analysis of susceptibility to infection, infected subjects must have had at least 1 positive blood culture for a Candida species while hospitalized at the participating center. Non-infected controls must have been hospitalized with no history or evidence of candidemia/invasive candidiasis or any invasive fungal infection. Non-infected controls were recruited from the same hospital wards as infected patients.
so that comorbidities and clinical risk factors for infection would be similar between groups. Intergroup comparisons between the 2 groups of non-infected subjects and between the 2 groups of infected subjects (at DUMC and RUNMC) were performed regarding similarity in genetic distribution of the studied SNPs prior to further statistical

Table 1  Baseline Patient Characteristics for DUMC Infected Cohort (n = 291) and Control Cohort (n = 300). Including Caucasian and African-American Adult Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Infected cohort</th>
<th>Control cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>55.9</td>
<td>57.8</td>
</tr>
<tr>
<td>Immunocompromised State</td>
<td>59%</td>
<td>38%</td>
</tr>
<tr>
<td>HSCT</td>
<td>1%</td>
<td>0%</td>
</tr>
<tr>
<td>Solid organ transplant</td>
<td>12%</td>
<td>2%</td>
</tr>
<tr>
<td>Active Malignancy*</td>
<td>32%</td>
<td>20%</td>
</tr>
<tr>
<td>Solid Tumor</td>
<td>23%</td>
<td>12%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>7%</td>
<td>5%</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>Chemotherapy within past 3 months</td>
<td>17%</td>
<td>11%</td>
</tr>
<tr>
<td>Neutropenia (ANC &lt;500 cells/mm³)</td>
<td>10%</td>
<td>4%</td>
</tr>
<tr>
<td>HIV-infected</td>
<td>2%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Surgery within past 30 days</td>
<td>43%</td>
<td>48%</td>
</tr>
<tr>
<td>Receipt of Total Parenteral Nutrition</td>
<td>19%</td>
<td>4%</td>
</tr>
<tr>
<td>Dialysis dependent</td>
<td>12%</td>
<td>7%</td>
</tr>
<tr>
<td>Acute Renal Failure</td>
<td>34%</td>
<td>22%</td>
</tr>
<tr>
<td>Liver Failure</td>
<td>25%</td>
<td>4%</td>
</tr>
<tr>
<td>Intensive Care Unit Admission within past 14 days</td>
<td>49%</td>
<td>34%</td>
</tr>
<tr>
<td>Bacteremia within past 48 hours</td>
<td>24%</td>
<td>2%</td>
</tr>
<tr>
<td>Median Baseline Serum Creatinine (mg/dL)</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Median Baseline WBC count (cells/mm³)</td>
<td>10.55</td>
<td>8.55</td>
</tr>
</tbody>
</table>

*Candida* spp. **
- *albicans*: 42%
- *glabrata*: 29%
- *parapsilosis*: 16%
- *tropicalis*: 13%
- *krusei*: 4%
- Other *Candida* spp.: 3%

NOTE. ANC, absolute neutrophil count; DUMC, Duke University Hospital; HIV, human immunodeficiency virus; HSCT, hematopoietic stem cell transplantation; WBC, white blood cell.
1 a Subjects could have 1 species isolated.
2 b Sixteen subjects had 1 species isolated.
analysis of infected versus non-infected subjects. Subjects were excluded from the study if insufficient volume of blood or clinical data were available to allow for analysis.

Genetic Analysis
Genomic DNA was isolated from whole blood using standard procedures. Genotyping for the DECTIN-1 Y238X (c.714T > G, rs16910526) and CARD9 S12N (c.35G > A, rs40777515) polymorphisms was performed by using the TaqMan single nucleotide assay C_33748481_10 and C_25956930_20, respectively, on the 7300 ABI real-time polymerase chain reaction system (all from Applied Biosystems).

Genetic Association With Clinical Outcomes
Due to the lack of detailed clinical data on the Dutch candidemia patients (n = 40) and controls (n = 51) recruited at RUNMC, the correlations of the genetic data with clinical outcome were performed with the North American patients (n = 291) and controls (n = 300) from DUMC only. Infected subjects at DUMC were followed prospectively for up to 12 weeks following diagnosis of candidemia to determine clinical outcome: [1] disseminated disease, [2] persistent fungemia, and [3] all-cause mortality at 30 days. Disseminated infection was defined as the presence of Candida spp. at normally sterile body sites outside the bloodstream (excluding the urine). Persistent fungemia was defined as ≥5 days of persistently positive blood cultures. This analysis was performed in the entire infected cohort, as the progression of the disease once occurring is not expected to differ between European and African-American patients, and race was considered a covariate in the analysis. Variables with \( P \) value <.2 were further assessed in a multivariable logistic regression model. Variables with \( P < .05 \) were retained in the final predictive model. Odds ratios and 95% confidence intervals were reported for variables that remained significant in the final multivariable model.

Cytokine Stimulation Assays
PBMCs were isolated from healthy volunteers by Ficoll-Paque gradient. Subsequently, stimulation with heat-killed C. albicans was performed for 24 hours, 48 hours, or 7 days. The stimulation time varied for each cytokine, being 24 hours for tumor necrosis factor (TNF)–α, interleukin (IL)–1β, IL-6, and IL-8, 48 hours for interferon (IFN)–γ, and 7 days for IL-17 production. For the 7-day culture, the medium was supplemented with 10% human serum. After the incubation time, the above-mentioned cytokines were measured in the supernatants by enzyme-linked immunosorbent assay (purchased from R&D Systems or Sanquin Research).

Serum Cytokine Measurements
Cytokine concentrations of IL-6, IL-8, and IFN-γ in plasma and serum samples obtained from infected patients from day 1 up to day 5 after initial positive blood culture were
measured by multiplex fluorescent bead immunoassay (xMAP technology, Bio-Rad) and a BioPlex microbead analyzer (Luminex) according to the manufacturer’s protocol.

**Bioinformatic Analysis**

In order to predict the effect on the conformation of the CARD9 protein given the S12N amino acid change, we used HOPE (http://www.cmbi.ru.nl/hope/), a web server that performs automatic mutant analysis. No homology model was produced because no template with enough identity to the CARD9 sequence exists [14]. We also used the Polyphen software in order to determine quantitatively the damage for the protein stability taking into account the amino acid change of the CARD9 SNP [15].

**Statistical Analysis**

For the analysis of SNPs, statistical comparisons of frequencies were made between infected versus non-infected subjects and of clinical parameters within the infected group using the $\chi^2$ test with SPSS version 16.0 software. Data were analyzed for Caucasian descendants separately from African-Americans, as allelic frequencies were expected to differ between these 2 populations. For the analysis of the in vitro PBMC stimulation and in vivo serum cytokine measurements, a Mann–Whitney U test was performed on the DECTIN-1 genotype experiment and a Kruskal–Wallis -way analysis of variance was performed on the CARD9 genotype experiment with the use of GraphPad Prism software version 4.00.

**Results**

**Genetic Analysis**

Data from adult patients ($n = 331$ (291 North American and 40 Dutch)) and controls ($n = 351$ (300 North American, 51 Dutch)) were included in the analysis for genetic association with susceptibility to infection. Data from children were excluded from this analysis because the number of children enrolled ($n = 27$) was insufficient to analyze this association extensively in a pediatric population, where factors relating to susceptibility to candidemia may differ from those for adults.

The intergroup comparison between the Dutch RUNMC and North American Caucasian DUMC non-infected controls and between the infected subjects recruited at DUMC and RUNMC revealed a similar genetic distribution of the genotyped SNPs, which allowed the groups to be merged into 1 group of non-infected controls and 1 group of infected subjects (data not shown). In contrast, the African-American patients from the DUMC cohort displayed a different genetic pattern, and they were analyzed separately. Genetic distribution of the DECTIN-1 Y238X and CARD9 S12N
polymorphisms in the studied patient groups is shown in Tables 2 and 3, respectively. The genotyping of candidemia patients and non-infected matched controls for polymorphisms in DECTIN-1 and CARD9 revealed no significant association with susceptibility to candidemia. After statistical analysis of the Dutch and the North American cohorts separately, again no significant differences were obtained (data not shown). Similarly, no differences were observed when data were analyzed separately in non-neutropenic and neutropenic patients (data not shown). A post hoc analysis of power for the dectin-1 polymorphism demonstrated that we had 86.7% power to detect a 10% difference in frequency in the Caucasian cohort, with 238 infected and 263 non-infected subjects, with a 2-sided $\alpha = .05$. For African-Americans, a post hoc analysis of power for dectin-1 polymorphism demonstrated that we had 76% power to detect a 10% difference in frequency, with 93 infected and 88 non-infected subjects, also with a 2-sided $\alpha = .05$.

In order to study the role of these genetic variants in the clinical outcome of candidemia, clinical parameters on dissemination, persistence, and 30-day mortality of infected patients were correlated with the DECTIN-1 and CARD9 genotype. Because these data were only available for the North American cohort, for these analyses the
Dutch patients were excluded. No significant associations between DECTIN-1 and CARD9 polymorphisms and clinical parameters were observed in these analyses (Table 4). Minor differences in the clinical characteristics of the control and candidemia groups have been observed. Although we cannot completely rule out that these differences might have caused the lack of effect of the polymorphisms, from the clinical point of view these differences were not as big as to expect major effects.

Table 4  Univariate Analysis of the Association Between the DECTIN-1 and CARD9 Polymorphisms With Disseminated Disease, Persistent Fungemia, and 30-day Mortality in the DUMC Infected Cohort (n = 291).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DECTIN-1 Y238X</th>
<th>CARD9 S12N</th>
</tr>
</thead>
<tbody>
<tr>
<td>No disseminated disease (n = 268)</td>
<td>11.6%</td>
<td>56.9%</td>
</tr>
<tr>
<td>Disseminated disease (n = 63)</td>
<td>15.0%</td>
<td>61.5%</td>
</tr>
<tr>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.46</td>
<td>.54</td>
</tr>
<tr>
<td>No persistent fungemia (n = 280)</td>
<td>12.1%</td>
<td>56.6%</td>
</tr>
<tr>
<td>Persistent fungemia (n = 51)</td>
<td>12.8%</td>
<td>64.3%</td>
</tr>
<tr>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.90</td>
<td>.35</td>
</tr>
<tr>
<td>Survivors (n = 238)</td>
<td>13.4%</td>
<td>56.8%</td>
</tr>
<tr>
<td>Non-survivors (n = 93)</td>
<td>9.0%</td>
<td>60.0%</td>
</tr>
<tr>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>.29</td>
<td>.61</td>
</tr>
</tbody>
</table>

**NOTE.** Numbers are the percentages of patients with each outcome who were found to bear the mutant allele of the particular polymorphism.  
<sup>a</sup> P values were calculated with the χ² test.

**Functional Analysis of Cytokine Profiles**

In order to assess the functional consequences of the SNPs in DECTIN-1 and CARD9, both in vitro and in vivo cytokine measurements were performed. The in vitro evaluation was performed by measuring cytokine production upon heat-killed Candida stimulation of PBMCs with different DECTIN-1 and CARD9 genotypes, obtained from healthy volunteers. The results of these in vitro assays are shown in Figure 1. Significant differences in cytokine production were observed between cells obtained from individuals with different DECTIN-1 genotypes, including decreased production of TNF-α (P = .003) and IL-1β (P < .0001), while differences in the production of IL-6, IL-8, IFN-γ (Figure 1), and IL-17 (data not shown) were not statistically significant. In contrast, no effect of the CARD9 polymorphism on C. albicans–induced cytokine production was apparent.
Figure 1 Functional analysis on the stimulation of the peripheral blood mononuclear cells (PBMCs) with heat-killed *Candida albicans* blastoconidia. Cytokine production capacity of tumor necrosis factor alpha (TNFα), interleukin (IL)–1β, IL-6, IL-8, and interferon (IFN)–γ was compared between the cells obtained from healthy volunteers bearing wild-type or variant *DECTIN-1* or *CARD9* single-nucleotide polymorphisms (SNPs). He, heterozygous; Ho, homozygous; Wt, wild type. Data are presented as mean ± SEM. *P < .05 (Mann–Whitney U test or Kruskal–Wallis 1-way analysis of variance).
Serum samples collected from infected patients during the first 5 days after initial positive blood culture were measured for concentrations of IL-6, IL-8, and IFN-γ (Figure 2). In general, the IL-6, IL-8, and IFN-γ production decreased over time. Correlation with the genotype of the patients revealed that patients heterozygous for the DECTIN-1 polymorphism had lower cytokine concentrations at the initiation of infection, which remained low throughout the follow-up period. No differences in cytokine-circulating concentrations were apparent between individuals bearing different CARD9 genotypes.
Discussion

In the present study, we assessed the influence of genetic polymorphisms in the genes encoding dectin-1 and CARD9 for susceptibility to candidemia. Patients heterozygous for the \textit{DECTIN-1} Y238X polymorphism exhibited moderately lower cytokine responses compared with individuals bearing wild-type \textit{DECTIN-1} genotypes, whereas no effects
of the CARD9 SNP on these responses were observed. Despite these differences, the genetic variation in DECTIN-1 and CARD9 was not associated with susceptibility to candidemia.

Polymorphisms in several PRRs have been suggested to be associated with a higher risk to fungal infections [16-19]. Recent studies have unveiled genetic variants of DECTIN-1 and CARD9 that predispose to mucocutaneous fungal infections [11, 12]. Individuals heterozygous for the DECTIN-1 Y238X allele have also been shown to display increased colonization with Candida when they suffer from hematological malignancies, leading to more frequent prescription of prophylactic antifungal therapy [20]. However, the role of genetic variability in these genes in susceptibility to candidemia was not known. Therefore, we have investigated whether SNPs in DECTIN-1 and CARD9 are associated with susceptibility to systemic Candida infection.

Dectin-1 is the most important receptor of β–glucans, and it plays an important role for induction of cytokines in human monocytes and macrophages [21, 22]. The DECTIN-1 Y238X polymorphism, the best characterized of the 2 SNPs analyzed in this study, clearly affects dectin-1 function [11], as confirmed by the in vitro cytokine data provided here. However, our data indicate that if patients are heterozygous for this DECTIN-1 polymorphism, neither susceptibility to Candida bloodstream infections, nor clinical parameters such as severity and dissemination of the infection or 30-day mortality, are affected. This might be due to the specific involvement of dectin-1 recognition pathway in mucosal immunity to C. albicans, reflected by the induction of cytokines that are crucial for Th17 responses, including IL-6 and IL-17. In contrast, for an effective immune response to prevent Candida bloodstream infections, proinflammatory cytokines such as TNF-α, or Th1 responses releasing IL-18 and IFN-γ, may play a more important role. Indeed, the production of proinflammatory cytokines after stimulation of cells in vitro with C. albicans was only moderately decreased in individuals heterozygous for the DECTIN-1 Y238X SNP, and these data are supported by cytokine stimulation experiments from individuals with complete dectin-1 deficiency [11]. Unexpectedly, serum cytokine measurements of IFN-γ were found to be lower in patients heterozygous for the DECTIN-1 Y238X polymorphism, although this genetic variant does not seem to confer an additional risk to develop or worsen the outcome of candidemia. Presumably, the relatively high residual circulating cytokine concentrations in individuals bearing the DECTIN-1 polymorphism were sufficient to afford protection against candidemia. It can be therefore concluded that other PRRs and mechanisms of antifungal host defense can compensate for the deficit of dectin-1 function, especially at the tissue level where Candida invasion takes place.
Several explanations are possible for why no association of the \textit{CARD9} S12N SNP with susceptibility to candidemia was observed. Because the consequences of the presence of the \textit{CARD9} polymorphism for its function have not been studied, one possibility is that this polymorphism only minimally affects the protein structure. A bioinformatic approach may assess the putative effect on the function of the molecule of the amino acid change from serine to asparagine in \textit{CARD9}. Since in the Protein Data Bank there is no 3-dimensional model of the \textit{CARD9} protein available, as well as no modeling template to build a homology model, the analysis was made by comparing the amino acid composition of the protein bearing the mutation with the wild-type protein with the Swift Yasara twinset via the HOPE server [14]. This analysis generated evidence that the amino acid change is important because the mutation is located on position 12 in the \textit{CARD} domain. These domains occur in a wide range of proteins and are thought to mediate the formation of larger protein complexes via direct interactions between individual \textit{CARD}s. Moreover, as serine and asparagine differ in size and hydrophobicity and the mutated residue is buried in the core of the protein, this change may also modify \textit{CARD9} function. Nevertheless, the Swift algorithm predicted that the polymorphism S12N is “allowed” [23], and the Polyphen algorithm predicts the effect of the serine to asparagine amino acid change as “benign” for protein function, taking into account that this residue position is not conserved through evolution [15], and this is supported by the lack of differences in cytokine production.

In conclusion, we demonstrate that genetic variations in SNPs of the dectin-1/\textit{CARD9} pathway have no major impact on the susceptibility to systemic infections with \textit{C. albicans}. The dectin-1/\textit{CARD9} recognition pathway seems therefore to be of high importance for mucosal antifungal host defense, whereas systemic immunity to \textit{Candida} largely relies on alternative immune recognition pathways.

\textbf{Funding}
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\textbf{Acknowledgments}
All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
References

CHAPTER 3

The impact of caspase-12 on susceptibility to candidemia
CHAPTER 3

Abstract

*Candida* is one of the leading causes of sepsis, and an effective host immune response to *Candida* critically depends on the cytokines IL-1β and IL-18, which need caspase-1 cleavage to become bioactive. Caspase-12 has been suggested to inhibit caspase-1 activation and has been implicated as a susceptibility factor for bacterial sepsis. In populations of African descent, *CASPASE-12* is either functional or non-functional. Here, we have assessed the frequencies of both *CASPASE-12* alleles in an African-American *Candida* sepsis patients cohort compared to uninfected patients with similar predisposing factors. African-American *Candida* sepsis patients (n = 93) and non-infected African-American patients (n = 88) were genotyped for the *CASPASE-12* genotype. Serum cytokine concentrations of IL-6, IL-8, and IFNγ were measured in the serum of infected patients. Statistical comparisons were performed in order to assess the effect of the *CASPASE-12* genotype on susceptibility to candidemia and on serum cytokine concentrations. Our findings demonstrate that *CASPASE-12* does not influence the susceptibility to *Candida* sepsis, nor has any effect on the serum cytokine concentrations in *Candida* sepsis patients during the course of infection. Although the functional *CASPASE-12* allele has been suggested to increase susceptibility to bacterial sepsis, this could not be confirmed in our larger cohort of fungal sepsis patients.
Introduction

One of the leading pathogens causing sepsis in immunocompromised hosts are *Candida* spp. [1, 2]. Medical conditions that lead to an immunocompromised state increase susceptibility to *Candida* sepsis [3]. In addition to exogenous factors, it is believed that genetic variation also plays an important role in susceptibility to sepsis [4-6]. Caspase-12 is an inflammatory caspase, in which a loss-of-function genetic variant has been fixed in some populations by still undefined evolutionary pressures [7-9]. This loss-of-function is due to the presence of a T/C single nucleotide polymorphism (rs497116) on nucleotide position 125 in the *CASPASE-12* gene [10]. Although the ancestral variant is still present in African and African-American populations, of which 20–30% express the active variant of caspase-12, it is absent in Asian and Caucasian populations [9, 10].

Functional studies have suggested that functional caspase-12 is a negative regulator of caspase-1 activation, which might result in less cytokine production in response to recognition through pattern recognition receptors. Thus, based on the proposed inhibitory effect on caspase-1 and, consequently, lower IL-1β and IL-18 production, functional caspase-12 may increase the susceptibility to severe sepsis and/or the clinical outcome of sepsis patients [10]. Therefore, it is compelling to assess whether genetic variation in *CASPASE-12* plays a role in the susceptibility to *Candida* sepsis. The aim of this study was to assess whether genetic variants of *CASPASE-12* influence the incidence, severity, and mortality of *Candida* sepsis in a cohort of African-American patients.

Materials and Methods

Subjects were enrolled between January 2003 and January 2009 after informed consent (or waiver, as approved by the Institutional Review Board) at the Duke University Hospital (DUMC, Durham, NC, USA). Infected subjects had ≥1 positive blood cultures for a *Candida* species while hospitalized. Non-infected controls were recruited from the same hospital wards as infected patients, with no history or evidence of *Candida* sepsis/invasive candidiasis or any invasive fungal infection.

Genomic DNA was isolated from whole blood using standard procedures. The region of interest of the *CASPASE-12* gene was amplified as described previously [10].

Circulating cytokine concentrations of IL-6, IL-8, and IFNγ in infected patients were measured by Multiplex Fluorescent Bead Immunoassays (xMAP technology, Bio-Rad, Veenendaal, the Netherlands), from day 0 up to day 5 after the initial positive blood culture.
Statistical comparisons of frequencies were made between infected versus non-infected subjects using Chi-square tests. Statistical analysis of the cytokine data was performed by using the Mann–Whitney $U$-test. Overall, a $p$-value < 0.05 was considered to be statistically significant.

**Results**

A total of 93 African-American patients and 88 non-infected African-American controls had genetic and clinical data available for the analysis. The demographic data for the study subjects are presented in Table 1.

No significant differences in the distribution of $\text{CASPASE-12}$ genotypes were seen when comparing infected patients (CC 3.9%, CT 25.3%, TT 72.4%) and non-infected controls (CC 2.9%, CT 30.0%, TT 66.1%) ($p > 0.05$). No associations between the $\text{CASPASE-12}$ genotypes and disseminated disease, persistent fungemia, or 30-day mortality were observed (data not shown).

Serum samples collected from infected patients during the first 5 days after the initial positive blood culture were measured for concentrations of IL-6, IL-8, and IFN$\gamma$. Also, measurements of IL-1$\beta$ and IL-18 were performed in these samples. However, the concentrations of these cytokines were too low to detect (data not shown). Cytokine concentrations decreased over time. No differences in cytokine concentrations were apparent between individuals bearing different $\text{CASPASE-12}$ genotypes (Fig. 1).

Caspase-12 has been suggested to inhibit caspase-1 processing of pro IL-1$\beta$ and pro IL-18 into the active cytokines. Genetic variation of $\text{CASPASE-12}$ in populations of African descent has been previously associated with susceptibility to bacterial sepsis [10]. The present study was performed in order to assess the role of caspase-12 in sepsis caused by $\text{Candida}$ spp. The results indicate that the $\text{CASPASE-12}$ genotype has no significant effect on the susceptibility and severity of systemic infections with $\text{Candida}$.

$\text{Candida}$ is one of the leading pathogens causing sepsis [2, 11, 12]. Pro-inflammatory cytokines such as IL-1$\beta$ and IL-18 are a crucial factor in eliciting an effective immune response to eradicate the infection. A modulatory step in the production of these cytokines is exerted at the level of caspase-1, a protease that cleaves the pro-form of these cytokines into shorter bioactive proteins [13, 14]. It has previously been reported that $\text{CASPASE-12}$ knockout mice were better capable of clearing both local and systemic bacterial infections compared to wild-type mice, through an improved
Table 1 Baseline patient characteristics of African-American patients with *Candida* systemic infection or uninfected controls recruited at the Duke University Hospital (DUMC, Durham, NC, USA) (*n* = 181).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Infected cohort (n = 93), %</th>
<th>Control cohort (n = 88), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>51.6</td>
<td>48.9</td>
</tr>
<tr>
<td>Female</td>
<td>48.4</td>
<td>51.1</td>
</tr>
<tr>
<td>Immunocompromised state</td>
<td>54.8</td>
<td>48.9</td>
</tr>
<tr>
<td>HSCT</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Solid organ transplant</td>
<td>7.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Active malignancy*</td>
<td>22.6</td>
<td>13.8</td>
</tr>
<tr>
<td>Solid tumor</td>
<td>14</td>
<td>8.0</td>
</tr>
<tr>
<td>Leukemia</td>
<td>5.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>3.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Chemotherapy within past 3 months</td>
<td>12.9</td>
<td>5.6</td>
</tr>
<tr>
<td>Neutropenia (ANC &lt;500 cells/mm³)</td>
<td>4.3</td>
<td>1.2</td>
</tr>
<tr>
<td>HIV-infected</td>
<td>5.4</td>
<td>0</td>
</tr>
<tr>
<td>Surgery within past 30 days</td>
<td>34.4</td>
<td>30.7</td>
</tr>
<tr>
<td>Receipt of total parenteral nutrition</td>
<td>19.4</td>
<td>5.75</td>
</tr>
<tr>
<td>Dialysis-dependent</td>
<td>15.1</td>
<td>8.0</td>
</tr>
<tr>
<td>Acute renal failure</td>
<td>36.6</td>
<td>33.0</td>
</tr>
<tr>
<td>Liver failure</td>
<td>25.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Intensive care unit admission within the past 14 days</td>
<td>39.8</td>
<td>31.8</td>
</tr>
<tr>
<td>Median baseline serum creatinine (mg/dL)</td>
<td>2.27</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Candida</em> spp.**</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>albicans</em></td>
<td>44.2</td>
<td>-</td>
</tr>
<tr>
<td><em>glabrata</em></td>
<td>23.7</td>
<td>-</td>
</tr>
<tr>
<td><em>parapsilosis</em></td>
<td>17.2</td>
<td>-</td>
</tr>
<tr>
<td><em>tropicalis</em></td>
<td>10.8</td>
<td>-</td>
</tr>
<tr>
<td><em>krusei</em></td>
<td>3.2</td>
<td>-</td>
</tr>
<tr>
<td>Other <em>Candida</em> spp.</td>
<td>0.9</td>
<td>-</td>
</tr>
</tbody>
</table>

*Subjects could have more than one active malignancy
**Sixteen subjects had ≥1 species isolated
inflammatory response [15]. The same authors described a similar effect of caspase-12 in patients with bacterial sepsis, with individuals bearing functional caspase-12 being more susceptible to this condition [10]. However, the role of \textit{CASPASE-12} genetic variants in fungal sepsis has not been addressed so far.

Firstly, the comparison of \textit{CASPASE-12} genotype frequencies in African-American patients with non-infected controls revealed no statistically significant differences. Secondly, no effects of the \textit{CASPASE-12} genotype was observed in relation to the clinical outcome of infection, assessed as disseminated disease, persistent fungemia, and

\textbf{Figure 1} IL-6, IL-8, and IFNy circulating concentrations in infected patients from day 0 up to day 5 after initial positive blood culture, in relation to the \textit{CASPASE-12} genotype. TC heterozygous, TT homozygous mutant. The data are presented as mean ± standard error of the mean (SEM).
30-day mortality. Furthermore, serum cytokine concentrations during the first 5 days of infection were shown to be unaffected by the CASPASE-12 genotype.

Our findings on the lack of influence of the CASPASE-12 genotype on fungal sepsis contrast with those of Saleh et al. [10, 15] who suggested an important role of this genetic variant in bacterial sepsis. Moreover, circulating cytokine concentrations in infected patients were also not influenced by the CASPASE-12 genotype. It should be emphasized that this is, in particular, true for IL-6 and IFNγ, cytokines that are induced by IL-1β and IL-18, respectively [16-18]. This provides indirect evidence that functional caspase-12 has no clear effect on the production of IL-1β and IL-18 in the context of Candida sepsis. One possible explanation for the discrepancy between this study and that of Saleh et al. [10] is represented by the different cause of sepsis in the two studies, fungal and bacterial, respectively. However, one has to concede that the pro-inflammatory cytokines, of which production is reportedly regulated by the CASPASE-12 genotype, exert similar protective effects in bacterial and fungal sepsis [18-21]. In this respect, a recent study has also failed to reproduce the inhibitory effects of the CASPASE-12 genotype of lipopolysaccharide and Gram-negative bacteria-induced cytokine production [22], bringing into question the biological activity of caspase-12.

In conclusion, although an effect of the CASPASE-12 genotype on the susceptibility to bacterial sepsis has been previously reported in a small cohort of African-American patients [10], this could not be confirmed in our larger cohort of fungal sepsis patients. Furthermore, clinical outcome and in vivo cytokine responses were not influence by the CASPASE-12 genotype. Therefore, we propose that caspase-12 is redundant for systemic host defense in sepsis.

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Conflict of interest
All authors declare no conflicts of interest.

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References


CHAPTER 4

Role of autophagy genetic variants for the risk of Candida infections
Abstract

_Candida albicans_ can cause candidemia in neutropenic and critically ill patients, and oropharyngeal candidiasis in HIV-positive patients with low CD4+ counts. However, not all patients at risk develop _Candida_ infections, and the genetic background of the patient might play a role in the susceptibility to infection. Autophagy mediates pathogen clearance and modulation of inflammation. The aim of this study was to assess the effect of genetic variation in the _ATG16L1_ and _IRGM_ autophagy genes on the susceptibility to candidemia and oropharyngeal candidiasis.

We assessed whether genetic variation in the _ATG16L1_ and _IRGM_ genes influences susceptibility to candidemia in a cohort of candidemia patients of both African and European origin. In addition, we assessed the effect of these polymorphisms for the susceptibility to oropharyngeal candidiasis in an HIV-positive cohort from Tanzania. Functional studies have been performed to assess the effect of the _ATG16L1_ and _IRGM_ genetic variants on cytokine production both _in vitro_ and _in vivo_. The results indicate that _ATG16L1_ variants modulate production of TNFα, but not other cytokines, while no effects were seen in the presence of _IRGM_ polymorphisms. In addition, no significant associations between the SNPs in the _ATG16L1_ and _IRGM_ genetic variants and the incidence of candidemia or oropharyngeal candidiasis were identified.

In conclusion, despite moderate effects on the modulation of proinflammatory cytokine production, genetic variation in the autophagy genes _ATG16L1_ and _IRGM_ has a minor impact on the susceptibility to both mucosal and systemic _Candida_ infections.
Introduction

*Candida albicans* is a dimorphic fungus that is commensal in immunocompetent individuals, colonizing the skin, gastrointestinal tract and oral and genital mucosa. However, under certain host immune defense conditions, *C. albicans* can become pathogenic, being the most common fungal pathogen in humans [1]. *C. albicans* can cause both mucosal and disseminated infections. Candidemia is a systemic infection with *Candida* spp., predominantly but not limited to *C. albicans*, that occurs mostly in patients that are immunocompromised due to neutropenia, in critically-ill patients with use of broad spectrum antibiotics, invasive surgery, or in patients receiving total parenteral nutrition [2, 3]. Moreover, other risk factors such as ethnic background might also play a role based on the genetic differences between individuals of European vs. African ancestry [4]. Oropharyngeal candidiasis (OPC) is a *Candida* mucosal infection that occurs very often as an opportunistic infection in HIV positive patients with a low CD4⁺ count (<200 cells/µL) [5-7]. In the absence of proper treatment, the chance of spread of the infection to the bloodstream increases [8].

However, not all patients at risk develop *Candida* infections, and part of the differential susceptibility may be explained by the genetic makeup of the patients, as exemplified by several reports published previously [9-12]. Autophagy is a highly conserved housekeeping molecular process important for cellular development, maintenance, energy turnover and antigen presentation. The autophagy machinery enables the formation of double membrane vesicles leading to the sequestration of components of the cytoplasm into double membrane vesicles that ultimately fuse with the lysosome for degradation of the autophagosomal content [13]. Furthermore, autophagy is enabling the lysosomal digestion and antigen presentation of phagocytosed materials such as fungal and bacterial pathogens, including *Candida* spp. [14].

Previous studies have demonstrated that genetic variation in autophagy genes affect the modulation of inflammation by autophagy [15]. A genetic variant in *ATG16L1* (Thr300Ala, rs2241880) has been associated with susceptibility to Crohn’s disease, with the *ATG16L1*³00A conferring a higher risk [16-18]. Moreover, recent studies have shown that this *ATG16L1* polymorphism not only affects the capacity to execute autophagy [19], but also to modulate proinflammatory cytokine responses, especially IL-1β [20], and influences host defense against microorganisms such as mycobacteria [21].

Similarly, genetic polymorphisms in the *IRGM* (Immunity-related GTPase family M protein) gene are associated with Crohn’s disease, including the SNPs rs13361189 and rs4958847 [22]. Interestingly, it has been shown that one of these *IRGM* SNPs, rs13361189,
is in perfect linkage disequilibrium with a 20 kb insertion/deletion polymorphism situated upstream of the \textit{IRGM} promoter region. Consequently, this genetic variant modulates the expression of the \textit{IRGM} gene [23]. Considering the data linking \textit{ATG16L1} and \textit{IRGM} SNPs with the modulation of immune responses by autophagy, it is compelling to assess whether these genetic variants could influence the susceptibility to fungal infections, and in particular oropharyngeal and/or disseminated candidiasis because of their functional effects on the autophagy machinery. Although in a recent study we were not able to identify a crucial role of autophagy in systemic \textit{Candida} infections [24], no information is available on the role of autophagy in mucosal infection with \textit{Candida}. Interestingly, \textit{Candida albicans} is also one of the immunogens for developing anti-\textit{Saccharomyces cerevisiae} antibodies (ASCA), which are regularly observed in patients with mucosal lesions in Crohn’s disease [25, 26].

In order to determine the role of the genetic polymorphisms in two autophagy genes in the susceptibility to both systemic and mucosal infections with \textit{Candida albicans}, we evaluated the frequency of the genotypes of three polymorphisms in the \textit{ATG16L1} and \textit{IRGM} genes in the predisposition to two different types of \textit{Candida albicans} infections: oropharyngeal candidiasis in HIV positive patients and disseminated candidiasis or candidemia in intensive care unit patients. In order to support the genetic analysis, functional assays were performed with peripheral blood mononuclear cells bearing different \textit{ATG16L1} and \textit{IRGM} genotypes and serum cytokine measurements were conducted by comparing the genotypes for their ability to produce cytokines upon \textit{Candida albicans} stimulation either \textit{in vitro} or \textit{in vivo}. Finally, a bioinformatic analysis was performed with the aim to further explain the possible functional consequences of the genetic variants in \textit{ATG16L1} and \textit{IRGM}.

**Patients and Methods**

**HIV positive patients**

The effect of the polymorphisms associated with the autophagy process in the predisposition to OPC was assessed in a group of 155 HIV-seropositive patients recruited at the Muhimibili National Hospital HIV-clinic in Dar-es-Salaam, Tanzania. The recruitment period took place between April 2007 and August 2008. The patients’ clinical examination was performed by an independent physician according to the WHO clinical staging criteria [27]. The oral examination was routinely performed at each visit according to WHO rules [28]. Variations in color, size and shape of anatomical areas of the intraoral tissue were taken as clinical sings for OPC. The clinical manifestations of OPC in these patients were diverse, presenting a pseudomembranous candidiasis
or a combination of pseudomembranous and erythematous, hyperplastic, or angular cheilitis. This cohort has been previously reported in genetic association studies by our group [29], and the study was approved by the Ethical Committee of the Hospital. Sampling was performed by firmly swabbing the lesion site with a sterile cotton wool swab. Immediate microbiological confirmation was performed, the samples were sent immersed in 10% potassium hydroxide (KOH). Within this cohort, 82 patients developed OPC, and 73 patients did not present with OPC.

**Candidemia patients**

Patients were enrolled after informed consent (or waiver as approved by the Institutional Review Board) at the Duke University Hospital (DUMC, Durham, NC, USA) and Radboud University Medical Center (RUMC, Nijmegen, The Netherlands). The study was approved by the Institutional Review Boards at each study center, and enrollment occurred between January 2003 and January 2009. To be included in the analysis of susceptibility to infection, infected subjects must have had at least one positive blood culture for a *Candida* species while hospitalized at the participating center. Non-infected controls must have been hospitalized with no history or evidence of candidemia/invasive candidiasis, or any invasive fungal infection. Non-infected controls were recruited from the same hospital wards as infected patients in such a way that comorbidities and clinical risk factors for infection would be similar between groups. The clinical characteristics of the patients have been previously reported in genetic association studies [9, 24, 30].

**Genetic analysis**

Genomic DNA was isolated from whole blood using standard procedures. Genotyping for the *ATG16L1* T300A (rs2241880) and the *IRGM* SNPs (rs13361189 and rs4958847) single nucleotide polymorphisms (SNPs) was performed by using the TaqMan single-nucleotide assay C_9095577_20, C_31986315_10 and C_1398968_10, respectively, on the 7300 ABI Real-Time polymerase chain reaction system (all from Applied Biosystems, CA, USA). Two of these polymorphisms (rs2241880 and rs4958847) were also part of the Sequenome (Sequenom MassARRAY®, Sequenom, San Diego, CA) analysis of the study of Smeekens at al [24]. However, we chose to assess it in the present study through TaqMan technology in the candidemia cohort, due to the fact that the quality of DNA necessary for Sequenome analysis was not sufficient for the entire cohort of patients. The assessment of all three polymorphisms in the studies on mucosal forms of infections has not been presented elsewhere.

**Cytokine stimulation assays**

Peripheral blood mononuclear cells (PBMC) were isolated from 73 healthy volunteers by Ficoll-Paque gradient. Subsequently, stimulation with heat-killed *Candida albicans*
blastoconidia was performed for 24 hours, 48 hours or 7 days. The stimulation time varied for each cytokine, being 24 hours for IL-1β, IL-6, IL-8 and TNFα, 48 hours for IFNγ and IL-10, and 7 days for IL-17. At the end of the incubation time, the above-mentioned cytokines were measured in the supernatants by ELISA (purchased from R&D systems Minneapolis, MN, or Sanquin Research, Amsterdam, The Netherlands). Comparisons of cytokine production between patients bearing the different ATG16L1 or IRGM genotypes were performed.

**Serum cytokine measurements**

Cytokine concentrations of IL-6, IL-8 and IFNγ in plasma and serum samples obtained from infected patients from day 0 up to day 5 after initial positive blood culture were measured by Multiplex Fluorescent Bead Immunoassays (xMAP technology, Bio-Rad, Veenendaal, The Netherlands) and a Bio-plex microbead analyzer (Luminex, Austin, TX, USA) according to the manufacturer’s protocol.

**Bioinformatic analysis**

In order to predict the effect on the conformation of the ATG16L1 protein given the T300A amino acid change we used HOPE (http://www.cmbi.ru.nl/hope/), a next generation web server that performs automatic mutant analysis. No homology model was produced because no template with enough identity to the ATG16L1 sequence exists [31]. The HOPE server assessment was only possible for the ATG16L1 SNP since it is the only SNP from this study that alters the amino acid sequence and the HOPE servers works exclusively in case of non-synonymous mutations and their effect on protein function.

**Statistical analysis**

For the genetic analysis, statistical comparisons of frequencies were made between infected and non-infected patients (with either OPC or candidemia) by performing the Chi-square test with SPSS version 20 (IBM SPSS Statistics for Windows Version 20.0. Armonk, NY: IBM Corp. For the analysis of the in vitro PBMC stimulation and in vivo serum cytokine measurements, a Kruskal-Wallis one-way ANOVA was performed with the use of GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA). Corrections for multiple testing have not been performed due to the absence of significant differences, and throughout the manuscript only uncorrected P-values are mentioned.
Results

Functional analysis of cytokine profiles
The cytokines measured in the in vitro assays are chosen based on their role in the anti-Candida immune response: TNFα, IL-1β, IL-6, IL-8 and IL-10 to monitor the response of monocytes, and IFNγ and IL-17 as T-cell derived cytokines to monitor the activation of the Th1 and Th17 responses, respectively. A significant difference in C. albicans-induced cytokine production was observed between cells isolated from individuals with different ATG16L1 genotypes for production of TNFα (P = 0.0039) (Figure 1). However, no differences were observed in the production of IL-1β, IL-6, IL-8, IL-10, IFNγ and IL-17. In addition, the effect of the genotype of the IRGM gene on the in vitro cytokine production was evaluated. These analyses revealed that the only statistically significant effect of the rs13361189 genotype was observed for production of IL-8. However, Candida induced IL-8 production was not affected by the other SNP in IRGM. Furthermore, TNFα, IL-1β, IL-6, IL-10, IFNγ and IL-17 production were not affected by any of the two investigated SNPs in IRGM.

Bioinformatic Analysis using the HOPE server
We also performed a bioinformatic analysis using the HOPE next-generation server [31]. The output indicates that the change from a threonine into an alanine at position 300 of the ATG16L1 protein introduces changes in the amino acid size and hydrophobicity. The mutant residue is smaller and more hydrophobic than the wild-type residue. Moreover, the server predicted that the polymorphism would cause an empty space in the core of the protein (or protein-complex). The change in the hydrophobicity between the wild-type and mutant-type amino acids would cause a loss of hydrogen bonds in the core of the protein (or protein-complex), and as a results it would disturb correct folding (Supplementary figure 1).

Oropharyngeal candidiasis
The distribution of the ATG16L1 and IRGM polymorphisms in the HIV-positive patient groups with or without OPC is shown in Table 1. No statistically significant differences were observed. Since the CD4+ count is considered as a confounder, we stratified the study subjects based on their CD4+ counts. No significant association was observed in either CD4+ count group (Table 2).

Disseminated Candidiasis
The intergroup comparison between the Dutch RUNMC and North-American Caucasian DUMC controls and patients revealed a similar genetic distribution of the genotyped SNPs, which allowed the groups to be merged into one group of subjects of European descent (data not shown). Genetic distribution of the ATG16L1 and IRGM
Figure 1 Functional analysis on the stimulation of peripheral blood mononuclear cells (PBMCs) with heat-killed C. albicans blastoconidia. Cytokine production capacity of TNFα, IL-1β, IL-6, IL-8, and IFNγ was compared between the cells obtained from healthy volunteers bearing wild-type or variant ATG16L1 or IRGM SNPs. Data are presented as means ± SEM, * p < 0.05. For the ATG16L1 rs2241880 polymorphism the antisense nucleotides are depicted.
ROLE OF AUTOPHAGY GENETIC VARIANTS FOR THE RISK OF CANDIDA INFECTIONS

polymorphisms in the studied patient groups is shown in Table 3. No significant associations of polymorphisms in ATG16L1 and IRGM with susceptibility to candidemia were revealed ($P > 0.05$).

Serum samples collected from patients with bloodstream Candida spp. infections during the first 5 days after initial positive blood culture were measured for concentrations of IL-6, IL-8 and IFNγ. The results are shown in Figure 2: IL-6, IL-8, and IFNγ circulating
Table 3  Frequencies of the genotype of the ATG16L1 SNP number rs2241880 and the IRGM SNPs number rs13361189 and rs4958847 and the incidence of candidemia in a cohort of hospitalized patients of African-American and European descent.

<table>
<thead>
<tr>
<th></th>
<th>African-American descent</th>
<th>European descent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Candidemia</td>
<td>TT* (%)</td>
</tr>
<tr>
<td><strong>ATG16L1</strong> rs2241880</td>
<td>Non-Infected (N = 52)</td>
<td>28 (53.85)</td>
</tr>
<tr>
<td></td>
<td>Infected (N = 62)</td>
<td>25 (40.32)</td>
</tr>
<tr>
<td></td>
<td>Candidemia</td>
<td>CC (%)</td>
</tr>
<tr>
<td><strong>IRGM</strong> rs13361189</td>
<td>Non-Infected (N = 52)</td>
<td>7 (13.46)</td>
</tr>
<tr>
<td></td>
<td>Infected (N = 62)</td>
<td>14 (22.58)</td>
</tr>
<tr>
<td></td>
<td>Candidemia</td>
<td>GG (%)</td>
</tr>
<tr>
<td><strong>IRGM</strong> rs4958847</td>
<td>Non-Infected (N = 52)</td>
<td>20 (38.46)</td>
</tr>
<tr>
<td></td>
<td>Infected (N = 60)</td>
<td>19 (31.67)</td>
</tr>
</tbody>
</table>

Oropharyngeal candidiasis = OPC
* For the ATG16L1 rs2241880 polymorphism the antisense nucleotides are depicted.
concentrations were not affected by any of the SNPs in ATG16L1 and IRGM. Other cytokines such as TNFα, IL-1β, IL-10 and IL-17 were not detectable in the serum samples (data not shown).

Figure 2  Cytokine concentrations of IL-6, IL-8 and IFNγ in plasma and serum samples from infected patients from day 0 up to day 5 after initial positive blood culture correlated with the ATG16L1 or IRGM genotype. WT: wild-type, HET: heterozygous. HOM: homozygous. Data are presented as means ± SEM, * p < 0.05.
Discussion

In the current study, we evaluated the effect of polymorphisms in the autophagy genes \textit{ATG16L1} and \textit{IRGM} [15, 23] on the susceptibility to OPC and candidemia, respectively. Upon stimulation with \textit{Candida} blastoconidia, the \textit{in-vitro} cytokine production capacity of primary immune cells with different \textit{ATG16L1} genotypes demonstrated a lower production of TNF\(\alpha\) by cells bearing the ATG16L1 300A allele. On the other hand, the variant allele of \textit{IRGM} rs13361189 moderately increased IL-8
production under similar conditions. Despite these differences, the genotype of the \textit{ATG16L1} and \textit{IRGM} SNPs did not affect the susceptibility of patients to either OPC or systemic candidiasis.

Genetic variation in genes coding for the autophagy regulatory proteins are known to alter the clearance of intracellular bacteria [23]. The SNP rs2241880 of \textit{ATG16L1} causes a partial loss of function in the \textit{ATG16L1} protein, inhibiting the \textit{Salmonella}-induced autophagy process, which in turn results in reduced clearance of the microorganisms in human epithelial cells [15]. In addition, a polymorphism in the promoter region of the \textit{IRGM} gene conferred an increased susceptibility to tuberculosis [32]. 21 different mouse IRG genes are located on chromosomes 11 and 18, and have been demonstrated to have a very powerful effect on the clearance of intracellular pathogens such as \textit{Listeria monocytogenes}, \textit{Toxoplasma gondii} [33, 34], \textit{Mycobacterium tuberculosis} [35], \textit{Salmonella typhimurium} and \textit{Chlamydia trachomatis} [34]. Furthermore, genetic variation in both \textit{ATG16L1} and \textit{IRGM} genes is associated with a higher susceptibility to Crohn’s disease [16-18, 22]. Conversely, the role of these genetic polymorphisms on the susceptibility to fungal pathogen has yet to be studied. Therefore, we have investigated whether SNPs in \textit{ATG16L1} and \textit{IRGM} are related to the propensity to develop oropharyngeal and/or systemic \textit{Candida} infections.

The effect of the T300A polymorphism on \textit{ATG16L1} function is the best characterized among the three SNPs evaluated in this study. The loss of function predicted with the HOPE server was evident on TNF\(_\alpha\) production, with a decrease paralleling the allelic dosage. This polymorphism is known to reduce autophagy [15], which is supported by a previous study [36]. Nevertheless, genetic variation in the \textit{ATG16L1} gene did not influence the susceptibility to either oropharyngeal or systemic \textit{Candida} infections.

In contrast to the \textit{ATG16L1} SNP, the \textit{IRGM} polymorphisms evaluated in this study do not affect the \textit{IRGM} protein sequence or structure, but modulate \textit{IRGM} gene expression. McCarroll and colleagues demonstrated that \textit{IRGM} haplotypes differentially affected gene expression in different cell types, and that the gene expression levels affected the efficiency of autophagy for eradicating intracellular bacteria [23]. Despite the possible consequences of the variation in the two \textit{IRGM} SNPs, the genotype of either polymorphism did not affect the predisposition to oropharyngeal or systemic \textit{Candida} infection.

The observation that the investigated SNPs in \textit{ATG16L1} and \textit{IRGM} are not associated with increased susceptibility to \textit{Candida} infections, but are involved in the pathogenesis of Crohn’s disease, may suggest that the link between \textit{Candida} colonization and Crohn’s disease, through the presence of ASCA antibodies, is not mediated by autophagy.
However, one has to realize that the mechanisms underlying Candida colonization vs. Candida infection are largely different, as recently shown by our group [32]. Therefore, although autophagy appears not to be involved in susceptibility to Candida infection as demonstrated by the present study, it still could be associated with Candida colonization. Future studies are warranted to assess this aspect.

Autophagy-mediated pathogen clearance is an important process mainly for host defense against intracellular bacteria such as mycobacteria or Salmonella typhimurium. Whereas the role of autophagy in the clearance of extracellular bacteria has been demonstrated [37], the role of autophagy for anti-Candida albicans host defense remains unclear. The lack of association of the SNPs in ATG16L1 and IRGM with susceptibility to Candida infections implies that host defense against Candida infections does not depend on these two genetic variants in autophagy genes. The results of the present study are strengthened by an additional report that showed that although Candida was able to induce autophagy in vitro, this was not necessary for host defense against disseminated candidiasis in mice [24]. Moreover, a set of polymorphisms in autophagy genes did not modulate susceptibility to candidemia [24]. The complementary results of these investigations strongly argue that autophagy is a redundant mechanism for host defense against both systemic and mucosal Candida infections.

Acknowledgements
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Conflicts of Interest
All authors declare no conflicts of interest.
References


Supplementary Figure 1  Bioinformatics analysis of the consequences of the SNP number rs2241880 in ATG16L1. A comparison was performed between the wild type threonine residue and the mutant type alanine residue at the position 300 of the protein. The server is available at: http://www.cmbi.ru.nl/hope/home.

Method
There is no structural information known for this protein. No solved 3D-structure or modelling template was found. Therefore, HOPE will use annotations from the Uniprot-database and predictions from a series of DAS-servers for mutational analysis. More information about your protein of interest can be found in Uniprot entry Q676U5. See the method page for more information.

Amino acids
You are interested in a mutation of a Threonine into a Alanine at position 300. The figure below shows the schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same for each amino acid, is coloured red. The side chain, unique for each amino acid, is coloured black.

Each amino acid has its own specific size, charge, and hydrophobicity-value. The original wild-type residue and newly introduced mutant residue often differ in these properties.

The mutant residue is smaller than the wild-type residue. The mutant residue is more hydrophobic than the wild-type residue.

The report will evaluate the effect of the mutation on the following features: Contacts made by the mutated residue, structural domains in which the residue is located, modifications on this residue and known variants for this residue. A feature will only be shown when information is available. A short conclusion based on just the amino acid properties is shown always. In case a 3D-structure/model is available you will also find images and animations in the report.

Variants
This mutation matches a previously described variant with the following description "Undefined effect." See the ExPASy site about this variant: VAR_021834. The variant is annotated with severity "POLYMORPHISM".

Conservation
The wild-type residue is not conserved at this position. Your mutant residue is among the observed residue types at this position in other homologous sequences. Therefore, the mutation is possible and damaging to the protein.

Amino acid properties
The wild-type and new mutant amino acids differ in size. The mutant residue is smaller than the wild-type residue. The mutation will cause an empty space in the core of the protein (or protein-complex). The hydrophobicity of the wild-type and new mutant residue differs. The mutation will cause loss of hydrogenbonds in the core of the protein (or protein-complex) and as a result disturb correct folding.

Please cite
CHAPTER 5

Variation in Genes of β-glucan Recognition Pathway and Susceptibility to Opportunistic Infections in HIV-Positive Patients
Abstract

Opportunistic infections are the main cause of morbidity and death among HIV-positive patients. Most of these infections are linked to the immunodeficiency due to low CD4+ counts. However, not all patients with low CD4+ counts are equally susceptible to infections, and we hypothesize that variability in genes of innate immunity may also play an important role. The dectin-1/CARD9 pathway is crucial for recognition of both fungal and bacterial pathogens. The aim of this study was to assess the possible association between the occurrence of opportunistic infections and single nucleotide polymorphisms in \textit{DECTIN-1} and \textit{CARD9} in a cohort of 187 HIV-infected patients. The incidence of oropharyngeal candidiasis and other opportunistic infections was not influenced by either the Y238X \textit{DECTIN-1} or the S12N \textit{CARD9} polymorphism. Surprisingly however, the prevalence of pneumonia was significantly higher in patients bearing the defective variant \textit{DECTIN-1} allele. These results suggest a role of dectin-1 in the host defense against respiratory bacterial infections, and future studies are warranted to confirm this association.
Introduction

Opportunistic infections caused by a large variety of viral, bacterial and fungal pathogens are the main cause of morbidity and death among HIV positive patients, and most of them are linked to the immunodeficiency due to the decrease in the CD4+ lymphocytes (<200 cells/µL) [1]. Oropharyngeal candidiasis (OPC), caused by the dimorphic yeast *Candida albicans*, is the most frequently observed infection among HIV-positive patients [2-11], 2006. Whereas the immunodeficiency predisposes to recurrence of OPC, this is not life-threatening [12]. However, untreated oropharyngeal candidiasis in HIV-infected patients may lead to more invasive diseases, including esophageal [13] or even systemic candidiasis [14].

*C. albicans* is recognized by the innate immune system of the host by pattern recognition receptors such as TLR2, TLR4, mannose receptor and dectin-1 [15]. Dectin-1 belongs to the C-type lectin receptor family, is present on dendritic cells, neutrophils and macrophages and recognizes β-1,3-glucan molecules on the cell wall of *C. albicans* and other fungi [16]. Moreover, the ability of the dectin-1 receptor to synergize with TLR2 and TLR4 promotes pro-inflammatory cytokine production and induction of Th1 and Th17 mediated host defense against *C. albicans* [17, 18]. In addition, dectin-1 seems to be also involved in recognition of bacterial pathogens such as mycobacteria [19].

An early stop codon polymorphism in the *DECTIN-1* gene leads to a truncated dectin-1 protein at amino acid position 238 [20]; hence, the mutant protein is ten amino acids shorter than the wild-type and this results in defective β-glucan recognition and decreased induction of Th17 responses. Clinical manifestations of individuals homozygous for the 238X allele include recurrent vulvovaginal candidiasis and onycomycosis [20]. Moreover, the mutant allele of this polymorphism is also associated with a higher susceptibility to mucosal colonization with *Candida* spp. in patients undergoing stem cells transplantation [21].

CARD9 (caspase recruitment domain family member 9), the adaptor protein of both the dectin-1 pathway and the peptidoglycan recognition receptor NOD2, links intracellular signals with Bcl10-Malt1-dependent NF-κB activation. Rare mutations in *CARD9* have also been identified as the cause of increased susceptibility to *Candida* infections [22]. Moreover, a common genetic variant of the *CARD9* gene, a substitution of a guanine with an adenine residue, has also been described in the human HapMap database and the 1000 genomes project database. This results in an amino acid change from a serine to an asparagine residue on position 12 of the protein [23]. So far no studies have assessed the impact of this polymorphism on susceptibility to infections.
The potential role of polymorphisms in *DECTIN-1* and *CARD9* for the susceptibility of HIV patients to infections is unknown. The aim of this study was to assess whether *DECTIN-1* and *CARD9* polymorphisms are associated with opportunistic infections in a cohort of 187 HIV-seropositive patients.

**Materials and Methods**

**Patients**

We evaluated a cohort of 187 HIV positive patients under follow-up at the Out-patients Department of Special Infections of the Attikon University General Hospital of Athens. Patient samples and clinical information were collected after informed consent and the study was approved by the institutional review board. CD4+ cell counts were measured by flow cytometry using microspheres and viral load by real-time PCR. Infections were diagnosed according to the CDC definitions. The causative pathogens for pneumonia could not be identified retrospectively. Empirical treatment with good clinical effectiveness was administered for both *Streptococcus pneumoniae* and atypical pathogens.

**Genotyping of *DECTIN-1* and *CARD9* polymorphisms**

Genomic DNA was isolated from whole blood using standard procedures. The genotype for the *DECTIN-1* Y328X (rs16910526) and *CARD9* S12N (rs4077515) polymorphisms in the patients was screened by the TaqMan SNP assay C_33748481_10 and C_25956930_20, respectively, on the 7300 ABI Real-Time polymerase chain reaction system (Applied Biosystems, CA, USA). Positive and negative controls were included in the assays.

**Bioinformatic Analysis**

To predict the effect on the conformation of the CARD9 protein given the S12N amino acid change, we used HOPE (http://www.cmbi.ru.nl/hope/), a next generation bioinformatics web server that performs automatic mutant analysis. No homology model was produced because no template with enough identity to the CARD9 sequence exists [24]. More information can be found on http://www.cmbi.ru.nl/~hvensela/CARD9/. We also used the Polyphen software in order to determine quantitatively the damage for the protein stability taking into account the amino acid change of the CARD9 SNP [25], and the SIFT server to predict whether the polymorphism was tolerated or not for protein function [26].

**Statistical Analysis**

For the analysis of SNPs, statistical comparisons of frequencies were made between infected versus non-infected subjects using the Chi-square test by SPSS software. Bonferroni corrections were incorporated in these analyses.
Results

Patient Characteristics
The clinical characteristics of the HIV patients are presented in a previous report by Papadopoulos and colleagues [27] and are listed in Table 1. The nadir CD4+ count and the peak viral load did not differ among the DECTIN-1 and CARD9 genotypes, thus it was not considered as a confounder.

Table 1  Clinical characteristics of HIV patients.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Characteristic</th>
<th>Homozygous</th>
<th>Heterozygous</th>
<th>Homozygous</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DECTIN-1 Y238X</strong></td>
<td>Male/Female</td>
<td>73/11</td>
<td>61/19</td>
<td>20/4</td>
<td>0.266</td>
</tr>
<tr>
<td><strong>CARD9 S12N</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DECTIN-1 Y238X</strong></td>
<td>Age (years, mean ± SD)</td>
<td>41.6 ± 12.1</td>
<td>41.6 ± 10.3</td>
<td>38.5 ± 10.2</td>
<td>0.414</td>
</tr>
<tr>
<td><strong>CARD9 S12N</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DECTIN-1 Y238X</strong></td>
<td>Nadir CD4+ count (copies/ml, median-range)</td>
<td>266 (8-658)</td>
<td>259 (1-624)</td>
<td>195 (6-525)</td>
<td>0.370</td>
</tr>
<tr>
<td><strong>CARD9 S12N</strong></td>
<td></td>
<td>252 (1-658)</td>
<td>261 (12-525)</td>
<td>250</td>
<td>0.430</td>
</tr>
<tr>
<td><strong>DECTIN-1 Y238X</strong></td>
<td>Maximum viral load CARD9 S12N (copies/ml, median-range)</td>
<td>66850 (3127-3600000)</td>
<td>75041 (470-1040000)</td>
<td>35399 (2794-51000000)</td>
<td>0.855</td>
</tr>
<tr>
<td><strong>CARD9 S12N</strong></td>
<td></td>
<td>64395 (470-5100000)</td>
<td>81847 (5187-1040000)</td>
<td>750000</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Fungal Infections
Statistical comparisons were performed to assess the association of the DECTIN-1 and CARD9 polymorphisms with the occurrence of fungal or bacterial opportunistic infections (Table 2). Because dectin-1 is mainly a fungal recognition receptor, we initially assessed the incidence of the genotype of the DECTIN-1 and CARD9 polymorphisms on the occurrence of the fungal infections oropharyngeal candidiasis (OPC), Pneumocystis carinii pneumonia (PCP) and cryptococcosis, which revealed no statistically significant differences. Similarly, when oropharyngeal candidiasis was considered separately from the other fungal infections, no significant differences were observed with either the DECTIN-1 or with the CARD9 polymorphism.
Table 2  Occurrence of fungal or bacterial opportunistic infections (OIs) in HIV patients in relation to the DECTIN-1 and CARD9 genotype.

<table>
<thead>
<tr>
<th>SNP</th>
<th>OI</th>
<th>Infection status</th>
<th>Homozygous wild-type (%)</th>
<th>Heterozygous (%)</th>
<th>Homozygous mutant type (%)</th>
<th>P-value* (OR; 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECTIN-1 Y238X</td>
<td>Fungal #</td>
<td>Infected</td>
<td>25 (86.2)</td>
<td>4 (13.8)</td>
<td>0 (0.0)</td>
<td>0.910 (1.17; 0.62-1.72)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>136 (86.1)</td>
<td>21 (13.3)</td>
<td>1 (0.6)</td>
<td>0.733</td>
</tr>
<tr>
<td>CARD9 S12N</td>
<td></td>
<td>Infected</td>
<td>12 (41.4)</td>
<td>12 (41.4)</td>
<td>5 (17.2)</td>
<td>(1.85; 0.47-2.74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>72 (45.6)</td>
<td>67 (42.4)</td>
<td>19 (12.0)</td>
<td>(1.24; 0.23-1.66)</td>
</tr>
<tr>
<td>DECTIN-1 Y238X</td>
<td>OPC</td>
<td>Infected</td>
<td>16 (84.2)</td>
<td>3 (15.8)</td>
<td>0 (0.0)</td>
<td>0.985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>145 (86.2)</td>
<td>22 (13.2)</td>
<td>1 (0.6)</td>
<td>(1.24; 0.23-1.66)</td>
</tr>
<tr>
<td>CARD9 S12N</td>
<td></td>
<td>Infected</td>
<td>8 (42.1)</td>
<td>6 (31.6)</td>
<td>5 (26.3)</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>76 (45.2)</td>
<td>73 (43.7)</td>
<td>19 (11.4)</td>
<td>(1.73; 0.72-3.27)</td>
</tr>
<tr>
<td>DECTIN-1 Y238X</td>
<td>Bacterial @</td>
<td>Infected</td>
<td>25 (73.5)</td>
<td>9 (26.5)</td>
<td>0 (0.0)</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>136 (88.9)</td>
<td>16 (10.5)</td>
<td>1 (0.6)</td>
<td>(2.26; 1.13-4.32)</td>
</tr>
<tr>
<td>CARD9 S12N</td>
<td></td>
<td>Infected</td>
<td>20 (58.8)</td>
<td>11 (32.4)</td>
<td>3 (8.8)</td>
<td>0.195</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>64 (41.8)</td>
<td>68 (44.4)</td>
<td>21 (13.7)</td>
<td>(0.53; 0.22-1.84)</td>
</tr>
<tr>
<td>DECTIN-1 Y238X</td>
<td>Pneumonia</td>
<td>Infected</td>
<td>17 (68.0)</td>
<td>8 (32.0)</td>
<td>0 (0.0)</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>144 (88.9)</td>
<td>17 (10.5)</td>
<td>1 (0.6)</td>
<td>(2.93; 1.53-8.48)</td>
</tr>
<tr>
<td>CARD9 S12N</td>
<td></td>
<td>Infected</td>
<td>14 (56.0)</td>
<td>8 (32.0)</td>
<td>3 (12.0)</td>
<td>0.273</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>70 (43.2)</td>
<td>71 (43.8)</td>
<td>21 (13.0)</td>
<td>(0.88; 0.43-1.73)</td>
</tr>
<tr>
<td>DECTIN-1 Y238X</td>
<td>Syphilis</td>
<td>Infected</td>
<td>7 (70.0)</td>
<td>3 (30.0)</td>
<td>0 (0.0)</td>
<td>0.278</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>154 (87.0)</td>
<td>22 (12.4)</td>
<td>1 (0.6)</td>
<td>(1.84; 0.62-2.98)</td>
</tr>
<tr>
<td>CARD9 S12N</td>
<td></td>
<td>Infected</td>
<td>7 (70.0)</td>
<td>2 (20.0)</td>
<td>1 (10.0)</td>
<td>0.249</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non infected</td>
<td>77 (43.5)</td>
<td>77 (43.5)</td>
<td>23 (13.0)</td>
<td>(0.71; 0.33-1.74)</td>
</tr>
</tbody>
</table>

# Fungal infections comprise oropharyngeal candidiasis (OPC), Pneumocystis jirovecii pneumonia (PJP) and cryptococcal infection.

@ Bacterial infections comprise pneumonia, Salmonella and syphilis

* Chi-square test.
To elucidate the impact of the studied genotypes on the occurrence of fungal infections in the event of decreased CD4+ cells, enrolled patients were divided into two subcategories according to their median CD4+ count, i.e., those with CD4+ more than 250/mm³ and those with CD4+ less than or equal to 250/mm³. No differences were found in the occurrence of fungal infections between genotypes when the CD4+ count was below the median value (data not shown). No effects of the DECTIN-1 and CARD9 genotypes on the CD4+ counts were observed.

Bacterial Infections

Dectin-1 has also been described to be involved in recognition of Mycobacterium tuberculosis [19], and CARD9 is an adaptor molecule also for the NOD2 recognition pathway of bacterial peptidoglycans [28]. Therefore, we hypothesized that the occurrence of bacterial opportunistic infections may be related to the genotype of the DECTIN-1 and CARD9 polymorphisms. Interestingly, these analyses revealed increased susceptibility towards bacterial infections of patients heterozygous for the DECTIN-1 polymorphism (Table 2). In contrast, no association was found between the CARD9 SNP and the occurrence of bacterial infections. Within the group of bacterial infections, DECTIN-1 was significantly associated with pneumonia, of which three patients had pneumococcal bacteremia, two among heterozygous patients and one among homozygous patients of every allele. No association between the tested polymorphisms and syphilis or salmonellosis was observed.

Discussion

In the present study, we demonstrate that heterozygosity for the Y238X DECTIN-1 polymorphism in HIV-infected patients predisposes to bacterial infections, and in particular to pneumonia. In contrast, variation in the DECTIN-1 and CARD9 genes was not associated with susceptibility to opportunistic fungal infections in HIV-positive patients.

These findings are somehow unexpected. Because polymorphisms in PRRs are associated with a higher incidence of fungal and bacterial infections [29, 30] it was no surprise that genetic variants of DECTIN-1 and CARD9 were reported to increase the susceptibility to mucocutaneous fungal infections [20, 22]. This leads to the question how the lack of an effect of genetic variants of DECTIN-1 and CARD9 on opportunistic fungal infections in HIV patients is to be explained. Firstly, although the variant allele of the DECTIN-1 Y238X polymorphism results in a loss-of-function of dectin-1 and consequently of IL-6 and IL-17 production [20], this defect is only partial in the case of DECTIN-1 heterozygosity.
In the present study, HIV seropositive patients heterozygous for the DECTIN-1 Y238X polymorphism do not display an increased susceptibility to develop fungal infections, indicating that a partial dectin-1 deficiency is possibly redundant within this context. It could be also hypothesized that the basic defect in HIV patients, the lack of CD4+ cells, already results in a defective IL-17 and IL-22 production, and this has an overriding influence. Second, one has to observe also the subtle differences between the localization of the fungal infections in HIV and dectin-1-deficiency patients: oropharyngeal cavity, and vulvo-vaginal, respectively. Thus, although dectin-1 is known to be expressed on epithelial cells [31], differences in the pathogenesis of OPC on the one hand and recurrent vulvo-vaginal candidiasis on the other hand, may explain why polymorphisms in DECTIN-1/CARD9 may be important for the latter, but not for the former.

Third, another possibility to explain the lack of effect of DECTIN-1/CARD9 polymorphisms on susceptibility to fungi is that the alternative β-glucan receptors, including the complement receptor 3, scavenger receptors, and CD5 [32, 33] may also recognize the fungi and induce host defense mechanisms. Finally, one also has to observe that the relatively low number of patients may also be a factor contributing to the lack of effects, and future studies are warranted to enable to draw a definitive conclusion.

T-cell immunity is crucial for antimicrobial host defense. A threshold number of CD4+ T cells is required to protect the host, and under that threshold host defense relies predominantly on innate host defense mechanisms [34-37]. We hypothesized that a genetic deficiency in the innate immunity may surface more prominently in HIV patients than in immunocompetent patients, due to the defective T-cell responses. In line with these arguments, a positive association between the DECTIN-1 polymorphism and the prevalence of pneumonia was found in the HIV-infected patients. Pneumonia is an inflammatory condition of the lung that can be caused by viral, fungal or bacterial pathogens.

Both the innate and the adaptive immune system are involved in the protection against the causal agents of pneumonia, and the increased prevalence of pneumonia in the hyper-IgE syndrome patients with STAT3 mutations demonstrated an important role of the Th17 produced cytokines IL-17 and IL-22 in host defense during bacterial pneumonia [38, 39]. IL-17 is involved in granulopoiesis and neutrophil recruitment [40], while IL-22 induces the production of β-defensins that improve the mucosal host defense against pathogens [41, 42].

Finally, IL-22 stimulates the lung endothelium regeneration and increases transepithelial resistance to injury [43]. It is important to underline one more time that it is likely that this effect of the DECTIN-1 polymorphism on susceptibility to pneumonia is
exerted mainly in conditions in which CD4+ dependent mechanisms are defective, as no increase in the susceptibility to pneumonia has been observed in dectin-1 deficient patients with a normal T-cell immunity [20].

No relation was found between the occurrence of salmonellosis and syphilis and the genotype of the DECTIN-1 Y238X polymorphism, but that may be related to the low number of infections with these microorganisms in the present study. However, due to the low prevalence of fungal infections in our cohort, at this stage it is not possible to draw definitive conclusions regarding the role of these polymorphisms on the susceptibility to fungal infections. However, the cohort provided sufficient power for the analysis of bacterial infections in relation to the host genotype (data not shown). Several explanations may be the cause of the lack of an association of the CARD9 S12N SNP with susceptibility to opportunistic infections. Since the consequences of the presence of the CARD9 polymorphism for its function have not been studied, one possibility is that this polymorphism affects only minimally the protein structure and function. Radboud University Nijmegen created the next generation bioinformatics server HOPE, which can predict the changes in protein structure given an amino acid change. Despite the lack of the homology model, the output of the server yielded that the mutation is located within the “CARD” domain (caspase recruitment domain). Although the mutation introduces an amino acid with changed hydrophobicity of the wild-type and mutant aminoacids, and the mutation causes loss of hydrophobic interactions in the core [24], the Harvard University’s Polyphen server predicts the effect of the serine to asparagine amino acid change as benign for its protein function [25], and the SIFT output show the polymorphism to be tolerated by the protein [26]. These bioinformatic tools have a high reliability based on sufficient sensitivity and specificity values (File S1).

In conclusion, the present study is the first to report the role of genetic variation in the dectin-1/CARD9 pathway in relation with the susceptibility to opportunistic infections in immunocompromised HIV patients, which revealed an association of the DECTIN-1 polymorphism with pneumonia. Further studies are warranted to confirm this association and to decipher the precise mechanisms through which this effect is exerted.

Acknowledgments
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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.
References


27. Papadopoulus AI, Ferwerda, B, Antoniadou, A. Association of toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms with increased infection risk in patients with advanced HIV-1 infection. Clinical Infectious Diseases 2010; 51: 242-247.


**Supplementary Figure 1** Output of the Polyphen server and multiple sequences alignment at the position 12 of the CARD9 protein regarding the S12N amino acid change of the SNP number rs4077515.

**Fragment of multiple alignment around position 12:**
**Supplementary Figure 2** Output of the SIFT server regarding the S12N aminoacid change of the SNP number rs4077515.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Amino Acid change</th>
<th>Protein</th>
<th>Amino Acid</th>
<th>Using orthologs in the protein alignment</th>
<th>Using homologs in the protein alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4077515</td>
<td>S12N</td>
<td>S</td>
<td>P</td>
<td>0.99</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*Low confidence means that the protein alignment does not have enough sequence diversity. Because the position artifically appears to be conserved, an amino acid may incorrectly predicted to be damaging.
Supplementary Figure 3  Output of the HOPE server regarding the S12N aminoacid change of the SNP number rs4077515.

Method

There is no structural information known for this protein. No solved 3D-structure or modelling template was found. Therefore, HOPE will use annotations from the Uniprot-database and predictions from a series of DAS-servers for mutational analysis. More information about your protein of interest can be found in Uniprot-entry Q9V297. See the method page for more information.

Amino acids

You are interested in a mutation of a Serine into an Asparagine at position 12.

The figure below shows the schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same for each amino acid, is coloured red. The side chain, unique for each amino acid, is coloured black.

Each amino acid has its own specific size, charge, and hydrophobicity-value. The original wild-type residue and newly introduced mutant residue often differ in these properties.

The mutant residue is bigger than the wild-type residue. The wild-type residue was more hydrophobic than the mutant residue. The report will evaluate the effect of the mutation on the following features: Contacts made by the mutated residue, structural domains in which the residue is located, modifications on this residue and known variants for this residue. A feature will only be shown when information is available. A short conclusion based on just the amino acid properties is shown always. In case a 3D-structure/model is available you will also find images and animations in the report.
CHAPTER 6

Gene Polymorphisms in Pattern Recognition Receptors and Susceptibility to Idiopathic Recurrent Vulvovaginal Candidiasis
Abstract

**Objective.** Approximately 5% of women suffer from recurrent vulvovaginal candidiasis (RVVC). It has been hypothesized that genetic factors play an important role in the susceptibility to RVVC. The aim of this study was to assess the effect of genetic variants of genes encoding for Pattern Recognition Receptors (PRRs) on susceptibility to RVVC.

**Study design.** For the study, 119 RVVC patients and 263 healthy controls were recruited. Prevalence of polymorphisms in five PRRs involved in recognition of *Candida* were investigated in patients and controls. *In silico* and functional studies were performed to assess their functional effects.

**Results.** Single nucleotide polymorphisms (SNPs) in TLR1, TLR4, CLEC7A and CARD9 did not affect the susceptibility to RVVC. In contrast, a non-synonymous polymorphism in TLR2 (rs5743704, Pro631His) increased the susceptibility to RVVC almost 3-fold. Furthermore, the TLR2 rs5743704 SNP had deleterious effects on protein function as assessed by *in-silico* analysis, and in-vitro functional assays suggested that it reduces production of IL-17 and IFNg upon stimulation of peripheral blood mononuclear cells with *C. albicans*. No effects were observed on serum MBL concentrations.

**Conclusion.** Genetic variation in TLR2 may significantly enhance susceptibility to RVVC by modulating host defense mechanisms against *Candida*. Additional studies are warranted to assess systematically the role of host genetic variation for susceptibility to RVVC.
Introduction

*Candida* microorganisms, especially *C. albicans*, often colonize the genital tract in women, and under certain conditions are responsible for mucosal inflammation [1-6]. Vulvovaginal candidiasis (VVC) is a frequent consequence of *Candida* infection, accompanied by variable pruritus, soreness, rash, and vaginal discharge, with patients experiencing a strong discomfort. Most women have at least one event of VVC in their lifetime, while up to 5-8% suffer from recurrent vulvovaginal candidiasis (RVVC), defined as at least three infections per year [4, 6].

Known risk factors of vulvovaginal candidiasis include diabetes, pregnancy and therapy with glucocorticosteroids, immunosuppressive drugs, and antibiotics [7-9]. However, the vast majority of RVVC patients are healthy women without any identifiable predisposing or episode precipitating factors [7, 8, 10-12]. Moreover, no distinct *C. albicans* strains, the species that causes more than 90% of the VVC episodes, have been described in RVVC patients, arguing against microbiological factors as the major determinants of VVC or susceptibility to recurrent disease [8, 12]. Thus, it has been hypothesized that host genetic factors may be a major component determining susceptibility to RVVC.

The innate immune system provides the first barrier against vulvovaginal *Candida* infection. Pattern recognition receptors (PRRs) on innate immune cells sense molecular moieties on the surface of microorganisms, and thereafter induce an intracellular signal that stimulates production of effector molecules such as cytokines or defensins. Two classes of PRRs have been reported to be the main recognition receptors for *C. albicans*: Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) [13]. TLR4 recognizes fungal cell wall mannans, while TLR2/TLR1 and TLR2/TLR6 heterodimers recognize *Candida* phospholipomannan [14]. Additionally, TLR2 synergizes with DECTIN-1, the receptor for β-glucan, for the induction of proinflammatory cytokines [15, 16]. DECTIN-1 can also induce TLR-independent signals for the production of IL-17, IL-6 and IL-10 through a Syk/CARD9-dependent pathway [17]. Single nucleotide polymorphisms (SNPs) in both TLRs and C-type lectin-like receptors (CLRs) have been described to be associated with an increased susceptibility to both systemic [18] and oropharyngeal [19] candidiasis, and we hypothesized that similar effects may be exerted on the susceptibility to RVVC.

The aim of this study was to assess the impact of the SNPs in the genes coding for DECTIN-1 (Tyr238X, rs16910526), CARD9 (Ser12Asn, rs4077515), TLR1 (Arg80Thr, rs5743611), TLR2 (Pro631His, rs5743704) and TLR4 (Asp299Gly, rs4986790; Thr399Ile, rs4986791) on the susceptibility to RVVC.
Materials and Methods

Ethics statement
The inclusion of patients and controls in this study was approved by the Institutional Review Boards of the Radboud University Medical Centre, Wayne State University School of Medicine and Hôpital Necker. Enrollment took place between January 2010 and December 2011. Patients gave written informed consent and the study in accordance to the declaration of Helsinki. Enrollment of healthy controls for blood donations was approved by the Institutional Review Board of the Radboud University Medical Centre.

Patients and control subjects
The patient cohort consisting of RVVC patients were recruited at Wayne State University School of Medicine (Detroit, MI, USA), Radboud University Nijmegen Medical Center (Nijmegen, The Netherlands) and Hôpital Necker (Paris, France). Patients were enrolled during episodes of acute vaginitis or, if asymptomatic, while receiving maintenance fluconazole therapy. Inclusion criteria for the study were: healthy women above 18 years of age, diagnosed with at least three documented episodes of VVC in a year, microbiologically validated and all caused by \( C.\ albicans \). Exclusion criteria were use of any immunosuppressive therapy (including steroids), diabetes, pregnancy and HIV infection. EDTA venous blood was collected after obtaining written informed consent. Asymptomatic healthy controls without a history of vaginal \( Candida \) infections (N=263) were recruited at Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands and gave written informed consent. All patients and controls were of Western-European genetic background.

Genotyping of the single nucleotide polymorphisms (SNPs)
Genomic DNA was isolated from whole blood using the Qiagen (Valencia, CA, USA) isolation kit 6 and following the standard protocol. The genotype for the \( CLEC7A \) (DECTIN-1) Tyr328X (rs16910526) and \( CARD9 \) Ser12Asn (rs4077515) polymorphisms in the patients was screened by the TaqMan SNP assay C_33748481_10 and C_25956930_20, respectively (Applied Biosystems, Foster City, CA, USA). The genotype for the \( TLR1 \) polymorphism Arg80Thr (rs5743611) was assessed with the TaqMan SNP assay C_27855269_10. The genotype of \( TLR2 \) Pro631His (rs5743704) was assessed by applying a predesigned TaqMan SNP assay C_25607736_10. The genotyping for the \( TLR4 \) polymorphisms Asp299Gly (rs4986790) and Thr399Ile (rs4986791) was performed with the TaqMan SNP assay C_11722238_20 and C_11722237_20, respectively. The TaqMan qPCR assays were performed on the 7300 ABI Real-Time polymerase chain reaction system (Applied Biosystems). Positive and negative controls were included in the assays.
**Bioinformatic analysis for the TLR2 polymorphism rs5743704**

The TLR2 protein information was obtained from Swissprot accession code O60603, and OMIM accession code 603028, using the MRS server (http://mrs.cmbi.ru.nl/mrs-web). We used the PROSITE server (http://expasy.org/prosite) to retrieve information about the TLR2 protein domains. No protein structure is available for the complete TLR2 protein at the moment. However, the cytoplasmic TIR domain is known and can be found as PDB-file 1O77 [20]. This structure in this file contains a mutation on position 713 and misses one loop, but the remaining residues are 100% identical to the sequence of TLR2.

The extracellular domain up to residue 509 is 100% identical to the protein in PDB file 2Z7X [21]. The Pro631His polymorphism is located in the region linking the TIR domain with the intracellular domain. We used the automatic modeling script in the YASARA & WHAT IF Twinset [22] to extend the known structures and to model missing loops in these structures. As a result, we produced models of the N- and of the C-terminal domain, which are both accurate, because they are based on the known structure of that protein. These structures are extended with residues of the linking region. Additionally, we used HOPE (http://www.cmbi.ru.nl/hope), a next generation bioinformatics web server that performs automatic mutant analysis [23]. Besides this, an analysis of the pathogenicity of the Pro631His polymorphism in TLR2 was performed using conservation and structural information. The Polyphen-2, SIFT, PANTHER, snps3D and the SNAP servers where employed for this analysis.

**Cytokine stimulation assays**

Peripheral blood mononuclear cells (PBMC) were isolated from 73 healthy volunteers by Ficoll-Paque gradient. All healthy volunteers gave written informed consent. 0.5x10⁶ PBMCs/well were plated in round bottom wells plates. Subsequently, incubation with *Candida albicans* blastoconidia (1x10⁶/ml, heat-killed at 100°C for 1h) was performed for 24 hours, 48 hours or 7 days. The incubation time varied for each cytokine: 24 hours for IL-1β and IL-6, 48 hours for IFNγ and IL-10, and 7 days for IL-17 (with the addition of 10% human serum). After the incubation time, cytokines were measured in the supernatants by ELISA (R&D Systems, MN, USA or Sanquin Research, Amsterdam, The Netherlands). Detection limits were 40 pg/mL, except for the IFNγ ELISA (12 pg/mL). Mannose binding lectin (MBL) concentrations in serum were measured by ELISA (Bioporto, Gentofte, Denmark).

**Statistical analysis**

For the analysis of the polymorphisms in the PRRs, statistical comparisons of frequencies were made between RVVC versus control subjects. We used SPSS 16.0 software to perform Chi-square tests and the 5% Confidence Intervals Odds ratio to
calculate the risk. Furthermore, for the analysis of the effect of the TLR2 genotype on the in vitro cytokine production, a non-parametrical Kruskal-Wallis analysis was performed, and for the circulatory MBL measurement we used the Mann-Whitney U test. Both analyses were done using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA.

Results

The influence of polymorphisms in PRR genes on the susceptibility to RVVC
As a polymorphism in the gene encoding the CLR DECTIN-1 (CLEC7A) has previously been shown to be associated with an increased susceptibility to fungal infections, a first set of comparisons assessed the prevalence of CLEC7A and CARD9 polymorphisms in control individuals and patients with RVVC (Table 1). No effects of CLEC7A (Tyr238X, rs16910526) and CARD9 (Ser12Asn, rs4077515) polymorphisms on the susceptibility to RVVC have been observed. In a second set of experiments, the prevalence of polymorphisms in the genes coding for TLR1 (Arg80Thr, rs5743611), TLR2 (Pro631His, rs5743704) and TLR4 (Asp299Gly, rs4986790; Thr399Ile, rs4986791) was also assessed. These analyses revealed that the polymorphisms in the TLR1 and TLR4 genes did not affect the susceptibility to RVVC. In contrast, the genotype for the Pro631His polymorphism on the TLR2 gene was associated with a 2.705-fold increase ($P$-value 0.046) in susceptibility to RVVC in the patient cohort. All genetic distributions are in Hardy-Weinberg equilibrium (Table 1).

Bio-informatic analysis TLR2 rs5743704 polymorphism
Because the TLR2 Pro631His polymorphism was associated with an increased susceptibility to RVVC, the functional effects of this genetic variation were studied. In a first analysis, an in silico evaluation of the effect of the Pro631His polymorphism on the TLR2 protein was performed. We evaluated its effect at the genomic level, by performing multiple sequence alignment of the homologous DNA sequences from 35 other species via the Ensembl server. The cytosine base is conserved at the chromosome 4 nucleotide position 154625451, being also the ancestral allele, evolutionary conservation that underlines a high likelihood that this position in the molecule is functionally important.

To analyze the effects of the single nucleotide polymorphism rs5743704 on the TLR2 protein, we analyzed the possible domains identified via the Prosite server. The amino acid change for the Pro631His polymorphism affects an amino acid residue that is located near the TIR domain (residue 639-784). To visualize the Pro631His polymorphism on the 3D structure of the TIR domain, we evaluated the homology model using the YASARA
<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Group</th>
<th>WT</th>
<th>HET</th>
<th>HOM</th>
<th>In HWE (Yes/No)</th>
<th>P-value*</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DECTIN-1 Tyr238Stop</strong></td>
<td>Controls (N=263)</td>
<td>219 (83.3%)</td>
<td>44 (16.7%)</td>
<td>0 (0%)</td>
<td>Yes</td>
<td>0.852</td>
<td>0.95 (0.53 – 1.70)</td>
</tr>
<tr>
<td></td>
<td>RVVC (N=119)</td>
<td>100 (84.0%)</td>
<td>19 (16.0%)</td>
<td>0 (0%)</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CARD9 Ser12Asn</strong></td>
<td>Controls (N=263)</td>
<td>90 (34.2%)</td>
<td>127 (48.3%)</td>
<td>46 (17.5%)</td>
<td>Yes</td>
<td>0.563</td>
<td>1.03 (0.76 – 1.40)</td>
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<tr>
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<td>RVVC (N=119)</td>
<td>43 (36.1%)</td>
<td>51 (42.9%)</td>
<td>25 (21.0%)</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TLR1 Arg80Thr</strong></td>
<td>Controls (N=263)</td>
<td>232 (88.2%)</td>
<td>29 (11.0%)</td>
<td>2 (0.8%)</td>
<td>Yes</td>
<td>0.46</td>
<td>1.41 (0.81 – 2.43)</td>
</tr>
<tr>
<td></td>
<td>RVVC (N=119)</td>
<td>100 (84.0%)</td>
<td>17 (14.3%)</td>
<td>2 (1.7%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>TLR2 Pro631His</strong></td>
<td>Controls (N=263)</td>
<td>252 (95.8%)</td>
<td>11 (4.2%)</td>
<td>0 (0%)</td>
<td>Yes</td>
<td>0.046</td>
<td>2.71 (1.15 – 5.85)</td>
</tr>
<tr>
<td></td>
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<td>107 (89.9%)</td>
<td>11 (9.2%)</td>
<td>1 (0.9%)</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TLR4 Asp299Gly</strong></td>
<td>Controls (N=263)</td>
<td>230 (87.5%)</td>
<td>32 (12.2%)</td>
<td>1 (0.5%)</td>
<td>Yes</td>
<td>0.791</td>
<td>0.90 (0.47 – 1.73)</td>
</tr>
<tr>
<td></td>
<td>RVVC (N=119)</td>
<td>105 (88.2%)</td>
<td>14 (11.8%)</td>
<td>0 (%)</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TLR4 Thr399Ile</strong></td>
<td>Controls (N=263)</td>
<td>230 (87.5%)</td>
<td>32 (12.2%)</td>
<td>1 (0.3%)</td>
<td>Yes</td>
<td>0.791</td>
<td>0.90 (0.47 – 1.73)</td>
</tr>
<tr>
<td></td>
<td>RVVC (N=119)</td>
<td>105 (88.2%)</td>
<td>14 (11.8%)</td>
<td>0 (%)</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as number of individuals (% major or minor allele), P-value and OR (95% CI).

*Chi-square (χ²) test. WT = Wild-type; HET = Heterozygous; HOM = Homozygous; HWE = Hardy-Weinberg equilibrium.
software. A screenshot of the 3D homology model is shown in Figure 1. The amino acid change is situated between the intracellular domain and the TIR domain, and might introduce a loss in rigidity when substituting proline by histidine.

**Figure 1** Screenshot of the 3D homology model of the TLR2 protein using the YASARA software. The side chains of the wild-type and mutant residues are shown in green and red respectively.

Finally, the HOPE server predicted that due to the fact that the polymorphism introduces an amino acid with different characteristics, this would affect the function of the molecule. The wild-type residue proline is rigid, resulting in a characteristic backbone conformation that might be required at this position. Additionally, proline is more hydrophobic than histidine, and this may also influence the function of the molecule through loss of hydrophobic interactions on the surface of the protein. This hypothesis was also tested through a series of web servers to predict the pathogenicity of the Pro631His amino acid change. As shown in Supplementary Table 1, according to the Polyphen-2, SIFT, PANTHER and snps3D, the effect of the Pro631His polymorphism is predicted detrimental for TLR2 function.
The effect of the TLR2 Pro631His polymorphism on the production of inflammatory mediators

In order to assess the functional consequences of the TLR2 Pro631His polymorphism in further detail, cytokine production of PBMCs stimulated with heat-killed *Candida albicans* was assessed in a group of healthy volunteers. The major limitation of this

**Figure 2** Functional analysis on the stimulation with heat-killed *Candida albicans* of peripheral blood mononuclear cells (PBMCs) with different TLR2 Pro631His genotypes. Cytokine production capacity of TNFα, IL-1β, IL-6, IL-10, IL-17 and IFN-γ was compared between the cells obtained from healthy volunteers bearing the wild-type or the variant allele of the TLR2 polymorphism. D1: Donor 1, D2: Donor 2. Data are presented as means ± SEM.
part of the study was that only 2 individuals in this group were heterozygous for the variant allele, which precludes drawing definitive conclusions. However, we observed that whereas the IL-1β, IL-6, and TNFα production was in the same range comparing the different genotype groups, both individuals bearing the mutated allele displayed a strongly diminished release of the T-cell derived cytokines IFNγ and IL-17 (Figure 2), known to be important for antifungal host defense [24-26]. In contrast, MBL circulating concentrations in the RVVC patients did not differ depending on the TLR2 genotype (Figure 3).

**Figure 3** Analysis on the Mannose Binding Lectin (MBL) concentrations in serum obtained from RVVC patients bearing the wild-type or the variant allele of the TLR2 polymorphism Pro631His. Wt: Wild-type, He: Heterozygous. Data are presented as means ± SEM.

Discussion

Vulvovaginal candidiasis is one of the most common infections in women. Despite known risk factors such as the hormonal status, diabetes, pregnancy or immunosuppressive therapy [7-9, 12, 27], the majority of the patients are healthy women who are not exposed to these conditions. In the present study, we explored whether genetic variation in genes coding for PRRs involved in *Candida* recognition [14] affects susceptibility to RVVC. We show that a non-synonymous polymorphism in TLR2 is associated with increased susceptibility to RVVC. *In-silico* assessment suggested functional consequences of the TLR2 polymorphism, and this hypothesis is supported by the decreased production of T-cell derived cytokines IFNγ and IL-17 in two individuals bearing the mutation. However, a definitive conclusion was not able to be drawn due to the low number of volunteers with TLR2 SNP identified in the functional studies.
Genetic variants in PRRs have been previously reported to influence predisposition to RVVC. This has been described in a family of patients with a complete deficiency of DECTIN-1, due to an early stop codon mutation in the CLEC7A gene coding for the DECTIN-1 receptor [28]. Functional consequences of DECTIN-1 deficiency were demonstrated to include impaired induction of both innate and adaptive Th17 immune responses. Furthermore, several other studies have shown the involvement of adaptive immunity in RVVC in humans [29, 30]. In addition to RVVC occurrence in two of the three patients with DECTIN-1 deficiencies, all suffered from onychomycosis. This mutation appears to be relatively common, up to 15% heterozygosity in populations of European origin, allowing epidemiological studies in heterozygous patients [28]. Moreover, heterozygosity in the CLEC7A gene is associated with increased mucosal Candida colonization [31]. Thus, we assessed its impact in the present study. The loss of one functional allele, however, does not seem to have a major impact on the susceptibility to RVVC. This implies that only a complete loss-of-function in the molecule is associated with a significant risk for infection. In addition, it has also recently been shown that this polymorphism does not affect the predisposition to disseminated Candida infections [31, 32].

CARD9 is an adaptor molecule mediating signals induced by DECTIN-1, but also other C-type lectin receptors. Its role in antifungal immunity has been demonstrated both in knock-out mouse studies [33], as well as by the increased susceptibility to severe fungal infections in patients defective in this molecule due to an early stop codon in position Gln295Stop [34]. However, this mutation is rare and has not been reported in other individuals. The evaluation of the effects of a known CARD9 Ser12Asn polymorphism did not yield a significant association with RVVC susceptibility in the present study. Similarly, we have previously shown that this polymorphism does not influence the susceptibility to candidemia [32].

In addition to C-type lectin receptors, TLRs are the second major family of PRRs involved in the recognition of fungi in general, and Candida in particular, TLR2, TLR4, and TLR1 being most important in antifungal immunity [35]. While TLR1 polymorphisms have been recently shown to influence susceptibility to candidemia [18], no such effect was apparent in patients with RVVC. Similarly, two TLR4 polymorphisms that have been previously associated with susceptibility to gram-negative sepsis and other infections did not have a significant effect on susceptibility to RVVC [36]. In contrast, the TLR2 Pro631His polymorphism induced an almost 3-fold increase in the susceptibility to RVVC. TLR2 is an important PRR for C. albicans recognition, activating innate immune responses both alone and in synergy with DECTIN-1 [37]. The deficiency of TLR2 influences susceptibility to systemic candidiasis in mice [38], but no studies have been performed in vaginal candidiasis models. The in silico analysis...
using homology modeling and conservation analysis suggests detrimental effects of the mutation on the function of the receptor. This is supported by the study of Etokebe and colleagues that the Pro631His polymorphism has a dominant negative effect on the TLR2 signaling in HEK-293T cells [39]. Finally, we studied the functional relevance of this polymorphism in primary cells from individuals bearing the various alleles. Although we were able to assess cytokine production in only two individuals with a mutant \textit{TLR2} allele, both of them consistently produced very low amounts of the T-cell derived cytokines IFN\(\gamma\) and IL-17, mediators that are crucial for mucosal antifungal defense [24, 28]. This observation is supported by the finding of Ben-Ali \textit{et al.} who demonstrated that the 631His \textit{TLR2} variant leads to reduced NF-\(\kappa\)B activation [40].

In addition to the effects of the \textit{TLR2} polymorphism on cytokine production, we have also assessed its influence on the concentrations of mannose-binding lectin (MBL). MBL is a circulating receptor for mannose residues which activates the complement system and opsonizes \textit{Candida} [30]. MBL deficiency is associated with an increased susceptibility to RVVC [41, 42]. However, no differences in serum MBL concentrations were found between individuals bearing different \textit{TLR2} alleles, showing that the effect of the \textit{TLR2} Pro631His polymorphism on the RVVC susceptibility is independent of the production of MBL. Ideally, the cytokine analyses should be extended to assessment in lavage fluids or cells. However, these materials were not collected from the patients presented in this manuscript, and future studies should address this aspect.

In conclusion, in this study we provide evidence that polymorphisms in PRRs may play an important role in susceptibility to RVVC in otherwise healthy women. While \textit{CLEC7A}, \textit{CARD9}, \textit{TLR1} and \textit{TLR4} polymorphisms had no impact, the \textit{TLR2} Pro631His polymorphism was associated with an almost 3-fold increase in susceptibility to RVVC. These data demonstrate the role of \textit{TLR2} genetic variation in innate immunity genes for RVVC, and future investigations are warranted in larger cohorts if possible to replicate and extend the results of the present study.

\textbf{Acknowledgments}

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\textbf{Conflicts of Interest}

All authors declare no conflicts of interest.
References

**Supplementary Table 1** Predictions on pathogenicity of the Pro631His polymorphism in the *TLR2* gene using different web servers.

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CHAPTER 7

Polymorphisms in innate immunity genes and susceptibility to recurrent vulvovaginal candidiasis


Diana Rosentul, Corine Delsing, Leo A.B. Joosten, Jos W.M. van der Meer, Bart-Jan Kullberg, Mihai G. Netea.
Summary

Vulvovaginal candidiasis (VVC) is a clinical syndrome caused by vaginal yeast infection with *Candida* species (mostly *C. albicans*), characterized by itching, soreness, bright red rash, and heavy white vaginal discharge, causing a strong discomfort in patients. Recurrent vulvo-vaginal candidiasis (RVVC) is defined as the occurrence of at least three episodes of VVC per year. Predisposing factors such as pregnancy, the use of antibiotics, contraceptive pills or hormone replacement treatment and diabetes mellitus have been reported to contribute to the susceptibility for RVVC. Nevertheless, in most patients suffering from this debilitating disease, none of these risk factors are present. Therefore, it is believed that genetic predisposition may also play a significant role for the susceptibility to RVVC. The present review summarizes the current knowledge concerning the gene polymorphisms that have been associated with RVVC. Several components of the innate immune system related to the recognition of *C. albicans* (mannose binding lectin, Dectin-1), or the induction of inflammation (the cytokine interleukin-4, the NLRP3 component of the inflammasome, IDO and IL-22) have recently been shown to influence susceptibility to RVVC. However, further studies are warranted in order to understand more thoroughly the spectrum of genetic variation inducing genetic susceptibility to RVVC in otherwise healthy patients.
Introduction

The dimorphic yeast *Candida albicans* inhabits the genital and gastrointestinal tract as a commensal microorganism [1-7]. However, under certain circumstances, *C. albicans* becomes a pathogen causing infections that can range from vulvovaginal candidiasis, to oropharyngeal candidiasis, and disseminated systemic infection [8]. The vast majority of women experience at least one episode of vulvovaginal candidiasis in their lifetime, while up to 5 to 8% of them will suffer from recurrent vulvovaginal candidiasis (RVVC), characterized by at least three infections per year.

Most patients with candidemia or disseminated candidiasis are neutropenic or have a history of recent invasive procedures [9]. The risk factors of vulvovaginal candidiasis are different, with diabetes, pregnancy, HIV, glucocorticosteroid therapy, immunosuppressive therapy, or antibiotic therapy playing a role [10-15]. However, most patients with RVVC are healthy women without identifiable risk factors. It is believed that genetic factors are at least partly responsible for the increased susceptibility to RVVC. In the present review, we will summarize the current knowledge on the genetic polymorphisms predisposing to RVVC.

Vaginal immune response to *Candida albicans* and RVVC

*C. albicans* yeast cells are initially recognized by the innate immune system and epithelial cells (EC) [16]. Toll-like receptor (TLR) and lectin-like receptors (LR) families are the main classes of pattern-recognition receptors that recognize *Candida* components [17]. Both TLR4 and mannose receptor interact with cell wall mannans [18], while TLR2 recognizes phospholipomannan [19]. In addition, TLR2 collaborates with the β-glucan receptor dectin-1 for the stimulation of proinflammatory cytokines [20-22]. Dectin-1 is a C-type lectin receptor that amplifies TLR2 and TLR4-induced cytokine production in both murine and human cells, while inducing TLR-independent signals for the production of IL-17, IL-6, and IL-10 through a Syk-dependent pathway [23] (Fig. 1).

In addition to the cell-membrane receptors, mannose-binding lectine (MBL) is a circulating protein that recognizes and binds mannose and N-acetyl-glucosamine residues on the surface of microorganisms, activating the complement system. The cells in the female genital tract that produce MBL have yet to be identified [24]. Furthermore, macrophages and dendritic cells contain cell surface receptors that recognize and bind MBL, promoting opsonization of MBL-bounded microorganisms [25]. Reductions in serum concentrations of MBL are commonly considered to contribute in some degree to susceptibility to infection, especially in the context of other defects of host defense [26].
In healthy women, the main immunological defense mechanism against *Candida* is the cell-mediated mucosal Th1 response, and especially the proinflammatory cytokines interferon-γ (IFNγ), interleukin-17 and interleukin-22 are considered as major effector molecules [27, 28, 29]. The elimination of *Candida* is mediated by the recruitment and activation of macrophages, which respond to a proinflammatory cytokine response. *Candida* recognition and binding by mucosal macrophages is mediated by TLRs and CLRs, and possibly MBL. Stimulation of macrophages results in the synthesis of NO and oxygen radicals, compounds toxic for *Candida*. Nitric Oxide (NO) is an important effector molecule of the innate immune defense against microorganisms [30]. However, its anti-*Candida* activity is strongly inhibited by IL-4, an anti-inflammatory cytokine with immunosuppressive effects. In addition, IL-4 blocks macrophage-mediated anti-*Candida* responses and the production of the Th1-derived proinflammatory cytokines [31, 32].
In addition to immune cells, *C. albicans* hyphae can also be detected by ECs, resulting in the production and release of proinflammatory cytokines (32). In addition, experimental evidences have shown that the interaction between ECs and *C. albicans* can impair its growth [34-36].

These host defense mechanisms are believed to be dysregulated in women suffering from RVVC. Defective mucosal defense mechanisms is likely to result either from a defective production of proinflammatory cytokines in response to *Candida*, or an overwhelming release of anti-inflammatory Th2-derived cytokines and prostaglandin E2 in the lower genital tract. A genetic propensity towards defective inflammatory responses, or towards the production of IL-4 and/or other anti-inflammatory mediators may thus decrease the capacity of the local cell-mediated immune system to contain *Candida* proliferation. It has been also proposed that this may lead to induction of a vaginal allergic response accompanied by histamine release and clinical symptoms [5, 37-39]. A limited number of studies have investigated the role of genetic variability of the host for the susceptibility to RVVC. An overview of these studies are presented here, supporting a role for genetic variability in several genes of innate immunity such as MBL, IL-4, dectin-1 and NLRP3 for the vaginal mucosal infections with *Candida*.

**Mannose Binding Lectin (MBL) Polymorphisms**

A decrease in circulating MBL concentrations correlates with the presence of mutant alleles in exon 1 of *MBL* gene. The best-studied mutation is the single nucleotide mutation at base 230 of exon 1, causing a change of codon 54 from GGC to GAC [25, 40, 41]. The substitution of glycine with an aspartic acid residue disrupts the fifth Gly-Xaa-Yaa repeat in the collagen-like domain of each 32 kD peptide chain and probably prevents the formation of the normal triple helix. As a result of this change, the assembly and stability of the final MBL protein decreases notably [25, 40, 41]. Serum concentrations of MBL are between 1.2 and 1.7 mg/mL in individuals who are homozygous for the wild-type (guanine) allele, 0.3 mg/mL in individuals who are heterozygous, and 0.01 mg/mL in individuals homozygous for the variant allele (adenine) [30, 40-42]. The codon 54 substitution has been demonstrated to be accompanied by an increased rate of various infection [40, 43].

Babula and colleagues reported an association between RVVC and the carriage of the Gly54Asp polymorphism of *MBL* gene. The variant allele was also related to a reduction in the vaginal concentration of MBL. However, not all women with RVVC in this study carried the Gly54Asp variant allele, although the level of MBL in their vaginas was reduced. Arg52Cys, and Gly57Glu are other polymorphisms in exon 1 of the gene coding for MBL that have been identified to be associated with reduced circulating MBL concentrations [41, 44-46].
There is some evidence linking the response to Fluconazol treatment in RVVC patients with the different genetic variation in codon 54, with the patients bearing the variants alleles more sensitive to the treatment [47]. Nevertheless, these findings are controversial since it has been suggested that there is no correlation between the Gly54Asp polymorphism and RVVC predisposition [48].

To conclude, the Gly54Asp MBL polymorphism may be a genetic determinant of susceptibility to RVVC in this population, but additional confirmation studies are needed.

Interleukin-4 (IL-4) Gene Polymorphism
IL-4 is one of the most important anti-inflammatory cytokines, and a high production capacity of IL-4 has been associated with immunosuppressive effects. Local vaginal IL-4 levels were higher and median NO levels were lower in patients with RVVC than in controls [24]. A single nucleotide polymorphism consisting of a cytosine to thymine variation in the promoter region of the IL-4 gene (C589T) has been reported [49], and this has been associated with increased IL-4 production by immune cells [50], and a 12-fold increase in the vaginal concentration of IL-4. Similarly, there was a strong inverse relationship between vaginal concentration of IL-4 and MBL, as well as between the presence of the C589T allele and vaginal NO production. These factors are likely to influence susceptibility to RVVC [24].

Dectin-1 early stop-codon polymorphism
One of the most important pathogen recognition receptors for C. albicans is represented by the β-glucan receptor dectin-1 [22]. Recently, an early stop-codon mutation in position 238 of the carbohydrate recognition domain (CRD) of dectin-1 has been described in a family of patients with RVVC and onycomycosis [51]. The mutated variant leads to the loss of the last nine amino acids of the CRD domain, and as a consequence of two main stabilization structural features, a cysteine disulfide bridge between the helix and the deleted strand and the glutamic interaction with calcium atoms.

The Tyr238Stop allele results in the loss of β-glucan recognition and impaired cytokine response [51, 52]. Binding of heat-killed fluorescent-labeled C. albicans to monocytes was significant lower in those patients who were homozygous for Tyr238Stop mutation compared to healthy controls. Particularly, the proinflammatory cytokine IL-17 response to Candida stimulation was lower in patients bearing the mutation. This study also revealed that the amplification effect of β-glucan/dectin-1 interaction on the TLR2 stimulation of cytokines was absent in individuals homozygous for the mutation. On the other hand, monocyte and neutrophil phagocytic and candidacidal functions were normal in the presence of live C. albicans, both in healthy and in
homozygous Tyr238Stop individuals, showing the redundant nature of the mechanisms leading to the phagocytosis and killing of live yeasts.

In line with the increased risk for mucosal infections in these patients, the frequency of mucosal *Candida* colonization and infection in a cohort of hematologic patients undergoing stem cells transplantation was three times higher in patients bearing the dectin-1 Tyr238Stop allele. In contrast, dectin-1 Tyr238Stop polymorphism did not correlate with an increased susceptibility to systemic candidiasis [53]. A recent study also demonstrated that the early stop codon polymorphism in dectin-1 is correlated with a 2.4-fold increased susceptibility to RVVC [77], validating the involvement of this genetic variant in host defense against mucosal fungal infections.

**NLRP3 gene polymorphism and susceptibility to RVVC**

Vulvar vestibulitis syndrome (VVS), also known as vulvodynia, consists of an intense pain syndrome confined to the vaginal vestibule, which is defined as erythema and pain during introduction of a vaginal tampon, while touching the vagina with a cotton swab, as well as during gynecological examination or sexual intercourse. The causes of this condition and possible therapy have not yet been clarified, but a relationship between VVS symptom initiation and vaginal infection with *C. albicans* has been proposed [54]. *C. albicans* infections in the mucosa leads the proinflammatory cytokine formation by epithelial cells for inactivation by phagocytic cells, and this may play a role in the local inflammatory reactions [33].

The “inflammasomes” are macromolecular structures that upon activation regulate the production and release of the proinflammatory cytokines interleukin IL-1β and IL-18 in response to microorganisms or endogenous danger signals [55]. An inflammasome is formed as a result of the interaction of several cytoplasmic proteins, among which a NACHT- LRR-PYD-containing protein (NLRP1 or 3) and apoptosis-associated speck-like protein (ASC) [56]. Following this, caspase-1 is activated, cleaving the precursor of IL-1β into the active form that is secreted from the cell. Cell-mediated immune response against *C. albicans* depends strongly on the production of IL-1β [33, 57].

Several polymorphism of the NLRP3 gene have been described in literature. A variable number of tandem repeat polymorphism in intron 4 of the CIAS1 gene influences the NALP3 production [58]. In 2009, Lev-Sagie and colleagues performed a study evaluating the relationship of the CIAS1 length polymorphism, RVVC and VVC in 143 women with VVS and 182 control patients with no history of vulvodynia. The NLRP3 gene presents four different tandem sequence repetition alleles; furthermore, those alleles could be combined into 10 different genotypes. In both VVS patients and control subjects, the homozygosity for the 12 allele repetitions in the intron was the most prevalent, whereas the presence of 6 allele repetitions was the least prevalent.
The 12,12 genotype was found in a higher percentage of control subjects than in patients with and VVS. On the other hand, the 7 repetitions allele was more frequently present in patients with VVS than in control subjects, being the genotype 7,7 more frequent in RVVC patients than in patients with no history of RVVC and controls subjects. Moreover, the *Candida* induced IL-1β production measured in whole blood culture of peripheral blood samples from 27 patients with VVS was higher in the samples collected from patients bearing the 12,12 genotype than in those from patients who were homozygous for the 7,7 genotype.

The possession of allele 7, which has 200 base pairs less than allele 12, may lead to the formation of an altered NLRP3-containing inflammasome, or an inflammasome with a reduced biological activity [57]. Homozygous 12,12 genotype is related to a higher expression of NLRP3 levels in the cell [58, 59]. It has been proposed that this genotype is optimal for the inflammasome function. However, the relationship between VVS and NLRP3 length polymorphism has not yet been completely clarified and, potential association between NLRP3 length polymorphism and RVVC in women who do not have VVS remains to be evaluated.

**IL-22 and IDO1 polymorphisms**

Another important cytokine released that contributes to host defense against mucosal candidiasis is IL-22 [60-62]. It has been reported that a low capacity to produce of IL-22 is correlated with chronic and recurrent mucosal candidiasis [63-66]. Recent findings using animal models have suggested the importance of tolerance in order to reduce the tissue damage caused both by the pathogen and by the host immune defense. During infections, this phenomenon is combined with the resistance mechanisms decreasing pathogen burden [67, 68] and increasing the fitness of host-defense against non virulent *C. albicans* [69]. In fact, failing to clearing the infection, an overwhelming inflammatory reaction including an important vaginal PMNs recruitment is associated with RVVC symptoms [70, 71].

An important regulator of mucosal antifungal tolerance is Indoleamine 2,3-dioxygenase 1 (IDO1) [72], a heme enzyme that catalyzes the first and rate-limiting step in tryptophan catabolism to N-formyl-kynurenine (entrez gene Gene ID: 3620 [73], which belongs to the kynurenine pathway [74]. Mice experiments have revealed that a IDO1 deficiency causes a pathogenic inflammatory response leading a greater susceptibility to aspergillosis [75] and mucosal candidiasis [76].

De Luca and colleagues have found that genetic variation in the *IL-22* and *IDO1* genes vary significantly between RVVC patients and controls [77]. In addition, since dectin-1 is known to mediate IL-22 synthesis in the event of mucosal candidiasis [78], they
tested the Y238X SNP in DECTIN1 in their cohort and found that heterozygous genotype increased the risk to RVVC (see also above). Both protective IL-22 and IDO1 genotypes were associated with higher IL-22 and IDO1 vaginal secretion, respectively. In patients carrying the latter genotype, a higher IL-22 production was observed [77].

The effect of the genetic polymorphism in the *IL-22* gene on the susceptibility to RVVC can be explained due to its effect on IL-22 expression in the vagina. IL-22 controls yeast growth by stimulating the secretion of antimicrobial peptides from epithelial cells. In addition, through the activation of the STAT3 pathway, IL-22 controls the integrity of the epithelium [60]. Subsequently, the mucosal damage is limited and the inflammation is kept under control [79].

The genetic polymorphism in *IDO1* gene described by De Luca and colleagues has a protective effect on the event of RVVC, by regulating IDO1 expression. The protective variant has a higher IDO1 and therefore kynurenines production. Subsequently, IDO1 limits tissue damage by inducing tolerance to *Candida albicans* by inhibiting the pathological immune reaction that causes the RVVC symptomatology [77].

In conclusion, *IL-22* and *IDO1* genetic variation affect the susceptibility to RVVC by modulating the resistance and tolerance, respectively.

**TLR2 Polymorphism and Susceptibility to RVVC**

Single nucleotide polymorphisms (SNPs) in both TLRs and C-type lectin-like receptors (CLRs) have been described to be associated with an increased susceptibility to both systemic [80] and oropharyngeal [81] candidiasis. The *TLR2* gene is located on the chromosome 4, and TLR2 can also recognize phospholipomannans of *C. albicans* [20].

A SNP in *TLR2* is mapped at the position 154625451, introduces a histidine instead of a proline at the position 631 of the TLR2 protein. In the present thesis I discussed how the mutated amino acid is located between the intracellular domain and the TIR domain, and may reduce the rigidity when substituting proline by histidine and how this nucleotide position on the TLR2 is conserved in many species. The *in silico* analysis using homology modeling and conservation analysis suggests detrimental effects of the mutation on the function of the receptor. Additionally, peripheral blood mononuclear cells from healthy volunteers bearing the heterozygous genotype secreted very low levels of the T-cell derived cytokines IFN-γ and IL-17, essential molecules for mucosal antifungal defense [51, 82]. This finding is in agreement by the observations of Ben-Ali *et al*. who have shown that the 631His *TLR2* variant performs a reduced NF-κB activation [83].
In the study described in the present thesis, the genotype for the Pro631His polymorphism of the $\text{TLR2}$ gene was associated with a 2.95-fold increase in susceptibility to RVVC in the patient cohort. This effect was most likely due to a reduced anti-$\text{Candida}$ cytokine response, as shown by reduced secretion of the T-cell derived cytokines IFN-$\gamma$ and IL-17. In addition, we have investigated the effect of the Pro631His polymorphism on the MBL levels. Nevertheless, no differences in serum MBL concentrations were found between individuals bearing different $\text{TLR2}$ alleles, suggesting that the effect of the $\text{TLR2}$ Pro631His polymorphism on the RVVC susceptibility does not rely on MBL production.

**Conclusions**

The data presented above supports the importance of polymorphisms of the genes of the innate immune system in the susceptibility to RVVC. MBL, dectin-1 and $\text{TLR2}$ polymorphisms are related to the recognition of $\text{Candida}$ yeasts by the innate immune system. As a result, defective recognition of mannans and $\beta$-glucans, is taking place in patients bearing these polymorphisms, followed by lower host defense reaction. Proinflammatory cytokine production has been also shown to be defective in individuals bearing these polymorphisms, as is the case with certain haplotypes of the $\text{NLRP3}$ gene that have been also associated with susceptibility to RVVC. Moreover, $\text{IL-22}$ SNPs that result in a decreased product [77]. In contrast, polymorphisms of the $\text{IL-4}$ gene that lead to increased production of the anti-inflammatory IL-4 cytokine can result in a diminished NO production, and this may lead to an increased susceptibility to RVVC. In contrast, IDO1 regulates kynurenines production and it mediates tolerance towards $\text{Candida albicans}$, thus reducing the symptoms in an RVVC context.

In conclusion, these studies suggest an important role of polymorphisms in the genes of the innate immune system for susceptibility to RVVC, and it is presumed that the relatively few studies performed to date represent only the tip of the iceberg. Future studies are warranted to evaluate the entire spectrum of genetic susceptibility to RVVC, in order to improve diagnosis and possible future therapy of this important clinical syndrome.

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References


CHAPTER 8

Summary and Conclusions
Opportunistic infections in general, and fungal infections in particular, are associated with immunosuppressive states. Several well-defined risk factors are known to be associated with an increased susceptibility to fungal infections, but not all patients at risk develop the disease. This led us to the hypothesis that the genetic make-up of the patient is an important component of the risk to acquire an opportunistic fungal infection. The aim of this thesis was to assess the role of polymorphisms in genes encoding proteins important for antifungal host defense for the susceptibility to infections caused by the dimorphic yeast *Candida albicans* and to opportunistic infections in HIV seropositive patients.

The host response against pathogenic microorganisms is initiated through recognition by the innate immune system. This recognition is facilitated by Pattern Recognition Receptors (PRRs) [1]. PRRs are proteins capable of detecting conserved molecular structures on the surface of microorganisms and that subsequently activate signal transduction through intracellular mediators in innate immune cells. Ultimately, this signal leads to the activation of NF-κB (and other transcription factors), which in turn mediates the expression and secretion of pro-inflammatory cytokines. Two of the most important proinflammatory cytokines, IL-1β and IL-18, are initially produced as inactive precursors and need to be enzymatically cleaved in order to become biologically active. This cleavage is performed by the cysteine protease caspase-1, which is in turn activated by a multiprotein complex known as the inflammasome [2-4]. The process of autophagy, that eliminates dysfunctional organelles and large protein complexes by engulfment into double membraned vesicles, influences both the antimicrobial activity of the cell and the activation of the inflammasome, crucial processes during host defense [5].

In this thesis I presented a series of studies that assessed the role of single nucleotide polymorphisms (SNPs) in the genes involved for the innate immune mechanisms described above for the susceptibility to *Candida* infections. The evaluation of the genetic susceptibility to opportunistic infections was complemented by functional assays with cultures of peripheral blood mononuclear cells, as well as by bioinformatic approaches to explain the aberrant function of the mutated protein.

In Chapter 2 of the thesis we evaluated the role of the Y238X SNP in the *CLEC7A* gene, coding for the archetypical β-glucan receptor dectin-1, as well as the genotype for the S12N SNP in its adaptor protein CARD9, in influencing susceptibility to candidemia and/or clinical parameters such as severity and dissemination of the infection in a cohort of *Candida* infected patients and non-infected matched controls. Neither of these two polymorphisms affected the predisposition to disseminated candidiasis or the severity of the disease. The most likely explanation for the lack of association lies in the relation of dectin-1 signaling pathway with production of predominantly IL-6.
and IL-17 upon recognition of *Candida albicans*. The production of these cytokines leads to a strong Th17 response, known to be critical for mucosal (rather than systemic) immune response against *Candida spp.* [6]. Functional studies revealed a lower cytokine production in the individuals heterozygous for the Y238X SNP in *DECTIN-1*. However, the remnant cytokine production still produced by cells of these patients seems to be sufficient to activate the immune response against *Candida* in the bloodstream. From this and our earlier studies we can thus conclude that defects in dectin-1 signaling affects mucosal, but not systemic *Candida* infections.

In **Chapter 3** we investigated the effect of genetic variation related to the inflammation-modulating gene *Caspase-12* for the susceptibility to *Candida albicans*-related sepsis. Caspase-12 has been reported to impair caspase-1 processing of pro-IL-1β and pro-IL-18 into the biologically active forms by inhibiting caspase-1 activity. However, our results indicate that the *Caspase-12* genotype has no significant effect on the susceptibility to and severity of systemic infections with *Candida* (assessed as disseminated disease, persistent fungemia, and 30-day mortality), and this lack of effect was supported by the fact that *Caspase-12* did not influence in-vivo cytokine production. In conclusion, despite a previous study that suggested that *Caspase-12* genotype affects the predisposition to bacterial sepsis [7], in our study this conclusion was not confirmed in a larger cohort of fungal sepsis patients. Based on these results, we suggest that caspase-12 is redundant for host defense against systemic *Candida albicans* infections.

Autophagy is an important molecular housekeeping process that allows the cell to optimize its energy turnover, phagocytosis and antigen presentation. Recent studies have also shown that autophagy modulates inflammation, and thus can have an additional effects on host defense [8]. In **Chapter 4** we evaluated the effect of polymorphisms in the autophagy genes *ATG16L1* and *IRGM* [5, 9] on the susceptibility to oropharyngeal candidiasis and candidemia, respectively. A lower in vitro TNFα production capacity was shown on primary human immune cells bearing the *ATG16L1* *300A* allele. Conversely, we obtained a slightly higher IL-8 production under similar conditions in cells of individuals bearing the variant allele of the *IRGM* rs13361189 polymorphism. Nevertheless, the genotype of the *ATG16L1* and *IRGM* SNPs did not affect the predisposition to either oropharyngeal or systemic candidiasis. In previous studies, genetic variation in genes coding for the autophagy regulatory proteins have been associated with intracellular bacteria clearance [9]. However, the lack of association of the SNPs in *ATG16L1* and *IRGM* with susceptibility to *Candida* infections suggests that the host defense against *Candida* infections does not rely on the autophagy process.
The effect of genetic variability of genes related to PRRs in the susceptibility to oropharyngeal candidiasis and other opportunistic infections on HIV patients has remained elusive. In Chapter 5 we assessed whether SNPs in CLEC7A and CARD9 genes are associated with susceptibility to oropharyngeal candidiasis and other opportunistic infections in HIV positive patients. As shown here, genetic variation in CLEC7A (Y238X) and CARD9 (S12N) are not associated with the occurrence of opportunistic infections in HIV positive patients. The CD4+ count is not significantly different among the CLEC7A and CARD9 genotypes, therefore it was not considered as a confounder. In contrast, a positive association between the CLEC7A polymorphism and the prevalence of pneumonia was found in these patients. This might be due to the fact that patients bearing the truncated form of dectin-1 produce lower amounts of IL-6 and IL-17 [6], and this is also accompanied by lower IL-22 production. IL-22 is an essential cytokine for host defense against pneumonia [13]. In an environment with reduced CD4+ T cell numbers and cellular immunity, defects of genes in the innate immune system are likely to become more important, as these defects are not masked by a potent adaptive immunity.

Polymorphisms in genes coding for PRRs have been associated with a higher susceptibility to infectious diseases, including systemic Candida infections. In Chapter 6, we evaluated whether SNPs in genes coding for various PRRs known to be involved in Candida recognition or the adaptor molecule CARD9 play a role in the susceptibility of otherwise healthy women to recurrent vulvovaginal candidiasis (RVVC). The results indicate that DECTIN-1, CARD9, MRC1, TLR1 and TLR4 SNPs had no impact on susceptibility of women to RVVC. In the case of the DECTIN-1 Y238X SNP, our results suggest that only a complete loss-of-function in the dectin-1 protein is associated with a significant risk for infection, as shown previously [6]. In contrast, the P631H SNP in TLR2, an important pattern recognition receptor for C. albicans which activates innate immune responses both by itself or in synergy with dectin-1 [12], is responsible for an almost 3-fold increase in the susceptibility to RVVC. This effect was most likely due to a dampened anti-Candida cytokine response, as shown by reduced secretion of the T-cell derived cytokines IFN-γ and IL-17. On the other hand, this effect was independent of the circulating MBL level, an important component of host defense against RVVC.
Final Conclusions

The goal of the investigations described in the present thesis was to assess the role of genetic polymorphisms in genes coding for proteins belonging to the innate immune system for the susceptibility to *Candida* infections: systemic infections, RVVC, and OPC (and other opportunistic infections) in HIV patients. What can be concluded from these studies? Earlier investigations have reported a role for TLR1 on the one hand, and cytokine polymorphisms on the other hand [14], for the susceptibility and outcome of candidemia. Less effects have been observed in the present studies of polymorphisms in the dectin-1/CARD9 pathway or the inflammasome genes, suggesting that redundancy in the pathogen recognition pathways may mask the effect of genetic variants in these genes, still allowing for appropriate and sufficient recognition and signal transduction in situations in which a certain pathway is less effective.

In contrast to candidemia, a much stronger effect of genetic variation has been observed for RVVC. The findings of our studies, coupled with the fact that co-morbidity and risk profiles do not explain susceptibility to RVVC in the majority of patients, imply that host genetic variants are an important component of the etiology of RVVC. The complementary role of genetic, immunologic and bioinformatic approaches was evident in the present thesis, since the outcome of each methodology was crucial to understand the complete effect of genetic variations in innate immune genes on the predisposition to opportunistic infections.

Although the results reported here are not definitive regarding the genetic burden of *Candida* infections, they provide the proof-of-principle that the genetic background of the host is an important risk factor for these infections. However, these studies also demonstrate the limits of candidate gene approaches, arguing for the extension of the genetic analyses to more comprehensive discovery-based approaches. Future studies are thus warranted to extend these results by using whole exome sequencing or performing Genome Wide Association Studies (GWAS) in large enough cohorts, in order to evaluate genetic variation as a whole, including epistatic interactions. Mapping the non-coding regions in genes may show the effect of VNTRs affecting gene expression, and it may be related to predisposition to opportunistic infections. Analyzing the role of structural variants, as well as epigenetic changes, may complement the investigation. Finally, the interaction between genetic variations and environment is necessary to bring the predictive model closer to reality.

In conclusion, variations in genes encoding proteins related to the innate immune system have an important effect on the susceptibility to infections. A combination of clinical, genetic and bioinformatic approaches is necessary to be able to understand
the underlying mechanisms of their effect. This thesis provides the proof-of-principle that genetic predisposition is an important component of the etiopathology of opportunistic infections, and future genome-wide genetic studies are warranted to extend these observations.
References

List of Publications
List of Publications


Curriculum Vitae
Curriculum Vitae

Diana Carolina Rosentul Amram was born in Caracas, Venezuela on August 19th, 1981. In 2006 she finished her Licenciate in Biology Diploma (a 5 years program with a thesis) at the Central University of Venezuela. Diana performed her internship at the private company “Empresas Polar” in the topic “Comparison of foodborne *Salmonella* rapid detection methods with the conventional culture method” under the supervision of MSc. Patricia Blasco and Dr. Adriana Bravo. In May 2007, Diana obtained the “Josefina Gomez Ruiz” Award (From the Venezuelan Microbiology Society) and the second place of the International Commission on Microbiological Specifications for Foods (ICMSF) Award for the research: “Immunomagnetic separation as a rapid foodborne *Salmonella* detection alternative”. The ICMSF is linked to the International Union of Biological Societies (IUBS) and to the World Health Organization (WHO) of the United Nations.


In February 2008, Diana started her Second year of Master’s in Basic and Applied Microbiology in the University of Western Brittany, Brest, France (she was excepted of doing the first year). Her internship was performed at the Invertebrates Physiology Laboratory, Microbiology at the French Research Institute for Exploitation of the Sea (IFREMER), Brest, France, under the supervision of Dr. Jean Louis Nicolas, from February 2008 until September 2008. The thesis topic was: *Vibrio aestuarianus* Metalloprotease expression under different culture conditions*. She obtained her Master’s diploma in November 2009. She was funded by The Venezuelan Ministry of Science and Technology Fellowship.

In June 2009, Diana started her PhD in the Internal Medicine Department of UMC St. Radboud with Prof. Dr. Mihai G. Netea and Prof. Dr. Bart-Jan Kullberg as promotors and Dr. Theo S. Plantinga as copromotor. The aim of the PhD thesis was to unravel the genetic susceptibility to opportunistic infections, particularly, *Candida albicans* infections. Diana Rosentul was part of the Marie Curie Initial Training Network “FINSysB” including Research Skills Training Workshops and Transferrable Skills Trainings.

From October 2012 until September 2013, Diana became Research Assistant in the Department of Biochemistry and Molecular Biology of the University of Southern Denmark, Odense, Denmark. The research topic was the Study of the methylation pattern in the small subunit of the mitoribosome. The research was performed under the supervision of Prof. Dr. Stephen Douthwaite.
In January 2014 Diana moved to Israel. Initially she studied the Hebrew language and did a Startup creation Internship at PenZa Perception Lab, Jaffa. In August 2014, Diana was appointed as a Postdoctoral Researcher in the Cell Research and Immunology Department of Tel Aviv University with the guidance of Dr. Irit Gat-Viks and Prof. Dr. Eran Bacharach. The aim of the investigation is to determine the genetic susceptibility of Influenza in mice using a systems genetics and immunology approach.
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