FINAL PUBLISHABLE SUMMARY REPORT

- ## Executive summary

The kingdom of fungi comprises over 1.5 million fungal species, but only a handful of species are associated with a wide spectrum of diseases in humans and animals, ranging from allergy and autoimmunity to life-threatening infections. Understanding the spectrum of immunological responses to fungi is perhaps the most important challenge in the field of medical mycology. Most fungi (such as *Aspergillus* spp*)* are ubiquitous in the environment. Some, including *Malassezia* spp. and *Candida albicans*, establish lifelong commensalism on human skin and body cavities without causing disease. The fact that fungi are capable of colonizing almost every niche within the human body suggests that they must possess particular adaptation mechanisms. However, the occurrence of fungal diseases in primary immune deficiencies suggests the pivotal contribution of the underlying deregulated inflammatory immunity to susceptibility to fungal infections and diseases. The most serious of the human diseases caused by fungi are the opportunistic fungal infections that occur in patients with defective immunity, including AIDS and leukaemia and in patients suffering surgical trauma and therapeutic immunosupression. Other fungal diseases include mucosal infections (i.e. thrush and chronic mucocutaneous candidiasis and both IgE and eosinophilic-driven hypersensitivity diseases, including severe asthma, allergic bronchopulmonary mycoses, chronic sinusitis, hypersensitivity pneumonitis, atopic eczema/dermatitis syndrome and gut inflammation. Although the mortalities and morbidities associated with fungal diseases are not often highlighted to the general public in reality their burdens exceeds those of many of the best known bacterial diseases.

The ALLFUN project focuses on yeasts, such as *C. albicans* and *Malassezia* spp and filamentous fungi, like *A. fumigatus* and others known to be associated with a number of inflammatory, autoimmune and allergic diseases affecting occupational and non occupational health leading to chronic diseases such as respiratory allergy and chronic mucosal inflammation.

The objectives of the ALLFUN cooperative project are to:

- Study the specific cellular and molecular mechanisms by which ubiquitous airborne or commensal fungi contribute to immune homeostasis and its dysregulation leading to inflammatory diseases.
- Identify genetic signatures of susceptibility to fungal-associated inflammatory and allergic diseases, such as inflammatory bowel diseases, pulmonary, vaginal and cutaneous allergy.
- Determine across diagnostic groups the distribution of the cross reactive antibodies to phylogenetically conserved fungal allergens, other conserved aeroallergens and structurally related human proteins.
- Identify bioactive fungal and human inflammatory targets as biomarkers of diseases and potential therapeutic targets.
- Act as a model for an integrated interdisciplinary approach to investigate complex disease processes and produce translatable scientific knowledge for the development of new reliable diagnostic tools and therapeutic interventions.

- **Summary description of project context and objectives**

The ALLFUN proposal aimed at defining the cellular and molecular mechanisms by which ubiquitous airborne or commensal fungi contribute to immune homeostasis and its dysregulation leading to allergy and inflammatory diseases. Breakthroughs in understanding how mucosal homeostasis is established, maintained or disrupted in the presence of fungi should be sources of new therapeutic targets and drugs (i.e. anti-inflammatory, immunomodulatory and anti-infectious molecules). European scientists representing the leading edge of this field have been brought together here in a unique synergistic and cross-cutting collaboration that addresses a major medical and economic problem of considerable importance to the health care sector.

The study was centered on yeasts and filamentous fungi known to be associated with a number of inflammatory, autoimmune and allergic diseases. Via a multidisciplinary systems biology study combining fungal genetics, clinical research and animal models in a systems biology approach, integrating traditional wet-lab methods with those of functional genomics, immunomics, allergomics and bioinformatics, the ALLFUN project met the criteria of the FP7 call HEALTH-2010.2.4.5-2, whose strategic objective was "to elucidate mechanisms by which infections may lead to aberrant activation of inflammation, the lack of resolution of which is responsible for inflammatory diseases". Understanding the spectrum of immunological responses to fungi is perhaps the single most important challenge in the field of medical mycology.

ALLFUN attacked the issue of fungus-driven allergy and inflammation via a **three**-pronged multidisciplinary approach:

1. The study of fungal pathogen interaction with the immune system aimed at producing a systems biology analysis of this interaction from experimental models to the different clinical settings (under WP1 and 3) from 1 to 42 months.
2. The incorporation of information gathered from the systems biology analysis of fungal-host interaction into new diagnostics (WP2), from 8 to 42 months.
3. The incorporation of information gathered from the systems biology analysis of fungal-host interaction into intervention strategies in allergic and inflammatory fungal diseases (WP4) from 1 to 42months.

1. In the first part of the project, mice with inflammatory fungal diseases and a cohort of patients presenting a panel of allergic and inflammatory, fungus-associated diseases were in parallel assessed for innate and adaptive immune parameters of immunity using high-throughput technologies, such as gene expression profiling, multiplex analysis of cytokine and chemokines, multiparameter flow cytometry and transcriptome analysis by deep sequencing. Comparison was done with the appropriate controls, i.e., mouse model of resistance to inflammation and allergy to fungi and cohorts of healthy subject, either colonized and/or exposed or not to fungi (**WP1, 3, 4**). Biosamples were shipped to the wet-lab Beneficiaries for the assessment of immune reactivity to fungal mutants and purified antigens/allergens.
2. At the same time, we have used fungal mutants and purified fungal antigens already available and we have assembled a collection of genetically-modified fungal mutants and purified fungal antigens produced through *in vitro* high-throughput screens (**WP2**). Based on information gathered in WP1 and 3, an array of avant-garde technologies were applied to achieve the diagnostic task of WP2. Phage surface displayed cDNA and antibody libraries were used to clone fungal allergens and human IgE-binding self-antigens and for the affinity selection of phage surface displayed antibody fragments. We obtained a long list of deliverable PCR primers, host and fungal components to be used to generate easy to use diagnostic kits to develop PCR and microarray-based diagnostic kits aimed at developing universal diagnostic approaches for fungal diseases which correspond to an urgent unmet medical need.
3. Knowledge gained in **WP1** and **WP3** were translated and validated into Intervention Strategies in WP4. Thus, **WP4** is a clear indication that all the Beneficiaries of ALLFUN consortium have a clear vision of the translational research approach, and, therefore, **WP4** represented a perfect platform for thorough blending of ideas and cooperation among all the Beneficiaries of ALLFUN. To this purpose, standardization SNPs primers and centralization of the SNP analysis carried out in WP4 for identification of patient at risk of developing chronic fungal diseases were prioritized immediately after the start of the project. This undoubtedly lead to better comparison of the results and huge cost reduction.
The results were transferred to an industrial platform, based on SMEs and Enterprises devoted to the development and production of immunodiagnostics assays and immunomodulatory strategies.
The project was broken down into 4 WPs for RTD activities (WP1-4). Project management (WP5) and Dissemination of datasets accumulated in this project to the public domain and training (WP6) are integral part of the project. All deliverables, including the mutant strain collection and purified fungal biomolecules, the biomarker knowledge base and predictive models have been disseminated through workshops and training activities, and are being used as intellectual breakthroughs for new applications within the Horizon 2020. **Indeed, a major intellectual breakthrough of the project was to discover which specific clinical settings result in an exaggerated inflammatory response that are likely to compromise the ability of a patient to cope with infecting/colonizing fungi. This vision contrasts with dogmas and paradigms that reply entirely on the 'intrinsic' susceptibility to infection that determines a state of chronic or**

intractable disease. It is expected that the information derived from the clinical component in ALLFUN will have a major impact on future research in fungal diseases, vaccine development and fundamental research of the immune system for years to come.

- **Main S & T results/foregrounds.**

Intervention strategies and diagnostics in fungal allergy, inflammation and autoimmunity: Translating basic science into clinical practice

The overall objective of this research proposal was the elucidation of the specific cellular and molecular mechanisms by which ubiquitous airborne or commensal fungi contribute to immune homeostasis and its deregulation leading to inflammatory diseases in mouse models of inflammation, such as respiratory allergy to airborne fungi and mucosal inflammatory PRR/PAMP signaling pathways and effectors mechanisms underlying the functional activity of responses to yeast commensals. Based on a systems biology approach through an interdisciplinary network within the ALLFUN project consortium, the project succeed in obtaining relevant information on common or unique immune cells that helped to: i) define the predictive mouse models within the human disease context; ii) design and validate intervention strategies in fungal allergy and inflammation through immunogenetic screening and anti-inflammatory strategies, and iii) identify, purify and characterize common immunogenic fungal molecules including proteins, cell wall polysaccharides and lipids, for fungal serology and development of ultrasensitive diagnostic kits.

- **Identification of "drugable" signaling immune pathways in innate immune cells in response to fungi at mucosal surfaces.**

Extensive studies performed on dendritic cells (DCs), epithelial cells and polymorphonuclear basophils have discovered the relevant signaling pathways activated by distinct pathogen–associated molecular patterns (PAMPs) or by tissue damage–associated molecular patterns (DAMPs) leading to protection from fungal infections and fungus-dependent inflammation and allergy at the different body sites. To this purpose, a set of fungal cell wall mutants with specific and defined alterations in the cell walls has been generated, in order to define the shared and unique PAMP repertoires and pattern recognition receptors (PRRs) involved in innate immune recognition of the major fungal pathogens. This collection represents the largest collection of fungal cell wall mutants world-wide. A major library of mutant fungal PAMPs was also generated. Evidence have indicated a role for fungal glycans in recognition of fungi, including innate B cells. These cells are well characterized in mice and less in humans due to the lack of highly specific markers. Isolation of human blood cells sub-populations have shown that B cells can be stimulated by glycans leading to the production of pro-inflammatory cytokines and immunoglobulin. β-glucans recognition by dectin-1 rather than N-mannan was influenced by the genetic background of the host. Dectin-1 was required for the proper control of gastrointestinal and vaginal candidiasis in C57BL/6 but not BALB/c mice, the latter actually showing increased resistance in the absence of dectin-1. Susceptibility of dectin-1-deficient C57BL/6 mice to infection was associated with defective IL-17A, aryl hydrocarbon receptor-dependent IL-22 production as well as adaptive Th1 responses. In contrast, resistance of dectin-1-deficient BALB/c mice was associated with increased IL-17A and IL-22 production, and the skewing towards Th1/Treg immune responses that provide immunological memory. Disparate canonical/noncanonical NF-κB signaling pathways downstream dectin-1were activated in the two different mouse strains. Thus, the net activity of dectin-1 in antifungal mucosal immunity is dependent on the host's genetic background that affects both the innate cytokine production as well as the adaptive Th1/Th17 cell activation upon dectin-1 signaling. The priming mechanism of *Candida* on PRR-induced inflammation has also highlighted the role of dectin-2. Further work has focused on the importance of chitin as an immunological relevant cell wall PAMP. Chitin is an essential structural polysaccharide of fungal pathogens and parasites and is the second most abundant polysaccharide in nature after cellulose and its derivatives. Chitin acted to block immune recognition of yeast cells by human monocytes. The NOD2, TLR9 and the mannose receptor were found as essential fungal chitin-recognition receptor that lead to the production of the anti-inflammtory IL-10. Thus, chitin recognition is critical for immune homeostasis and is likely to have a significant role in infectious and allergic disease

(Figure 1).Chitin has also been found to influence the maturation of FoxP3+ regulatory T (Treg) cell differentiation on the one hand, and the ability of fungi to prime immune cells to subsequent stimulation with TLR ligands. This capacity to prime innate immune cells to other receptor ligands is mediated by more efficient activation of MAP-kinase pathways and it is suggested to play an important role in the amplification of inflammation in diseases associated with fungi, such as Crohn's Diseases (CD). In addition, significant progress has been made in assessing the role of the C-type lectin receptor mannose receptor for the susceptibility to systemic candidiasis. Complementary experiments carried out with *A. fumigatus* has generated the most extensive collection of cell wall mutant constructed to date in this fungus. α-1,3 glucans, galactomannan (GM) and galactosaminogalactan (GAG) are the three polysaccharides present at the surface of the cell wall of germinated conidia and in the extracellular matrix embedding the hyphae in vitro under static and aerial conditions, or in vivo in aspergilloma and invasive aspergillosis (GAG and GM). These polysaccharides are amorphous and show adhesive and immunological properties in vitro and in the relevant in vivo models. For instance, a tripe alpha glucan synthase of *A. fumigatus* was found to be avirulent in vivo. TLR1 was found to be important in the innate immune detection of the fungus in mouse and humans, and human TLR1 variants modulated innate immune responses to it.

Despite the identification of specific signaling pathways negatively regulating responses to fungal PAMPs, the unexpected convergence of molecular pathways responsible for recognition of PAMPs and DAMPs raised the question of whether and how the host discriminates between PAMPs and DAMPs and the relative contribution of either one to inflammation, immune homeostasis and mechanisms of repair during fungal infection and inflammation. The definition of pathways of inflammation resulting from either recognition of PAMPs or DAMPs or both have been recently defined in infection and allergy caused by the fungus *A. fumigatus* in mice and humans. S100 proteins represent important danger signals that, although primarily intracellular, mediate inflammatory responses through autocrine/paracrine interactions with receptor for advanced glycation end–products (RAGE). The mechanism exploits a previously unrecognized role for the danger molecule S100B that, by forming complexes with various TLR ligands, exhibited promiscuous activities at the extracellular and intracellular levels. Namely, S100B inhibited TLR2 via RAGE, through a paracrine epithelial cells/neutrophil braking circuit, and this accounted for its anti-inflammatory activity. However, the ability of S100B to bind nucleic acids resulted in the activation of intracellular TLRs converging on TRIF and eventually resolving danger-induced inflammation via transcriptional down-regulation of S100B expression (Figure 2).

- **Identification of "drugable" signaling immune pathways for personalized antifungal vaccine**

Fungal vaccine are expected to alleviate morbidity and mortality from fungal infections and diseases that remain unacceptably high. Major challenges in the development of human fungal vaccines are the differences in pathogenic mechanisms and fungal strategies to escape from immune surveillance and the wide spectrum of target populations not necessarily sharing the same risk factors. Progresses for fungal vaccines demand for the integration of advanced immunology approaches, systems biology, immunogenetic profiling and bioinformatics in the areas of fungal biology, host biology, and the interaction between the two. Bridging the fields of basic immunology and vaccine research is needed to create individualized immune profiles that have the potential for directing the rational development of customizing adjuvants and vaccines with predicting efficacy or an adverse response in each target population. We have determined the molecular mechanisms leading to CD4+ or CD8+T cell steering in *Aspergillus* infection and the possible dysregulation in Chronic Granulomatous diseases (CGD), a primary immunodeficiency caused by the inherited disorder of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) that is associated with infections by *Aspergillus* spp. Although considered an "innate immunity" disease, the defect in antigen presentation described in patients with CGD may reveal an unsuspected role for T cell and adaptive immunity in the pathogenesis of aspergillosis in this immunodeficiency. We found that the activation of either T cell subset is contingent upon the nature of the fungal vaccine, the involvement of distinct innate receptor signaling pathways and the mode of antigen routing and presentation in DCs. Specifically, *Aspergillus* conidia activated CD8+ T cells upon sorting to the Rab14+ endosomal compartment required for the alternative MHC class I presentation (Figure 3). Cross-priming of CD8+ T cells failed to occur in mice with CGD due to defective DC endosomal alkalinization and autophagy. However, long-lasting antifungal protection and disease control could be successfully achieved upon vaccination with purified fungal antigens activating CD4+ T cells through the endosome-lysosome pathway. Cross-priming of CD8+ T cells also

failed to occur in TLR3-deficient mice and TLR3 deficiency was a risk factor for infection in high-risk patients. These studies reveal that a defective CD8+ T cell response may predispose to fungal infections and is a proof-of-concept demonstration that understanding memory at basic levels could be exploited to personalize vaccination strategies against fungal diseases.

- **Description of the microbiome associated with allergic fungal diseases**

Allergic Bronchopulmonary Aspergillosis (ABPA), severe asthma, and asthma with fungal sensitisation (SAFS) are debilitating and chronic diseases associated with high morbidity and mortality whose pathogenesis is only partially understood. Although some fungi like *A. fumigatus* in ABPA or *M. sympodialis* in Atopic Dermatitis (AD) have been demonstrated to play a pivotal role on the pathogenesis of the diseases, the involvement of other fungal species is still largely unknown. We have evaluated the microbiome composition in airways of ABPA, SAFS and asthma patients compared to normal healthy individuals (Table 1) using culture-independent molecular techniques from bronchioalveolar lavage or cotton skin swaps from patients affected by the diseases. Each sample was divided into 3 portions for following analyses: (i) culture, (ii) molecular (DNA extraction and PCR) and (iii) FACS analysis for Tregs/Th17 lymphocytes. Extracted DNA was subjected to Illumina MiSEQ/Nextera XT and QIIME for (i) bacterial 16S and 23S regions, (ii) fungal ITS1-5S and 28S regions and (iii) archeal 16S region. The application of advanced culture-independent molecular techniques for the identification of microorganisms has contributed to our knowledge on the role of microbial colonization in health and disease. A vast variety of microorganisms, including several pathogenic and non-pathogenic species were detected as exemplified for the bacterial microbiome in healthy individuals (Figure 4A) and significant differences in composition of the mycobiome were observed when fungal disease and control samples were compared. The burden of fungal DNA was significantly higher in samples obtained from ABPA patients when compared to those of patients with mild asthma (Figure 4B). An enormous amount of data was collected amplifying by PCR the bacterial 16S and 23S regions and the fungal ITS1-5S and 28S regions, and archeal 16S region followed by multiplex library construction using the bacterial 16S and fungal ITS universal primers and MiSeQ of the relevant amplicons. Analysis of the sequencing data using the UNITE database as reference is still ongoing. However, significant differences in composition of fungal and bacterial biomes were observed when fungal disease and control samples were compared indicating a correlation between the severity of the symptoms and the lung microbiome composition.

- **Introducing the challenging concept that an hyper-inflammatory response may promote fungal virulence**

Infections by opportunistic fungi have traditionally been viewed as the gross result of a pathogenic automatism which makes a weakened host more vulnerable to microbial insults. However, fungal sensing of a host's immune environment might render this process more elaborate than previously appreciated. We have provided evidence that IL-17A binds fungal cells, likely acting on both host and fungal structures in experimental settings of host colonization and/or chronic infection. Global transcriptional profiling revealed that IL-17A induced artificial nutrient starvation conditions in fungi, resulting in a down-regulated target of rapamycin (TOR) signaling pathway and in increased autophagic responses and intracellular cAMP. The augmented adhesion and filamentous growth (Figure 5) eventually translated into enhanced biofilm formation and resistance to local antifungal defenses. This might exemplify a mechanism whereby fungi have evolved a means of sensing host immunity to ensure their own persistence in an immunologically dynamic environment. The data also highlight how the TOR nutrient sensing pathway is successfully exploited by fungi to ensure survival in an immunologically reactive environment.

- **Identification of a tryptophan metabolic pathway as a key regulator of resistance and tolerance to fungi**

Resistance and tolerance are two alternative but complementary host defence mechanisms to reduce pathogen burden and mitigate the negative impact of infections on host. The ability to tolerate *C. albicans*, a human commensal of the gastrointestinal tract and vagina, implicates immune strategies that favors the induction of protective-non-sterilizing-antifungal immunity. *Candida* species are the causative agents of vulvovaginal candidiasis (VVC) and recurrent vulvovaginal candidiasis (RVVC), two forms of disease that affect a large number of otherwise healthy women. We have evaluated mechanisms of resistance and tolerance to the fungus in experimental and human VVC and RVVC. Both defense mechanisms were

activated in murine and human VVC. IL-22 was involved in mechanisms of resistance, whereas IDO1-deficient mice, while apparently resistant to VVC in the early phase, became susceptible in the later phase of the infection and this was associated with the failure to activate IL-10-producing Treg cells capable of restraining inflammatory Th1/Th17 cells. IDO1 was promptly induced in infection at the protein and gene expression levels, and was associated with increased levels of kynurenines, downstream products of IDO1 with immunoregulatory functions and increased kynurenine to tryptophan ratio. The kynurenines were functionally active as replacement therapy with a mixture of L-kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid, all molecules downstream of the IDO1 pathway, restored immunoprotection to VVC. These data suggested that IDO1 mediates the production of tolerogenic kynurenines in VVC. Thus, IL-22 and tryptophan metabolites mediate antifungal resistance and tolerance in *Candida* vaginal infection and their deficiencies are risk factors for RVVC (see below).

Similar findings were obtained in murine and human CF, a disease characterized by an exaggerated yet ineffective airway inflammation that fails to eradicate pulmonary pathogens. Thus, deciphering the cellular and molecular pathways leading to chronic inflammation could lead to preventive anti-inflammatory strategies in CF. Decreased tryptophan kynurenine metabolism, as a result of defective IDO1, was causally linked to susceptibility to *Aspergillus* infection and sensitization in murine and human CF through the generation of a Th17/Treg imbalance. Treatments with IDO1 agonists or prevention of Th17 cell activation restored antifungal protective immunity and improved lung inflammation and function, suggesting a therapeutic potential for IDO1 mimetic drugs in CF**.** Thus, that Th17/Treg imbalance to *A. fumigatus* in CF is linked to IDO1 deficiency and corrected by kynurenins is a proof-of-concept demonstration that targeting pathogenic inflammation via IDO agonists could have therapeutic effects in CF. Additionally, we also found that hypoxia regulated the inflammatory/anti-inflammatory balance in the lung via IDO1 and RAGE. Sustained expression of RAGE and its ligand S100B exerted a proximal role in promoting inflammation in murine and human CF, as revealed by functional studies and analysis of the genetic variability of *AGER* in patients with CF. Both hypoxia and infections contributed to the sustained activation of the S100B/RAGE pathway, being RAGE up-regulated by hypoxia and S100B by infection via TLRs. Targeting pathogenic inflammation via soluble (s)RAGE alleviated inflammation in murine CF while measurement of sRAGE levels predicted RAGE-dependent inflammation in CF patients. Thus, we have identified a novel molecular pathway that contributes to the heightened inflammation in CF and provided evidence that this pathway could be a useful therapeutic target and biomarker of lung inflammation in this disease (Figure 6).

- **A bench-to-bedside approach: From the identification of innate immune receptors involved in the activation of immune protection to fungi to the immunogenetic screening of high-risk patients by SNP analysis.**

Table 2 is a list of major putative genes and SNPs selected on the basis of the association with immune recognition of *Candida* yeasts. These have been tested in a cohort of patients comprising 800 CD patients, very well phenotyped and genotyped for a large number of SNPs and "serotyped" for reactivity against fungal glycans and the presence of anti-*Candida* antibodies. Considering the frequency of the SNPs within the population examined, several SNPs have been shown to be highly represented within the cohort. This is the case for the original SNPs altering Dectin-1, TLR4 or NLRP3, all associated with cellular responses to fungi. Two, not previously described, polymorphisms altering NOD2 and Dectin-1, were also found in up to 25% of the patients examined (unpublished observations).

The influence of a number of these SNPs on the susceptibility to *Candida* colonization and intra-abdominal candidiasis in high-risk surgical ICU patients (in collaboration with the Fungal Infection Network of Switzerland, FUNGINOS) was also studied in a prospective observational cohort study that included a cohort of eighty-nine patients at high risk for intra-abdominal candidiasis (68 with recurrent gastrointestinal perforation and 21 with acute necrotizing pancreatitis). Eighteen single-nucleotide polymorphisms in 16 genes previously associated with development of fungal infections were analyzed from patient's DNA by using an Illumina Veracode genotyping platform. *Candida* colonization was defined by recovery of *Candida* species from at least one nonsterile site by twice weekly monitoring of cultures from oropharynx, stools, urine, skin, and/or respiratory tract. A corrected colonization index greater than or equal to 0.4 defined "heavy" colonization. Intra-abdominal candidiasis was defined by the presence of clinical symptoms and signs of peritonitis or intra-abdominal abscess and isolation of *Candida* species either in pure or mixed

culture from intraoperatively collected abdominal samples. SNPs in three innate immune genes were associated with development of a *Candida* corrected colonization index greater than or equal to 0.4 (TLRs *4986790*, hazard ratio = 3.39; 95% CI, 1.45-7.93; p = 0.005) or occurrence of intra-abdominal candidiasis (tumor necrosis factor-α *rs1800629*, hazard ratio = 4.31; 95% CI, 1.85-10.1; p= 0.0007; β-defensin 1 *rs1800972*, hazard ratio = 3.21; 95% CI, 1.36-7.59; p = 0.008). This finding highlights the relevance of the **TNF-α** functional polymorphism in immune response to fungal pathogens.

Given the findings in experimental VVC, genetic deficiencies of IL-22 or IDO1 were assessed as risk factors for RVVC in a cohort of more than 900 vaginal samples from women attending the S. Maria Della Misericordia Hospital in Perugia, Section of Microbiology, including 145 samples from women with RVVC and 293 from women with VVC. Within the set of SNPs tested, the genotype frequencies for *rs2227485* in IL22 and *rs3808606* in IDO1 were significantly different between controls and RVVC, but not VVC, patients. Specifically, the TT genotype at rs2227485 in IL22 was significantly associated with a decreased risk for RVVC [12.4% in RVVC vs. 22.8% in controls; odds ratio, 0.48; 95% confidence interval (CI), 0.27–0.85; P = 0.012]. Likewise, the TT genotype at *rs3808606* in IDO1 also correlated with a minor susceptibility to RVVC (13.8% in RVVC vs. 24.0% of controls; OR, 0.51; 95% CI, 0.29-0.88; P=0.020). Functional analyses confirmed that protection afforded by carriage of the TT genotype at *rs2227485* in IL-22 correlated with high levels of vaginal IL-22 and decreased levels of pro-inflammatory IL-17A, TNF-α and calprotectin. Likewise, high levels of IL-22 and decreased levels of IL-17A and TNF-α were observed in women carrying the protective TT genotype at *rs3808606* in IDO1 and they were associated with enhanced IDO1 expression in vaginal cells and increased kynurenine-to-tryptophan ratio. Altogether, these data provide evidence that common genetic variants leading to enhanced expression phenotypes of IL-22 and IDO1 may contribute to protection in RVVC.

Whether the inability to properly handle respiratory pathogens is secondary or primarily responsible for the state of chronic inflammation seen in CF patients is an important challenge with important clinical implications. Th17 cells and their effector cytokines mediate host defensive mechanisms to extracellular bacterial infections, but they are also involved in the pathogenesis of many inflammatory and autoimmune diseases. Th17 cells are in balance with the Treg cells through IDO1. A case-control study on the susceptibility to infection in CF patients and genotype distribution of SNPs in IL17A and IL17F genes. Was performed. The *rs8193036* polymorphism (T-737C) in IL17A was found to be significantly associated with *P. aeruginosa* colonization in CF patients (odds ratio 3.73, 95% CI, 1.18–11.8; p=0.03), independently of the CFTR mutation (Table 3). This variant has been previously linked with the development of ulcerative colitis, an autoimmune condition in which increased levels of IL-17 are pathogenic. Evidence for such an association was also obtained in CF patients with *Aspergillus* colonization, although the size of the patient cohort is presently too small to reach statistical significance.

It was also found that the expression of IDO1 is under genetic and epigenetic control in CF. Defective IDO1 expression was revealed by functional studies in humans where IDO1 was found to significantly modulate predicted FEV1 values, thus suggesting a role in controlling disease severity in CF patients. Similarly, the tryptophan/kynurenine pathway was down-regulated in bronchial epithelial cells from CF patients.

Based on the experimental evidence on the role of RAGE in aspergillosis, studies have been undertaken to define whether polymorphisms within regulatory elements or ligand binding sites may potentially orchestrate RAGE's functional activity as well as ligand accumulation. Indeed, the nonsynonymous G82S polymorphism has been found to promote glycosylation and increased ligand affinity, up-regulating intracellular signaling pathways linked with modulation of pro-inflammatory genes. A gain-of-function effect was also demonstrated for the -374T/A polymorphism, resulting in enhanced transcriptional activity by impairing the binding of regulatory elements to the gene promoter. Elevated serum concentrations of S100B, associated with certain immune-mediated diseases, have been linked with genetic variants in the S100B gene. We studied the association of these genetic variants with susceptibility to invasive aspergillosis in a cohort of 223 patients undergoing allogeneic stem cell transplantation. Susceptibility to aspergillosis was significantly associated with the -374T/A polymorphism in RAGE and with the +427C/T polymorphism in S100B, the relative contribution of each depended on their presence in both transplantation counterparts or in donors only, respectively. Functional assays demonstrated again-of-function phenotype of both variants, as shown by the enhanced expression of inflammatory cytokines in RAGE polymorphic cells and increased S100B secretion in vitro and in vivo in the presence of the S100B polymorphism. Similar findings were recently obtained in CF patients. These findings point to a relevant role of the danger sensing signaling in human antifungal immunity and highlight a possible contribution of a genetically determined hyper function

of the S100B/RAGE axis to susceptibility to invasive aspergillosis in high-risk patients, such as stem cell transplanted patients and CF patients. TLR3 deficiency was also associated with increased susceptibility to aspergillosis in transplanted patients.

By using a comprehensive list of 24 polymorphisms from 21 genes previously associated with susceptibility to fungal infections, an association study with mold colonization and invasive mold infection (IMI) among 1101 Caucasian solid organ transplant recipients (715 kidneys, 190 liver, 102 lungs, 79 hearts, 15 other; cohort#2) from the Swiss Transplant Cohort Study was performed. Mold colonization (N=45) and proven/probable IMI (N=26) were associated with polymorphisms in IL1beta, IL1RN (interleukin-1 receptor antagonist) and DEFB1. Moreover, the associations with IL1beta and DEFB1, but not IL1RN, remained independently significant with IMI in a multivariate stepwise regression model. Presence of two copies of rare allele of IL1beta or IL1RN SNPs was associated with reduced levels of IL-1β and TNF-α in *Aspergillus*-induced PBMCs compared to controls. This suggests that polymorphisms in IL1B and IL1RN influence cytokine responses that are associated with antifungal host defense, thereby influencing the susceptibility to colonization and/or infection.

Studies to evaluate association between SNPs and susceptibility to ABPA and chronic cavitary pulmonary aspergillosis (CCPA) were conducted on candidate genes selected following extensive literature review and in-house work investigating gene expression by macrophages from ABPA, CCPA, healthy and asthmatic subjects. SNPs were selected from the online database genome variation server (GVS), which uses the HapMap data. 251 SNPs in 30 genes were selected for analysis in 95 ABPA, 112 CCPA, 152 atopic asthmatics and 279 healthy controls. SNP genotyping was performed using the Sequenom Mass Array iPLEX Gold platform. Association tests and Benjamini-Hochberg correction for multiple testing were completed using SVS, SPSS and Stata. Frequencies in ABPA were compared to frequencies in asthmatic controls, and frequencies in CCPA were compared to frequencies in healthy controls. The work into genetic susceptibility to CCPA has disclosed the role of receptors and other proteins in CCPA and described associations of CCPA with SNPs in TLR1, CLEC7A (Dectin-1), PLAT (2 SNPs), VEGFA and DENND1B. Macrophages from CCPA subjects showed low TLR3 and TLR10 expression and high TREM1 expression at baseline, compared with macrophages from healthy subjects. The differences in baseline expression between the healthy and CCPA groups suggest roles for TLR3 and TLR10 in susceptibility to CCPA, while association of SNPs in PLAT, VEGFA and DENND1B supports novel roles for plasminogen activation and angiogenesis and of these genes specifically in susceptibility to CCPA. Additionally, compared with macrophages from healthy subjects, CCPA macrophages showed unrestrained rises in IL1alpha, IL1beta, IL6, IRAK2, and TRAF6 throughout the experiment. SNPs associated with CCPA were found in IL1beta (2 SNPs), IL1RN and IL15 (3 SNPs). Uncontrolled expression of IL1 and IL6 and continuing high levels of these cytokines may result in continuing cellular influx and pro-inflammatory responses, inhibiting disease resolution and contributing to disease progression in CCPA. The association of SNPs in IL1beta, IL1RN and IL15 with CCPA supports a role for the IL1 pathway, as well as implicating the IL15 gene, in susceptibility to CCPA. The work into genetic susceptibility in ABPA identified 4 SNPs, IL13 and IL4R, IL6 and TLR3, that remained significant after correction for multiple testing. Time-dependant deficient IL1 and IL6 responses in the ABPA group compared to the asthmatic control group were demonstrated in the macrophage gene expression work. This works identified multiple SNPs as associated with ABPA. IL6 and TLR3 are novel associations implicating these cytokine pathways and receptors in the aberrant response to *A. fumigatus* and susceptibility to ABPA. Responses associated with diminished IL1 and IL6 production offer insights into the pathogenesis of ABPA and/or its complications, which may translate to improved therapeutics for this disease, eventually. Finally, investigations on 62 patients confirmed with AD have contributed to a better definition of the different patterns of the inflammatory response and the correlation with the general inflammatory and hormonal conditions. This has led to a further refinement of criteria for AD classification.

- **A bench-to-bedside approach: From the identification of signaling pathways leading to the activation of inflammatory responses to fungi to anti-inflammatory therapeutic protocols.**

The groundbreaking concept of an inflammatory pathway paradoxically promoting, rather than limiting, fungal infections and/or diseases, has led to the development of anti-inflammatory protocols that target pathogenic inflammatory responses against fungi. As already briefly mentioned, these included:

i) Treg-based molecules such as IDO agonists or kynurenins in experimental models of RVVC and CF.

ii) Blocking pathogenic Th17 cell responses by siRNA inhalation to silence IL-17A in murine CF. The results show that siIL-17A reduced the activation of Th17 in the lung, decreased inflammatory cell influx in the BAL and lungs and ameliorate tissue inflammatory. Thus, a proof-of-concept demonstration that targeting the Th17/Treg cell imbalance could have therapeutic and immunorestoration potential in CF has been obtained.

iii) Establishing the therapeutic utility of blocking the S100B/RAGE axis in aspergillosis in stem cell transplanted or CF patients.

iv) Development of novel CCR4 antagonists as therapeutic agents in respiratory fungal allergy. Following pathogen exposure or vaccination, DCs uptake the antigens and release, among others, CCL17 and CCL22 that will attract Th2 and CD4+CD25+ Tregs to the site of activation. Tregs, in turns, will down-regulate the expression and uptake of antigens via C-type lectin-like receptors CD206 and DC-SIGN, restraining the pinocytosis process of DCs and augmenting the expression of FcγRIIB, an inhibitory Fcγ receptor the engagement of which by IgG-bound antigens leads to inhibition of DC activation. As CCL22 and CCL17 mediate migration of both Th2 and Tregs, our results provide an additional insight on the pertinence of strategies aimed at using CCR4 antagonists. Because these antagonists are fully protected by PCT/GB2009/001475 (WO/2009/150433), peptide-based CCR4 antagonists based on the selection of novel sequences have been produced. The novel peptides were synthesized by a Fmoc/tBu solid-phase peptide strategy, purified and characterized by LC-MS. Five novel CCR4 antagonists were tested in the murine ABPA model. Briefly, mice with ABPA were treated with different doses of the different compounds (referred to as 1,2,3,4,5) and evaluated for parameters of inflammatory allergy in terms of fungal growth in the lung and brain, cell recruitment in the BAL, inflammatory tissue pathology and mucus secretion in the lung. The results of a preliminary experiment are shown in Figure 7. Among the 5 compounds, some of them (4 and 5) exerted a protective effect against ABPA, as observed by the control of fungal growth (A), eosinophil recruitment (B) and lung pathology (C). In contrast, compounds 1 and 3 failed to show a protective effect, and compound 3 actually appeared to exacerbate ABPA.

v) Identifying novel mechanisms of action of IVIg on inhibition of Th17 cells and expansion of Tregs and therapeutic utility in fungal inflammatory conditions. IVIg, a therapeutic preparation of polyclonal IgG, increasingly used in the treatment of diverse autoimmune and allergic diseases, may target Th17 cells to exert therapeutic effects. Th17 cells were differentiated from naïve human CD4+ T cells in the presence of TGFβ and IL-21. Th17 cells were amplified by stimulating memory CD4+ T cells in the presence of IL-1beta and IL-6. The effect of IVIg was examined on differentiation and amplification of Th17 cells, expression of the Th17 lineage-specific transcription factor RORC, secretion of Th17 effector cytokines and phosphorylation of STAT3, a transcription factor that plays an important role in Th17 development and function. IVIg inhibited the differentiation and amplification of human Th17 cells, as well as the production of their effector cytokines IL-17A, IL-17F, IL-21 and CCL20. Also, IVIg significantly enhanced Foxp3+ Treg cells among the memory CD4+ T cells. These results reveal a novel mechanism of action of IVIg in achieving therapeutic effect in autoimmune and allergic diseases, where Th17 cells play a key modulatory role in sustaining the chronic inflammatory response. However, the cellular and molecular mechanisms by which IVIg expands Tregs are relatively unknown. As Treg expansion in the periphery requires signaling by antigen-presenting cells such as dendritic cells (DCs) and IVIg has been demonstrated to modulate DC functions, it has been hypothesized that IVIg induces distinct signaling events in DCs that subsequently mediate Treg expansion. We demonstrated that IVIg expands Tregs via induction of cyclooxygenase (COX)-2–dependent prostaglandin E2 (PGE2) in human DCs. Inhibition of PGE2 synthesis by COX-2 inhibitors prevented IVIg-mediated Treg expansion in vitro and in vivo. IVIg-mediated COX-2 expression, PGE2 production, and Treg expansion were mediated in part via interaction of IVIg and F(ab′)2 fragments of IVIg with DC-specific intercellular adhesion molecule-3-grabbing nonintegrin. The prophylactic and/or curative effects of IVIg on both colonic inflammation and *C. albicans* clearance was then evaluated in experimental DSS colitis. IVIg was administered daily for one week, immediately after or before the infection. As

C57BL/6 and BALB/c mice are basically Th1- and Th2-type mouse strains respectively, we used these two strains of mice. IVIg administration had no effect on BALB/c mice in terms of clinical signs of inflammation, *C. albicans* colonization and mouse mortality rate (Figure 8). In contrast, administration of IVIg to C57BL/6 mice reduced the intestinal inflammation and *C. albicans* colonization (Figure 9). These results show that the effect of IVIg is likely Th1 mouse strain dependent, and the curative treatment with IVIg via intraperitoneal injection exerts beneficial biological activities in terms of reducing intestinal inflammation and *C. albicans* colonization. Finally, IVIg was protective both under prophylaxis and therapeutic schedules in murine aspergillosis.

- **A bench-to-bedside approach: From cloning fungal allergens and cell wall components to the development of multi-analyte label-free biosensors for advanced fungal diagnostics.**

Overall, the results obtained during the whole duration of the ALLFUN project surpassed the expectations in generating an incredible number of fungal molecules now ready to be implemented in the development of more sophisticated methods for a rapid *in vitro* diagnosis of fungal infections. This corresponds to an unmet medical need because the diagnosis of invasive fungal infections based on classical methods is still slow, unsatisfactory and in many cases come too late for the patient. The tools and the molecules developed by the ALLFUN project form a solid basis for the development of a fast and reliable diagnosis of invasive fungal infections. A list of purified fungal molecules (proteins, cell wall proteins, polysaccharides and lipids)–representing the worldwide largest collection of fungal allergens–has been generated. These include 20, 10 and 8 different allergens cloned, expressed, and purified from *A. fumigatus*, *M. sympodialis*, and *T. rubum*, respectively. Most of these allergens have been investigated for their capability to bind IgE from sensitized patients *in vitro*, and some also for their capability to induce positive skin test reactions *in vivo*. Additionally the major allergens of *A. alternata* (Alt a 1) and *C. herbarum* (Cla h 8) of *C. herbarum*as as well as Hps90 (heat shock protein), Als3 (a cell wall adhesin), Hyr1 (a cell wall proteins expressed in hyphae) and Eno1 (enolase) from *C. albicans* have been cloned produced and purified. The availability of the sequences contributed to speed up cloning of potential cross-reactive allergens from other members of the fungal kingdom. Cross-reactive allergens have been grouped in the following families:

- -5 Peroxisomal proteins (*A. fumigatus* (Asp f 3), *C. boidinii* (PMP20A, PMP20B), *M. furfur* (Mala f 2, Mala f 3).
- -5 Cyclophilins (*A. fumigatus* (Asp f 11, Asp f 27) *M. sympodialis* (Mala s 6), *S. cerevisiae* Cyp, and *C. albicans* Cyp.
- -5 P2 ribosomal proteins (*A. fumigatus* (Asp f 8), *A. alternata* (Alt a 5), *C. herbarum* (Cla h 5), *T. rubum* (Tri r 2), and *S. cerevisiae* P2 protein.
- -4 MnSOD's (*A. fumigatus* (Asp f 6), *M. sympodialis* (Mala s 11), *T. rubum* (Tri r 1)
- -6 Thioredoxins (*A. fumigatus* (Asp f 28, Asp f 29), *M. sympodialis* (Mala s 13), *C. comatus* (Cop c 2), *S. cerevisiae* (ScTRX).

The number of cell wall proteins identified and purified surpassed all optimistic expectations. Some of these proteins have been shown to represent IgE-binding proteins potentially involved in fungal allergy and need to be further investigated for their clinical relevance. Very promising results have been obtained demonstrating the IgE-binding capacity of *Aspergillus* polysaccharides and lipids. More effort will be required to demonstrate the clinical relevance of these reactions and to evaluate their diagnostic value.

Other cell wall proteins lacking IgE-binding properties were able to induce immune responses of different isotypes and might open new possibilities for an early diagnosis of fungal infections. Among others it was shown that deglycosilation of PPMs and anti-PPMs, already used as markers for the diagnosis of invasive candidiasis, was necessary to obtain acceptable sensitivity/specificity ratios for discrimination between *Candida* colonisation and invasive candidiasis that occurs in 30 to 40% of high-risk surgical intensive care unit (ICU) patients. In these patients (four hundred thirty-four consecutive adults with abdominal surgery or acute pancreatitis and ICU stay 72 hours or longer were screened: 89 (20.5%) at high risk for IAC were studied (68 recurrent gastrointestinal tract perforation, 21 acute necrotizing pancreatitis)., the assessment of accuracy of 1,3-β-d-glucan antigenemia (BG) for diagnosis of infection revealed that BG antigenemia wass superior to *Candida* score and colonization indexes and anticipated diagnosis of blood.

The Evanescence biosensor technology: technological background

The *EVA-Biosensor Technology* is an innovative biosensor technology consisting of two components: 1) The EVA-biosensor Chip and 2) The EVA-Reader.

1) The EVA-biosensor Chip

The "EVA"-Chip (Figure 10) is made by injection molding of polystyrene, identical to the material used to produce classical ELISA plates, the today's standard technology for the development of immunoassays. A high density binding polystyrene surface is obtained by irradiation of the molded chips with a Co-60-γ source at 25 kGray, a standard procedure also used to obtain ELISA plates with a high binding capacity. The "EVA" Chip has two areas: the upper part consisting of eight fully separated polystyrene wells and the lower part consisting of an optical prism with a surface of high optical quality with a special design perfectly fitting to the inlet of the EVA-Reader. Each well of the EVA-Chip can be bio-functionalized for the detection of an analyte of choice based on fluorescence-linked immunosorbent assays (FLISA) in analogy to, and following the principles used to develop the state of the art enzyme-linked immunosorbent assays (ELISA). In contrast to ELISA tests which are end point measurements, the innovative EVA-technology allows kinetic measurements which are practically background-free.

2) The EVA-Reader

The evanescence biosensor reader is based on evanescence excitation of fluorophors bound to the EVA-Chip surface (Figure 11). The interface between the liquid in the upper well and the lower prism is called the evanescent surface. A diode laser light beam with a wavelength of 635 nm is directed against the side wall of the prism where it is refracted in function of the refractive index of air (n = 1.0) and polystyrene (n = 1.595) according to classical geometrical optics. The light beam further travels in the prism, hits the evanescent surface at the bottom of the well, where total internal reflection takes place, and exits on the other side of the optical prism (Figure 11). The laser light does not enter the liquid above the bottom of the well following the classical optic rules of total internal reflection. Fluorescently labelled molecules present in the thin 200 nm layer at the bottom of the well are excited by the 635 nm laser beam. The set up of the EVA-Reader is conceived in such a way that only the photons emitted through the bottom of the prism are collected, filtered with interference filters down to 600 +/- 5 nm and counted in a time dependent mode (Figure 12). Laser, filters and detector are fixed and the EVA-chip is mechanically moved from well to well allowing consecutive cycles of measurement for each individual well. In a first detection cycle the fluorescence of the first well is measured for 1 s followed by well 2 and so on until all 8 wells are measured. The successive measurement cycles restart with well 1 with identical time intervals as for cycle 1 during 10 min resulting in a total of 40 single measurements for each well. Photon counts for each well are automatically plotted in 8 different graphs as function of the time on the X-axis and cumulative photon counts on the Y-axis. The fluorescence signal in each single well increase only if the immobilized ligand and the labelled analyte in solution form a complex at the sensor surface as explained in detail in Figure 13. For diffusion limited assays where all parameters are kept constant the Fick's second diffusion law apply and the slope (dFt/dt) is directly proportional to the concentration of the analyte. Theoretically the innovative evanescence-based biosensor technology has the capacity to replace all ELISA-based immunoassays. The *EVA-Biosensor* overcomes the troublesome disadvantages of ELISA − especially the long measurement time is shortened to 10 minutes and the multiple washing and manipulation steps are reduced to few simple steps. The ELISA advantages, however − high sensitivity and specificity − are maintained. This technology is suitable for simultaneous multiple parameter testing as required in many biosensor tests. The *EVA-Biosensor* chips use a design with 8 independent wells avoiding biochemical crosstalk and mixing up of reagents between the wells and have been tested in many different assays.

- **Potential impact and main dissemination activities and exploitation of results**

<u>Potential Impact</u>

The overreaching objective of ALLFUN is to orchestrate a consortium of leaders in the fields of fungal pathology and immunopathology, functional genomics, proteomics, immunomics, allergomics, bioinformatics and data management with cutting-edge laboratory instrumentation. A major theme in ALLFUN is to challenge existing theories with new perspectives and bring forward possible new players

either promoting or protecting from fungal diseases.
Through an integrated interdisciplinary approach, the ALLFUN Consortium has successfully investigated complex disease processes and produced translatable clinical data for new diagnostics and treatments. Namely:

- The purification, characterization and cloning of common immunogenic fungal molecules and fungal allergens for the development of more sophisticated diagnostic tools.
- The generation of fungal mutants and purified fungal PAMPs (made available to the international scientific community) for a comprehensive genome-wide examination of the interaction of fungi with the mammalian host. The ultimate goal of generating bio-engineered antigens to exploit the broadness and specificity of human T cell repertoire against fungi in the different clinical settings has been achieved.
- The exploitation of immunogenetics to identify new susceptibility genes in high-risk patients. Such knowledge is being currently introduced into the clinical practice to improve patient's risk stratification, therapy optimization and antifungal-tailored treatments.
- The use of the ability of patients' immune system to identify more accurate biomarkers predicting inflammatory disorders has been already successfully achieved. The kynurenines-to-tryptophan ratio has proved to be a good indicator of the patient's ability to cope with fungus-driven inflammation and it is ready for introduction in clinical practice.
- The design and validation of intervention strategies in fungal allergy and inflammation, including medical treatments that increase host resistance, such as antibiotics, place selective pressures on pathogens. As tolerance mechanisms are not expected to have the same selective pressure on pathogens, new drugs that target tolerance will provide therapies to which low-virulence fungi will not develop resistance. The proof-of-concept demonstration that targeting tolerance may be beneficial in fungal inflammatory diseases has been obtained. Novel compounds that target pathogenic inflammation to fungi have been already developed and more will be developed.

Dissemination activities

Since the beginning of the project, the dissemination activities were aimed at enhancing visibility of the consortium and of the project, mainly for informing scientific community about the project objectives and for communicating the value of the research funded by the European Community through FP7. The target groups for dissemination were identified as: **general public**; **secondary disseminators** (*e.g.* teachers, students, educators, journalists, national and international foundations for the diffusion of information on health protection, scientific associations) aiming at reaching patients suffering from allergies and autoimmune diseases; **decision-makers and leaders** of health policies and legislation (*e.g.* national and supra-national health protection agencies, ministries of health, medical associations, NGO (Non-governmental organizations associated with the United Nations), to demonstrate the efforts of the EC in promoting the development of innovative methodologies and approaches for non-invasive prediction, diagnosis and monitoring of allergies and autoimmune diseases; **health operators** (hospitals, associations of preventive medicine, therapists, general practitioners, field operators) to raise awareness on new tools for diagnosis and potential therapies for allergies and autoimmune diseases; **pharmaceutical and biotech companies** to facilitate and develop transfer of knowledge and technology, in compliance with the Consortium Agreement, thereby further promoting commercial exploitation of the results while protecting intellectual property.

The main dissemination approaches from the project start included:

1. The presentation of the ALLFUN results at several national and international congresses and conferences in fungal and immunological research (as listed in Template A2).
2. The organization of the following scientific events:

    - David Denning and colleagues from the University of Manchester organized the **6th Advances Against Aspergillosis (AAA) meeting in Madrid (Spain), 27 February-1 March, 2014** (http://www.advancesagainstaspergillosis.org/2014/goals.htm).
    - **SM5-Satellite meeting-ALLFUN meeting ICI held in Perugia (Italy), August 29-30 2013,** organized by Luigina Romani. During this event, ALLFUN has supported the participation of 10 invited speakers:

- *Antonio Cassone*
- *Axel A. Brakhage*
- *Bruce Klein*
- *Franco Aversa*
- *Gordon Brown*
- *Hermann Einsele*
- *Jay Kolls*
- *Paolo Puccetti*
- *Stuart M. Levitz*
- *Teresa Zelante*

- **Gordon Research Conference: Immunology of Fungal Infections held in Texas in January 13-18, 2013**, where Luigina Romani was one of the co-organizers. During this event, ALLFUN has supported the participation of 10 invited speakers:
  - *Agostinho Carvalho*
  - *Axel Brakhage*
  - *Frank van de Veerdonk*
  - *Jean Paul Latgé*
  - *Karl Kuchler*
  - *Luigina Romani*
  - *Mihai Netea*
  - *Neil Gow*
  - *Teunis Geijtenbeck*
  - *Thomas Harrison*

- David Denning and colleagues from the University of Manchester organized the **5th Advances Against Aspergillosis (AAA) meeting in Istanbul (Turkey), January 26-28th, 2012.** AAA is an alternate year meeting attracting 350-450 participants from around the world. In Istanbul, 352 participants spent 3 days listening to about 50 talks and interacting over 149 posters. The focus of the meetings was on translational research and covered numerous topics ranging from basic pathogenesis to clinical management. The program of this and all prior meetings is available here: www.advancesagainstaspergillosis.org/2012/index.htm.

- The Infectious Diseases Service at CHUV has held two 2-day international meetings "**Center of excellence on invasive fungal infections" on May 30th-31st 2011 and February 9-10th 2012**, supported by an unrestricted educational grant from MSD. The objective of these meetings was to provide a comprehensive up-to-date training including ex-cathedra lectures, case studies, journal club and mycology lab demonstrations on the epidemiology, pathogenesis, diagnosis, prevention and treatment of invasive fungal infections in ICU patients. Target audience were invited physicians (15 in 2011 and 21 in 2012) of different specialties (intensivists, infectious diseases, microbiology) working in teaching hospitals from the European Community and East-European countries.

- Institut Pasteur has organized the **2nd International Fungal Cell Wall Meeting** held on **October 8-10, 2011** in **Presqu'île de** *Giens in Southern France*.
  The focus of this second meeting was on the critical importance of the fungal cell wall in the biology, pathogenesis, and antifungal treatment of invasive fungal infections. With newer cell wall-directed antifungals now in development and new insights into cell wall structure and dynamic function through advanced genomic and proteomic methods, the fungal cell wall is seen as the most important structure in medical mycology as the foundation for growth, disease, and immune recognition.
  The program has included 3 days of invited speakers and purpose fully devoted considerable time to allow participants and faculty to interact and develop collaborations between sessions, during poster sessions, and over meals. The two first days of the meeting has

included topics such as cell wall structure and biofilm, polysaccharide biosynthesis and remodelling, regulation of growth polarity, antifungal drug targeting and combination therapies. The last day of the meeting was devoted to cell wall immunology and host recognition. ALLFUN has supported the participation to this meeting of the following invited speakers belonging to the ALLFUN project:

- *Luigina Romani*
- *Jagadeesh Bayry*
- *Daniel Poulain*
- *Neil Gow*
- *Jean Paul Latge*
- *Anne Beauvais*
- *Reto Crameri*
- *Thierry Calandra*

3. **Development of products as:**

- ✓ Project dedicated website.
  The project website has been created with its own domain: www.allfun-fp7.eu using the predominant color of the project: orange and grey. The homepage summarizes the main focus and structure of ALLFUN. Using the menu of the home page it is possible to access main sessions of the **public area**:
  The **partners** page describes the composition of the ALLFUN consortium and contains email address of the scientists involved and a link to their organization.
  The **events** and **training and dissemination** pages list the main activities organized to favour training and dissemination actions.
  The **publications** page list all the papers derived from the ALLFUN project with the possibility of downloading some of them. This page complies also with the Special Clause 39 of Grant Agreement allowing the deposit of manuscripts in an electronic repository.
  On the lateral column, the latest news about ALLFUN can be found.
  The **reserved area** is intended as a tool of information and updating for scientists; the access is restricted with a password in order to protect and dedicate to internal project communication. Inside this area it is possible to upload and download documents and files regarding technical annexes, workpackages and reports.
- ✓ Press release from Prof. Romani (coordinator)
- ✓ Brochure of the project.
- ✓ Aspergillus web site. P7-UNIMAN has created an *Aspergillus* website (www.aspergillus.org.uk) and blog (www.aspergillusblog.blogspot.com). The web site acts as a major early source of dissemination by uploading all conference abstracts, presentations and papers in the topics covered by ALLFUN, with particular reference to *Aspergillus*. Newsletters are also published montly on the *Aspergillus* web site.Press releases and papers of great topical interest are put on the blog, which gives the possibility to interested people to comment the articles and have an answer to their questions.
  Current figures for 2013 show that an average of 52,000 individual computers accessed **The Aspergillus Website** alone per month (this is a low estimate of individual users), and there were 80,000 visits per month, 90,000 in January. In terms of quantity of downloaded data, 70Gb of data was downloaded in May 2013 and averaged 900,000 page downloads per month. Our files are stored on multiple servers, some of which do not provide usage data, so these stats are certainly an underestimate of usage.
  The Aspergillus Website is listed at number 1, 1, 1 and 2 (up 1) in Google.co.uk, number 2, 3, 5 and 3 in Google.com, number 1 in Bing and Yahoo! for 'aspergillus', 'ABPA' 'aspergillosis' and 'aspergilloma'respectively. If 'aspergillus' is searched in Google, there are over 4 million results. Many of these ranking have improved over the last 12 months, none have worsened. The weekly blog (www.aspergillusblog.blogspot.com) has 5 -7000 page requests per month this year and has recently passed 250,000 page accesses since it began in 2007.
  Monthly newsletters are sent out to >20,000 (free) subscribers. This figure has started to rise as an upsurge in newly registered members has been noted thanks to the 'articles' library being

made ‘members only’. There are nearly 44,000 people registered on the site (31% medics, 24% scientists, 6% vets and 39% laypeople).

There are 212,000 links from other websites to **The Aspergillus Website** according to Google records and in the last 3 months to 15th October 2013 **The Aspergillus Website** was listed 1,000,000 times in Google searches alone, attracting 75,000 clicks as people come to our website for content. In the last 3 months up to the 17th October 2012 **The Aspergillus Website** was listed 2,800,000 times in Google searches alone, attracting 140,000 clicks.

4. **Publications/chapter of books/reviews**

A huge number of publications (including still in press papers and chapter of books) have been produced since the beginning of the project acknowledging ALLFUN funding. Several other papers and reviews are in various stages of the submission and peer review process. Template A1 below lists the most important scientific publications on the basis of their impact factor.

5. **Training**

From its start the ALLFUN project has sustained the participation of PhD and PostDoc scientists belonging or not to the ALLFUN Consortium to several and important scientific events through the award of fellowships summarized below (for a total of EUR 40.200, 82 fellowships). **These training initiatives have also allowed the dissemination of ALLFUN activities and results to a large community of young scientists.**

Exploitation activities

Several of ALLFUN deliverables lend themselves clearly to successful exploitation. Namely:

- The purification, characterization and cloning of common immunogenic fungal molecules and fungal allergens for the development of more sophisticated diagnostic tools.
- Development of a novel allergen and cell wall component-based Evanescence biosensor in fungal diagnostics.
- The generation of fungal mutants and purified fungal PAMPs (made available to the international scientific community) for a comprehensive genome-wide examination of the interaction of fungi with the mammalian host. The ultimate goal of generating bio-engineered antigens to exploit the broadness and specificity of human T cell repertoire against fungi in the different clinical settings has been achieved.
- The use of the ability of patients’ immune system to identify more accurate biomarkers predicting inflammatory disorders has been already successfully achieved. The kynurenines-to-tryptophan ratio has proved to be a good indicator of the patient’s ability to cope with fungus-driven inflammation and it is ready for introduction in clinical practice. Knowledge base of biomarkers of fungal disease susceptibility will thus become an invaluable resource to researchers in the fields of antimicrobial immunity and infections. This deliverable has the capacity to become a central point within the fungal research community, and will have high dissemination impact.
- The exploitation of immunogenetics via Illumina Veracode technology for haplotype tagging SNPs and common non-synonymous single polymorphism. to identify new susceptibility genes in high-risk patients. Such knowledge is being currently introduced into the clinical practice to improve patient’s risk stratification, therapy optimization and antifungal-tailored treatments.
- The design and validation of intervention strategies in fungal allergy and inflammation, includingmedical treatments that increase host resistance, such as antibiotics, place selective pressures on pathogens. As tolerance mechanisms are not expected to have the same selective pressure on pathogens, new drugs that target tolerance will provide therapies to which low-virulence fungi will not develop resistance. The proof-of-concept demonstration that targeting tolerance may be beneficial in fungal inflammatory diseases has been obtained. Novel compounds that target pathogenic inflammation to fungi have been already developed and more will likely be developed. In this regard, the formulation of novel CCR4 antagonists developed through the invaluable network between basic scientists and SMEs within the ALLFUN consortium is a realistic achievement.

The overreaching objective of ALLFUN is to orchestrate a consortium of leaders in the fields of fungal pathology and immunopathology, functional genomics, proteomics, immunomics, allergomics, bioinformatics and data management with cutting-edge laboratory instrumentation. A major theme in ALLFUN is to challenge existing theories with new perspectives and bring forward possible new players either promoting or protecting from fungal diseases. Through an integrated interdisciplinary approach, the ALLFUN Consortium has successfully investigated complex disease processes and produced translatable clinical data for new diagnostics and treatments. We obtained a long list of deliverable PCR primers, host and fungal components to be used to generate easy to use diagnostic kits to develop PCR and microarray-based diagnostic kits aimed at developing universal diagnostic approaches for fungal diseases which correspond to an urgent unmet medical need.

- **Project website and relevant contact details**

The project website has the address: http://www.allfun-fp7.eu/

Contractors involved:
- Università degli Studi di Perugia ITALY): Luigina Romani
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