UNIVERSITY OF IOANNINA
SCHOOL OF HEALTH SCIENCES
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DEPARTMENT OF INTERNAL MEDICINE
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The participation of *Malassezia* genus in the pathobiology of seborrheic dermatitis

CHRISTOFOROS VLACHOS

DERMATOLOGIST VENEREOLOGIST

Doctor of Philosophy Thesis

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Μέλη Τριμελούς Συμβουλευτικής Επιτροπής:
Επιβλέπων
Μπασούκας Ιωάννης Αναπληρωτής Καθηγητής Δερματολογίας του Τμήματος Ιατρικής της Σχολής Επιστημών Υγείας του Πανεπιστημίου Ιωαννίνων
Μέλη
Παπαδοπούλου Χρυσάνθη Αναπληρώτρια Καθηγήτρια Μικροβιολογίας του Τμήματος Ιατρικής της Σχολής Επιστημών Υγείας του Πανεπιστημίου Ιωαννίνων
Βελεγράκη Αριστέα Επίκουρη Καθηγήτρια Μικροβιολογίας του Τμήματος Ιατρικής της Σχολής Επιστημών Υγείας του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών

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Κωνσταντόπουλος Σταύρος	Ομότιμος Καθηγητής Παθολογίας – Πνευμονολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Μαρσέλος Μάριος –Αθανάσιος	Καθηγητής Φαρμακολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Μπασούκας Ιωάννης	Καθηγητής Δερματολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Παπαδοπούλου Χρυσάνθη	Καθηγήτρια Μικροβιολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Παππάς Περικλής	Αναπληρωτής Καθηγητής Φαρμακολογίας του Τμήματος Ιατρικής του Πανεπιστημίου Ιωαννίνων
Βελεγράκη Αριστέα	Επίκουρη Καθηγήτρια Μικροβιολογίας του Τμήματος Ιατρικής του Εθνικού και Καποδιστριακού Πανεπιστημίου Αθηνών
Γαϊτάνης Γεώργιος	Επίκουρος Καθηγητής Δερματολογίας του Τμήματος Ιατρικής Πανεπιστημίου Ιωαννίνων

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Ανδρέας Φωτόπουλος
Καθηγητής Πυρηνικής Ιατρικής

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Dedicated to my Mentors

PROLOGUE

Skin's fungi microflora is rather limited and the yeast *Malassezia* comprises the main representative. Although *Malassezia* is implicated in various skin diseases with high prevalence, the main principles of the fungi physiology and pathobiology are yet to be unraveled. This thesis was based on innovative perspectives, regarding the role of the yeast in the skins homeostasis and disease that were provided by Professor Ioannis Bassukas and Associated Professor George Gaitanis. The Implementation of those ideas required a wide cooperation of people and laboratories. In my belief the outcome justifies the involved efforts.

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Abbreviations

ACTH: adrenocorticotropic hormone
AD: atopic dermatitis
AhR: aryl hydrocarbon receptor
Ahrr: aryl hydrocarbon receptor repressor
AOAC: association of Official Analytical Chemists
Arnt: aryl hydrocarbon receptor nuclear translocator
BDCA1: blood dendritic cell antigen 1 (also known as CD1C)
bHLH: basic Helix-loop-Helix
CD: cluster of differentiation
Chs: chitin synthase
CLSM: confocal laser scanning microscopy
CXCL: Chemokine (C-X-C motif) ligand
CYP: cytochrome p450
Dapi: 4',6-diamidino-2-phenylindole
DDCs: dermal dendritic cells
DGGE: denaturing gradient gel electrophoresis
dNTP: Deoxynucleotide Triphosphates
FDA: foods and drugs administration
FICZ: 6-formylindolo[3,2-b]carbazoleGM-CSF: Granulocyte/macrophage colony stimulating factor
Gsta: Glutathione S-transferase A
Gstp: glutathione S-transferase P
HaCaT: Cultured Human Keratinocyte
HBD: human beta defensins
Hepa: mouse hepatoma cells
HLA: human leukocyte antigen
HMBS: hydroxymethylbilane synthase.
Hsp: heat shock protein
ICZ: Indolocarbazole
iDECs: inflammatory dendritic epidermal cells
IGS: intergenic spacer
IkBa: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
ITS: internal transcriber
KCs: keratinocytes
LCs: langerhans cells
LMI: leukocyte migration inhibition
LPS: lipopolysaccharides
LT: leukocyte transformation
MHC: major histocompatibility complex
MLR: mixed lymphocyte reaction
moDCs: monocyte derived DCs
MSH: melanocyte-stimulating hormones
MyD88: Myeloid differentiation primary response gene (88)
MyDCs: myeloid derived dendritic cells
NES: nuclear export signal-sequence
NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells
NLS: nuclear localization signal-sequence

PBGD: Porphobilinogen deaminase
PBMCs: peripheral blood mononuclear cells
PCR: polymerase chain reaction
pDCs: plasmacytoid dendritic cells
PFGE: pulsed field gel electrophoresis
PG: prostaglandin
PMNCs: polymorfonuclear cells
POMC: propiomelanocortin
PV: pityriasis vercicolor
Pz: precitpitation zone (ratio)
R848: Resiquimod
RFLP: restriction fragment length polymofism
SD: seborrheic dermititis
Sfp: sun protection factor
sp1: Specificity Protein 1
SRC1: steroid receptor coactivator-1
SSCP: Single-strand conformation polymorphism
STR-PCR: Short tandem repeat polymerase chain reaction
TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin
Tgf: Transforming growth factor
Th: T helper cell
TLR: toll like receptor
Tnf: tumor necrosis factor
UGT: coli uridine phosphorylase glycosyl transferase
UOI: University of Ioannina
UPGMA: Unweighted Pair Group Method with Arithmetic Mean
UV: ultraviolet

Introduction

The microorganisms that reside on the skin, comprising the skins' microbiota, display an impressive variety. This variety can be attributed to the fact that skin, as an ecosystem, exhibits a physiological and topographical diversity (Grice et al, 2009). The descriptive comparison of the axilla with the tropical forests and the dry forearms with the deserts (Marples, 1965) pictures this diversity.

Most of the microorganisms that comprise the skin microflora are bacteria that are classified in nineteen different phyla (Grice et al, 2009) and more than one thousand different species (Pennisi, 2008). However, regarding the fungal microflora this diversity is rather restricted. *Malassezia* is the predominant yeast species, isolated from healthy or diseased skin, especially in the anatomical areas that have ample sebaceous glands, as is the head and the trunk (Oyeka et al, 2002; Luciana et al, 2006; Zhang et al, 2011). Other fungal species are found in areas where the microenvironment is significantly different, as are the toe webs where non-dermatophytic molds are more common (49% vs 27% yeasts) (Oyeka et al, 2002).

Since *Malassezia* is the predominant eukaryote of the skin microflora (Rosenthal et al, 2011), at least unidirectional biological interactions with its multicellular organ environment, i.e. the skin, are anticipated. Thus, *Malassezia* yeasts can harvest biomolecules of human origin (lipids of sebum origin, amino acids) and assimilate them not only as an energy source but also to synthesize parts of their cellular wall (lipid cover), melanin (Mittag et al, 1995; Gaitanis et al, 2005) or biologically active substances (indoles) (Gaitanis et al, 2008).

Since the first identification of *Malassezia* species by Malassez, more than 140 years have intervened. Within this period *Malassezia* has been the focus of significant scientific interest that resulted not only to the identification of 14 species, but also to the exploration of the yeast's biological characteristics and its association with various skin diseases. *Malassezia* is implicated in the appearance of pityriasis vercicolor and regarding seborrheic dermatitis it is considered at least as an aggravator of the disease. However, to date in neither condition a conclusive pathophysiological mechanism that explains the pathogenic or triggering potential of the yeast has been established.

Aim of this work was improve the understanding regarding the input of specific *Malassezia*'s 'virulence' factors to the pathogenesis of the related human diseases and especially seborrheic dermatitis. In the present Thesis *Malassezia* strains were isolated from patients and their close relatives and were characterized to species level. Subsequently, the isolated

strains were studied *in vitro* for the production of lipolytic enzymes. Moreover, known *Malassezia*-associated bioactive indoles were studied for their effects on certain functions of human dendritic cells.

History and taxonomy

Malassezia is named after the 19th century histologist and anatomist Malassez, who in 1874 described the typical round and oval budding yeast cells in the stratum corneum of Pityriasis vercicolor (PV) patients (Asbee and Evans, 2002). However, this is not the earliest report of *Malassezia* that exhibits a history almost one and a half century long (Figure 1). Eichstedt and Robin described, in 1846 and 1853 respectively, the organism in the skin scales of PV patients (Moore, et al 1940). Nevertheless, Malassez was the one to distinguish this yeast from the agents of dermatophytic infections and in recognition of his work, Baillon proposed the name *Malassezia furfur* (1889) to describe the fungi that caused the furfuraceous nature of the lesions observed in pityriasis versicolor patients (Morrien, 1962).

The name *Malassezia* was not directly established. The term *Pityrosporum* was proposed in 1904 by Sabouraud to describe the yeast cells, without hyphal elements, isolated from healthy skin and scalp (Sabouraud, 1904). Castellani and Chalmers, who were able to consistently culture *Malassezia* yeast cells from human skin scales, also used the name *P. ovale* (Tompson and Colvin, 1970). In 1951 Gordon managed to isolate from pityriasis versicolor lesions, a spherical to oval yeast with double contour cell wall and filaments and he named the organism *P. orbiculare* (Gordon 1951).

By 1970, three *Pityrosporum* species were recognized, *P. ovale*, *P. orbiculare*, and *P. pachydermatis* (Sloof, 1970). However, most of the researchers accepted a relationship between the mycelia form, denoted as *Malassezia* and yeast form denoted as *Pityrosporum*. In 1977, three research groups showed that the phases could be interconverted (from yeast to hyphae and *vice versa*) (Salkin and Gordon 1977; Dorn and Roehnert 1977; Porro et al 1977) leading to the recognition of the dimorphism of the organism. In 1986 Cannon (Cannon, 1986) proposed the unification of the genus under the name *Malassezia,* employing *M. furfur* for the lipophilic strains and *M. pachydermatis* for the non-strictly lipophilic isolates. In 1989, Simmons and Gueho recognized *M. sympodialis*, on the basis of its lower G_C content (54% vs 66% for *M. furfur*) and the presence of sympodial budding (Simmons and Gueho, 1990). In the same period, Cunningham described 3 serovars A, B, C based on the surface antigens (Cunningham et al, 1990). In 1995 Guillot and Gueho assembled 104 isolates of *Malassezia* species encompassing all the different classifications favored by different groups and carried out sequencing of the large-subunit rRNA and nuclear DNA complementarity studies. On the basis of their results, they recognized, and

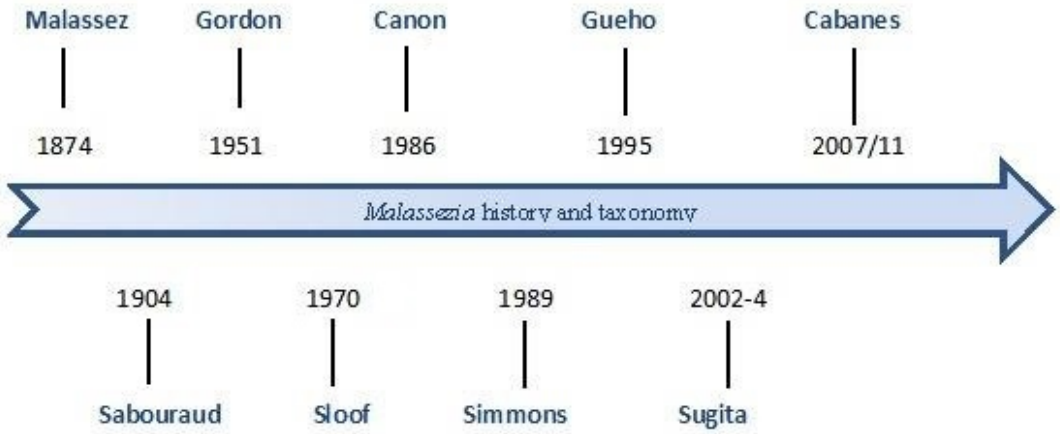
later named, seven *Malassezia* species: *M. furfur*, *M. sympodialis*, *M. obtusa*, *M. globosa*, *M. restricta*, *M. slooffiae*, and *M. pachydermatis.* Additionally, they provided a system for species identification based on the ability of distinct species to assimilate lipids (Gulliot et al, 1995; Guillot et al, 1996).

Few years later, in 2003 Sugita isolated the novel species of *M. dermatis* from the skin of patients with atopic dermatitis (Sugita et al, 2003). Species distinction was based on the analysis of r-DNA gene sequences (D1 and D2 of 26 rDNA and ITS 1 regions) and the new strains clustered with *M. sympodialis* with high bootstrap values.

The same researcher isolated, in 2004 two more new species *M. japonica* and *M. yamatoensis* (Sugita et al, 2004). Again analysis of D1/d2 of 26S rDNA and ITS1 regions was used.

Accordingly, Hirai (Hirai et al, 2004) isolated *M. nana* from the ear canals of cats and cows with and without external otitis. Additional research of the skin microflora in domestic animals led to the discovery of two more species from Cabanes in 2007. The species were named *M. caprae* and *M. equine* due to their origin (Cabanes et al, 2007).

M. cuniculi is the last identified species, isolated just in 2011. So far it has only been isolated from rabbit skin (Cabanes et al, 2011) raising the preferentially isolated from animal species to five and the total species number to 14.



Malassez, 1874	*Malassezia* was recognized as a different organism from dermatophytes
Sabouraud, 1904	Proposed the genus name *Pityrosporum*
Gordon, 1951	Yeast isolation from pityriasis versicolor lesions
Sloof, 1970	Detailed description of the genus *Pityrosporum* and recognition of 3 species (*P.ovale, P. orbiculare, P. pachydermatis*)
Canon, 1986	Proposition of the genus name *Malassezia* and description of *M. furfur* and *M. pachydermatis*
Simmons et al, 1989	Description of *M. sympodialis*
Gueho et al, 1996	Description of 4 new *Malassezia* species. Recognition of *P. orbiculare* as *M. globosa*
Sugita et al, 2002-2004	Description of 3 new species *(M. dermatis, M. japonica, M. yametoensis)*
Cabanes et al, 2007	Description of 3 new species *(M. caprae, M. equine, M. cuniculi)*

Figure 1: Key dates in the history and taxonomy of the genus *Malassezia*. From the first report by Malassez up to the recognition of the 14th species (current situation) 140 years intervened.

Biology of *Malassezia* yeast

Yeast and colony morphology

The morphology of the yeast varies between different species both at cell and colony levels. However, this variation is not enough to allow direct species identification through colony and microscopic characteristics. The **cell shape** can be spherical, like in *M. restricta* or *M. globosa*, elongated as in *M. furfur*, cylindrical in *M. pachydermatis* or *M. sloffiae* or oval in *M. yamatoensis* (Ashbee, 2007)(Figure 2). The small size of the cells, in the case of *M. nana,* gave it its species name (Hirai et al, 2004). In addition, the **budding pattern** is different between the species. *M. sympodialis* was named after its characteristic sympodial budding (Figure 2C). Moreover, *M. furfur, M. pachydermatis, M. slooffiae* and *M. obtusa* are characterized by a broader bud base, in contrast to the remaining of the species.

Malassezia species present also differences in the **macroscopic appearance** of their colonies in culture. In general, when cultured in Dixon's agar for 7 days at 32 ^{o}C, colonies have a smooth texture and a creamy color. However, they display differences as well (Ashbee et al, 2007): *M. obtusa* and *M. sympodiallis* colonies appear more flat, while those of *M. furfur* and *M. globosa* are rougher and umbonated. The colonies of *M. pachydermatis* are convex, while those of *M. slooffiae* are folded (Figure 3).

M. dermatis, *M. yamatoensis* and *M. japonica* colonies when cultivated in Leeming-Notman agar (LNA) at 32 ^{o}C for a week, appear yellowish-white, semi-shiny to dull and butyrous. Filaments sometimes are formed at the area of the origin of the bud. (Sugita et al 2003; Sugita et al, 2004)

In general all *Malassezia* species grow *in vitro* in the yeast form. However, when special media are employed, the formation of the mycelial form is also possible (Saadatzadeh et al, 2001). Enrichment of the culture medium with ammonium phosphate salts and Tween 80 (Dorn and Roehnert, 1977) or cholesteryl and glyceryl monostearate (Porro et al 1977) is needed in order to induce hyphae formation *in vitro*.

Table 1: Macroscopic and microscopic characteristics of *Malassezia* species. Despite the recorded differences, these characteristics are not sufficient for species identification according to the current taxonomy. Culture medium defines the macroscopic characteristics of the culture as well as the yeast or hyphae form.

	Colony morphology	Colony color	Cell Shape	Budding pattern	G+C content	Catalase reaction	b/glucosidase reaction	Growth at 37	Growth at 40	Tween ass imilation
M. furfur	Umbonate, usually smooth, soft, friable	Cream	Elongated, oval or spherical	Broad bud base	66.4	+	-	Good	+	20/40/ 80/CREL
M. symp/lis	Flat, smooth, shiny, soft	Cream to buff	Ovoid, globose	Sympodial budding	62.2	+	+	Good	+	40/80
M. pach/tis	Pale convex, smooth, soft, friable	Cream	Cylindrical	Broad bud base	55.6	+/-	+/-	Good	+	20/40/ 80/CREL
M. globosa	Rough, course, brittle	Cream to buff	Spherical	Narrow bud base	53.5	+	-	Poor	-	-
M. sloofiae	Finely folded, brittle	Cream to buff	Cylindrical	Broad bud base	68.7	+	-	Good	+	20/40
M. restricta	Dull, smooth, hard and brittle	Cream	Spherical	Narrow bud base	59.9	-	-	Poor	-	-
M. obtusa	Smooth, flat, sticky	Cream	Cylindrical	Broad bud base	60.7	+	+	Poor	-	-
M. dermatis	Convex	Yellow-ish white	Spherical, oval or ellipsoidal	Narrow bud base, fillaments	60,4	+	-	Good	+	20/40/ 80
M. japonica	Wrinkled and boutyrous	Yellow-ish	Spherical, oval or ellipsoidal	Sympodial budding	60,4	+		Good	+	40
M. yam/nsis	Wrinkled or folded	Yellow white	Oval to ellipsoidal	Narrow bud base		+		Good	-	20/40/ 80
M. nana	Smooth, convex	Cream to yellow	Ovoid to globose	Narrow - monopolar		+	-	Good	+/-	20/40/ 80
M. carpae	Convex	Whitish to cream-colored, smooth	ellipsoidal to subglobose	Narrow base - monopolar		+	+	Poor	-	40/60/ 80
M. equina	Convex	Whitish to cream-colored, smooth	Ovoidal	Narrow base monopolar		+	-	Poor	-	40/60/ 80
M. cuniculi	Raised-to moderately convex with entire margins	Dull white to cream	Spherical	Buds with monopolar pattern and narrow base		+	B gds+	Good	+	-

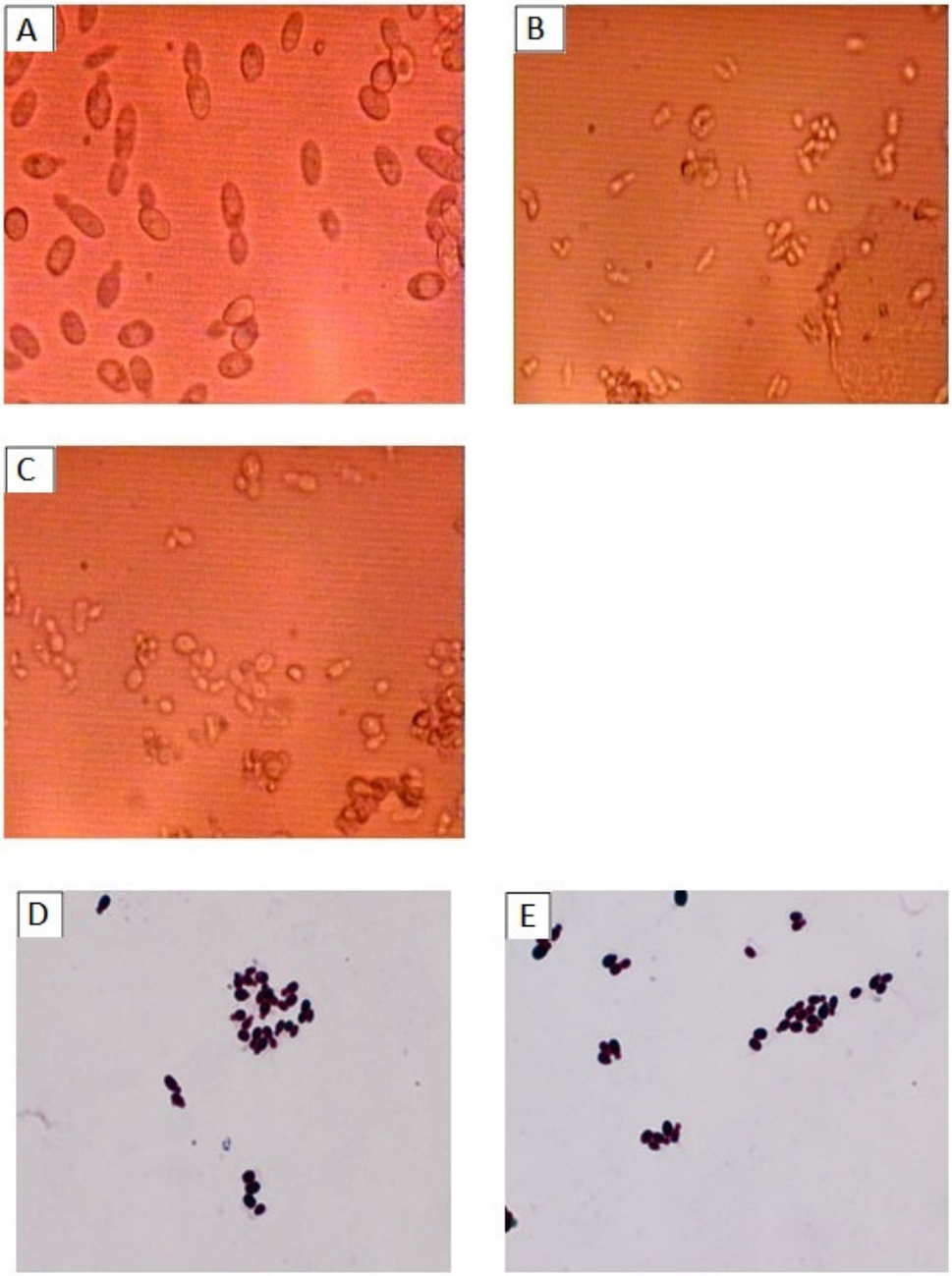


Figure 2: Examples of the microscopic appearance of selected *Malassezia* species after growth in Dixon's agar for 7 days. Panel A: *M. furfur,* Panel B: *M. pachydermatis,* Panel C: *M. sympodialis* (eosin stain x400 magnification). Panel E, F: *M. furfur* (Gram stains, x100 magnification).

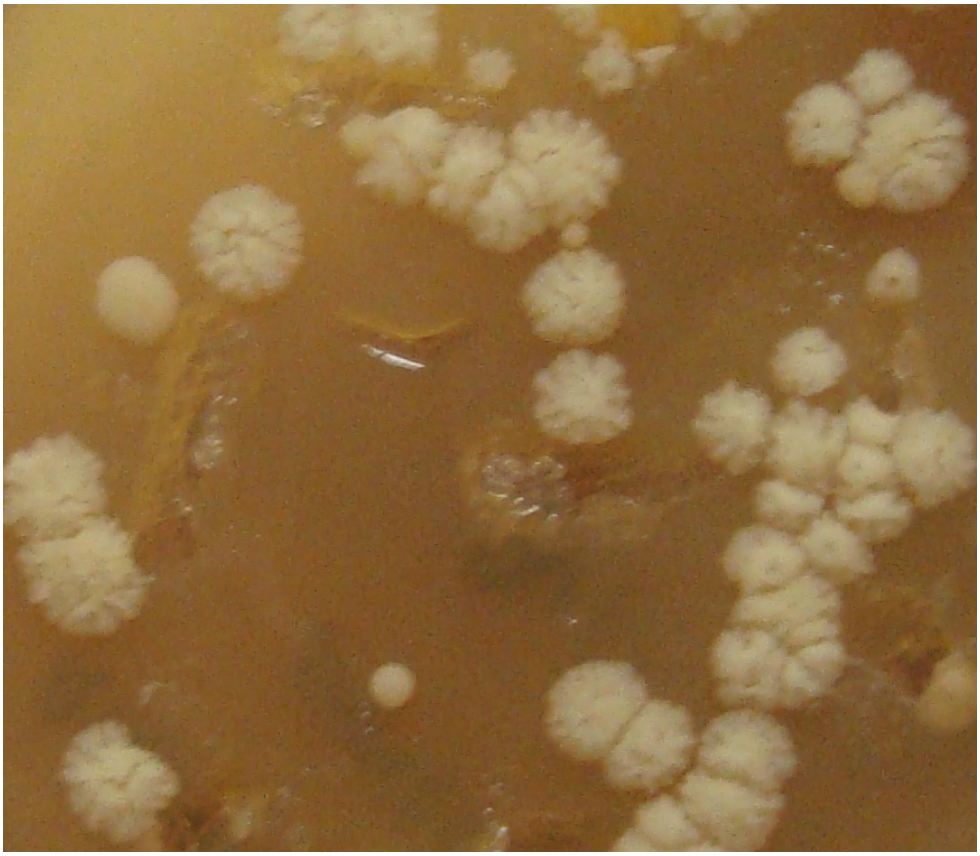


Figure 3: Stereoscopic view of *M. slooffiae* colonies on Dixon's agar after 7 days growth at 32^{o}C. The yellowish-white color, the creamy texture and the folded surface are evident.

Cell characteristics

Malassezia belongs to the group of Exobasiodiomycetes among with Tilletiales, Georgefisheriales, Microsomatales, Doassansiales, Ceraceosorales, Entylomatales, and Exobasidiales (Begerow et al, 2006). Today, it is accepted that Exobasiodiomycetes along with Entorrhizomycets and Ustilaginomycetes comprise the subphylum Ustylagomycotina. In this subphylum belong typically dimorphic yeasts (Begerow et al, 2006). The main cytomorphological characteristics of *Malassezia* are the multilaminar cell wall and the monopolar budding. Studies with transmission electron microscopy revealed that the multi-layered wall is covered by a fibrillar material (Winiarczyk S, 1992). This cell wall is thought to arise from indentations of the plasma membrane that form a spiral pattern. The configuration of the wall is the same in both the yeast form and in *Malassezia* filaments, an observation that was used to support the identical nature of *Pityrosporum* and *Malassezia* (Keddie et al, 1966). The characteristic cell wall morphology was first identified in *M. pachydermatis* but was later confirmed in additional species like *M. sympodialis* and *M. restricta* (Simmons et al, 1990; Gueho et al, 1996). The thickness of the cell wall is different from one species to another; however, it also depends on the age of the cells and the growth conditions. Among all *Malassezia* species, *M. globosa* has the most distinct cell surface morphology (Breathnach et al, 1976)). In contrast to the rest species, which are characterized by a double helicoidal cell surface, the cells of *M. globosa* present with a pattern of rather shallow grooves.

The buds arise in *Malassezia* from the inner layer of the cell wall and leave behind a distinct collarette scar- on the mother cell surface after the release of the daughter cell. Budding is usually monopolar but sympodial budding maybe be observed in *M. sympodialis* (Simmons et al, 1990).

Finally, a particular ultrastructure characteristic of this genus is the presence of endospores. This is especially obvious in *M. furfur* and could suggest a connection to the teliospore forming yeasts, as it has been suggested that the phenomenon of endosporualtion might represent an initial step towards basidium development (Mittag, 1994).

Malassezia ecology

Ten out of the fourteen *Malassezia* species (*M. furfur, M. globosa, M. symbodialis, M. restricta, M. slooffiae, M. nana, M. yamatoensis, M. obtusa, M. japonica, M. dermatis*), known to date, are found mainly in humans. These species have been isolated from the human scalp, face, chest, back, extremities but also from nails, nasal cavity and under certain circumstances from blood and urine specimens (Oliveri et al, 2011; Larocco et al, 1988). Other thermophilic animals harbor on their skin strains these 'anthropophilic' lipid-depended *Malassezia* species, but also distinct *Malassezia* species that are mostly considered zoophilic, as *M. pachydermatis* (Raabe et al, 1998). The aforementioned zoophilic species can be transient members of the human flora too (Prohic et al, 2009). The opposite is also true for 'anthropophilic' species. Thus, *M. furfur, M. sympodialis* and *M. slooffiae* have also been detected in many domestic animals like cats, dogs, pigs or horses (Crespo et al, 2000; Crespo et al, 2002; Guliot et al, 1998). Additionally, some anthropophilic species are preferentially isolated from lesional than from healthy human skin, like *M. yamatoensis* (Sugita et al, 2004).

Regarding the preferentially zoophilic isolates, there is a distinct degree of yeast species predilection for the colonization of certain animals: *M. carpae* has been isolated from goats and horses (Cabanes et al, 2007), *M. ecquina* from horses and cows (Cabanes et al, 2007) and *M. nana* from cats and cows (Bond et al 2008; Hirai et al, 2004). *M. pachydermatis,* which is the most frequent animal isolate, is also found in humans. Nevertheless, isolation of this latter species from humans is considered to reflect a rather transient colonization, probably as a result of direct yeast inoculation from animals, like pets (Chang et al, 1998).

Malassezia metabolism

Lipolytic activity of *Malassezia* and other yeasts

The lipid dependency of *Malassezia* yeasts is attributed to their deficiency in the synthesis of the myristic acid, a precursor of long chain fatty acids (C 12 or greater) (Porro et al, 1976). Meanwhile, this has been also confirmed through *M. globosa* and *M. furfur* genome analysis that revealed lack of the genes encoding fatty acid synthase (Xu et al, 2007). Nevertheless, lipid metabolism in *Malassezia* is not absent. Longer-chain fatty acids can be synthesized from intermediate/long ones and saturated can be transformed to corresponding unsaturated (Porro et al, 1976). This fact is further underlined by the diversity of the encoded lipases (N=13) and phospholipases (N=9) in *Mallasezia* genome (Xu et al, 2007). *Malassezia* yeasts do not assimilate lipids solely as an energy source but they use them also for the formation of the outer lipid layer of their cell walls. This latter need for lipid sources also expands the requirements for lipid supplementation in the culture medium of this yeast (Porro et al, 1976; Mittag, 1995).

Phospholipases mainly hydrolyze glycerophospholipids and can degrade neutral lipids (Riciputo et al, 1996). On the contrary, lipases mainly hydrolyze neutral lipids and release consequently free fatty acids, while esterases hydrolyze fatty acid esters. In most cases, triacylglycerides, free fatty acids or polyoxysorbitan fatty acid esters (i.e., Tween) have been used as substrates to support *Malassezia* growth *in vitro*. Additionally, the assimilation of cholesterol and cholesterol esters has been shown to lead in the induction of hyphae form in cultures (Porro et al, 1976).

Egg yolk assimilation method

The genome of *M. globosa* encodes 14 lipases, 13 of which are predicted to be secreted like the previously described LIP1 (Xu et al, 2007; Deangelis et al, 2007). Additionally, it encodes genes for 6 phospholipases C, 2 phospholipases D and 1 phospholipase B (Xu et al, 2007) (Figure 4A). So far, one *Malassezia* lipase secreted by *M. furfur, M. globosa, M. sympodialis* and *M. pachydermatis* has been isolated from the human scalp (Deangelis et al, 2007), while the genes of three other potential lipases that could act as virulence factors (Mgl0797, Mgl0798, and Mg13326) were shown to be highly expressed in strains isolated from HIV positive and negative SD patients (Patino-Uzcategui et al, 2011). Fermentation of egg yolk suspension for *in vitro* determination of phospholipase activity has been initially used by Habermann and Hardt for the assessment of bee *Crotalus terrificus* venom phospholipases (Habermann and Hart, 1972). Phospholipases C and B produced turbid areas in egg yolk plates, which were related to the secreted quantity of this enzyme. Phospholipase B catalyzes the cleavage of lyso-compounds to highly hydrophobic glycerophosphorylcholines and hydrophobic fatty acids. Phospholipase C releases lipophilic diglycerides and the hydrophobic phosphorylcholine (Habermann and Hart, 1972). The end result of the action of both enzymes is the increased turbity of egg yolk in the medium and the formation of a dense zone (Figure 4B). Price in 1982 (Price, 1982), introduced the egg yolk plate method for the detection of phospholipase activity in *Candida albicans* and verified the technique by correlating the results with the hydrolysis of phosphatidilocholine. Later the method was further improved (Samarayanake et al, 1984) and was used to assess the lipolytic activities of other organisms like *Cryptococcus neoformans* (Vitoddo et al, 1998; Chen et al, 1997) or other yeasts, including recently the animal isolate *M. pachydermatis* (Cafarchia et al, 2004).

The composition of egg yolk is rich in different lipids (65% triglycerides, 5% cholesterol, 30% phospholipids) (Ramesh et al, 1978) but also contains smaller quantities of other fat substances as well as proteins (phosvitin with ratio to lipids 1:2). Vitamins and xanthophylls are also present. Above composition, makes obvious that phenotypic changes in the medium can result not only from phospholipase activity but also from lipases and proteinases that are present.

Whether the dense precipitation zone that is observed in the presence of phospholipases solely reflects phospholipase activity has been questioned in the literature (Juntachai et al,

2009; Williamson et al, 1986). Nevertheless, the precipitation zone surrounding the colony growth is generally considered to result from lecithinase activity and the iridescent, «oil on water" area seen on the surface of the colonies, as the result of lipase action (AOAC international, 2000). Moreover, the amount of the secreted phospholipase can be easily quantified with the egg-yolk assimilation method using the 'precipitation zone ratio' (Pz), i.e. the ratio of the colony diameter to the diameter of precipitation zone. Linear regression analysis showed a correlation between Pz and Nmoles of phosphatidylcholine (for Pz values up to 0.75) (Price et al 1982). Conclusively, the egg yolk assimilation method provides a fast and inexpensive screening tool for the detection of secreted phospholipase activity in a yeast culture.

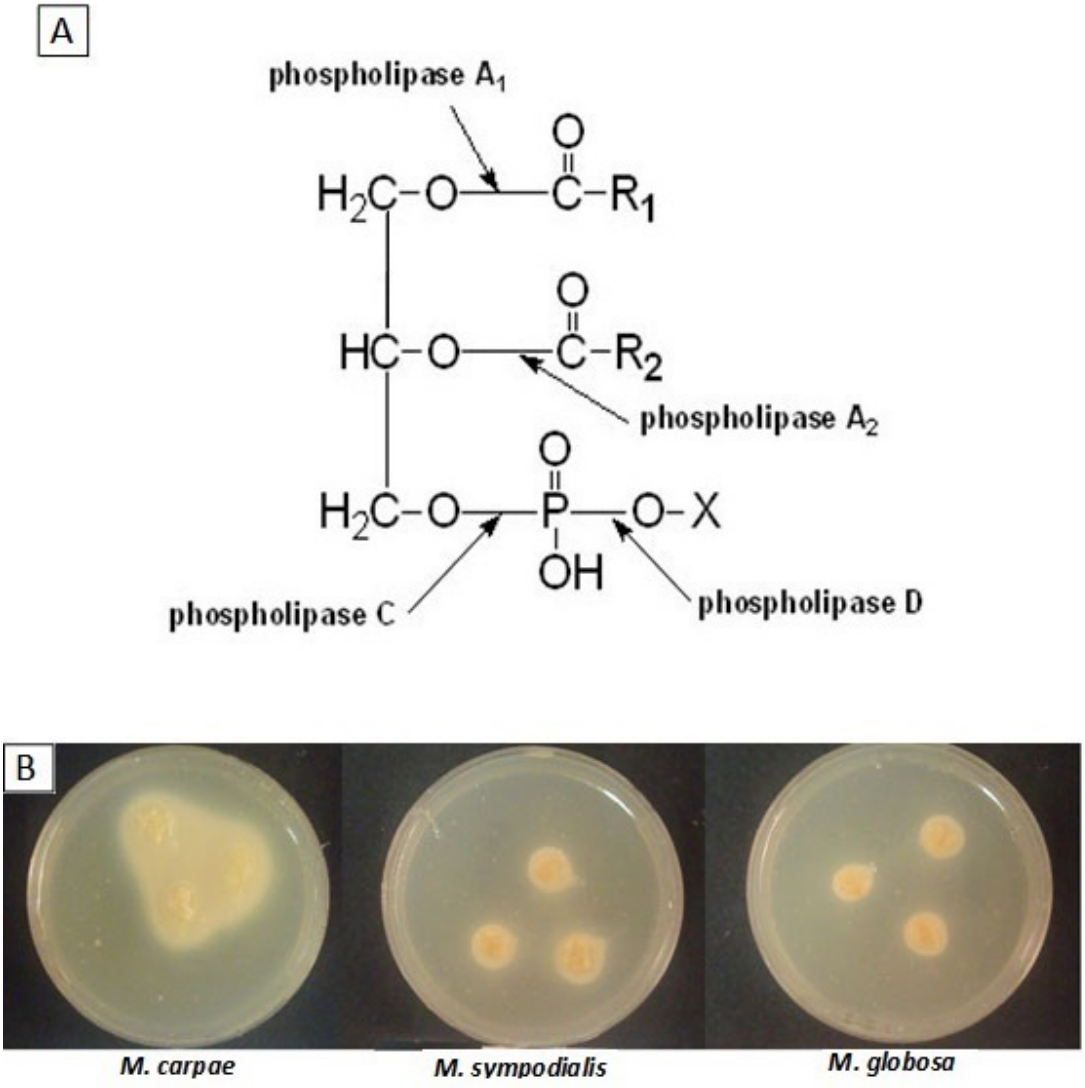


Figure 4: A) Schematic representation of the cleavage sites on phospholipids by the 4 different phospholipase families (A, B, C and D). Phospholipase B, not shown, displays both the activity of phospholipases A1 and A2. The genome of *M. globosa* encodes different phospholipases B, C and D. B) Assimilation of the egg yolk lipids gives a characteristic phenotypic result. Examples of three different *Malassezia* species are displayed.

Malassezia indoles production

Apart from lipid assimilation, *Malassezia* yeasts use additional resources for forming cell structures or acquiring energy. *M. pachydermatis* can assimilate carbohydrates like sorbitol or mannitol (Hossain et al, 2007). On the contrary, none of the other *Malassezia* species can ferment sugars, a characteristic common for basidiomycetous yeasts (Weary, 1970).

Malassezia can utilize both inorganic and organic nitrogen sources (Sloof, 1970). Mayser (Mayser et al, 1998) developed a minimal essential medium that could support the growth of *M. furfur* (only). With the use of this medium and a nitrogen auxanogram, it was proved that *Malassezia* could assimilate the essential amino acids, with the exception of cysteine. Nine additional nitrogen sources (ammonium salts, urea, creatine, creatinine, uric acid and allantoin) could also support *Malassezia* growth. When *Malassezia furfur* yeast is cultured in a medium with tryptophan as the single nitrogen source and a varying lipid source, it produces an array of indolic pigments (Gaitanis et al, 2008) (Figure 5).

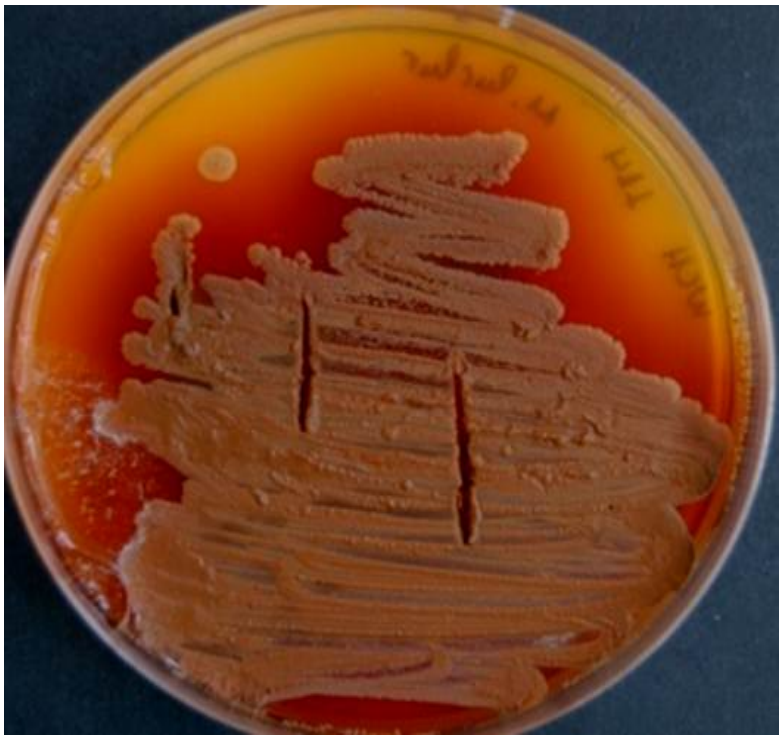


Figure 5: *Malassezia furfur* cultured in minimal growth medium with L-tryptophan as the single nitrogen source. The production of indolic compounds is responsible for the characteristic red color on the medium.

Biochemical and pharmacological characteristics of these indoles were linked to clinical characteristics of PV and SD and were correlated to the pathogenity of the yeast. More specifically the isolated and characterized indolic compounds are:

Pityriacitrin, a yellow indolic compound was identified by Mayser (Mayser et al, 2002) (Figure 5). Pityriacitrin had been shown to have UV absorbing properties in bacteria (Nagao et al, 1999). The production was proved for *M. furfur* and was confirmed that the UV protective effects of pityriacitrin are also expanded to the rest of the skin microflora, including *Candida albicans* and *Staphylococcus epidermidis* (Machowinski et al, 2006). Pityriacitrin was implicated in the causation of the hypopigment lesions in PV alba, however, the fact that the sun protection factor (spf: amount of UV radiation required to cause sunburn on skin with the sunscreen on, as a multiple of the amount required without the sunscreen- FDA, 2009) of topically applied pityriacitrin does not exceed 1.7, weakens the likeness of pityriacitrin involvement in the observed PV hypopigmentation (Gambichler et al, 2007).

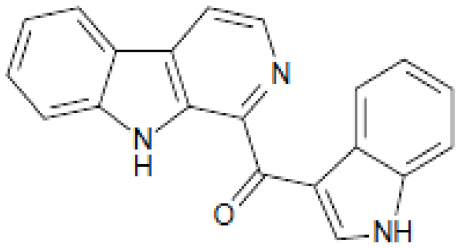


Figure 5: **Pityriacitrin**, an indolic compound with UV filtering characteristics.

Pityrialactone (Mayser et al, 2003) (Figure 6) has a pale yellow color and exhibits strong green yellow fluorescence under Wood's light. It is considered to be implicated in the fluorescence of the PV lesions under Wood's light and also to demonstrate UV protective abilities (Mayser et al, 2003).

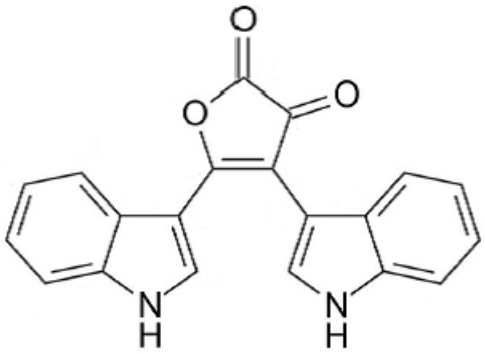


Figure 6: The molecular structure of **Pityrialactone.** This molecule is considered to participate in the fluorescence of PV lesions.

Pityriarubins have a red color and are structurally related to bis(indolyl)maleimides, which have attracted attention as protein kinase inhibitors (Davis et al, 1992). Interestingly, among the isolated pityriarubins, the novel Pityriarubins A, B and C were able to suppress the release of reactive oxygen species from lymphocytes that had been activated by calcium ionophore A23187, formyl-metleu-phe (FMLP) and interleukin 3 (Krämer et al, 2005) (Figure 7). The aforementioned properties were related to the absence of infiltrating granulocytes in the lesions of PV (Wroblewski et al, 2005).

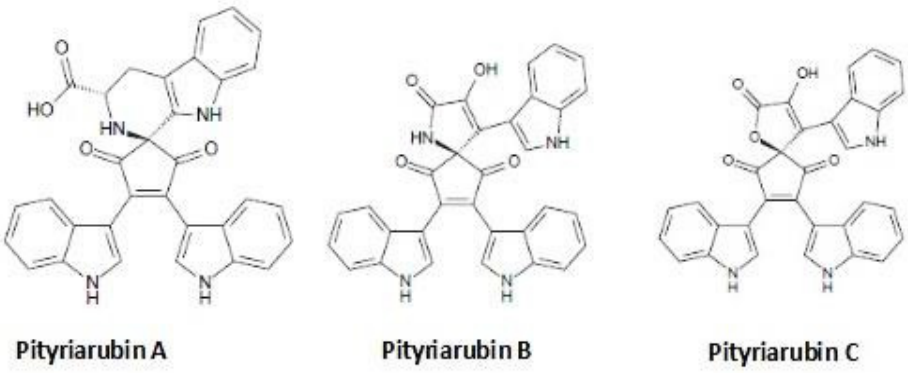


Figure 7: Molecular structure of **pityriarubins A, B, C**.

Malassezin is a colorless compound that is produced during *Malassezia* metabolism and has been shown to act as an Aryl hydrocarbon Receptor agonist (Wille et al, 2001) (Figure 8). The agonistic potency was evaluated by measuring the EROD (Ethoxyresorufin-O-deethylase)

activity. Similarly it induced cytochrome P450 (EC50 = 1.57 mM) in rat hepatocytes *in vitro* (Wille et al, 2001). Interestingly, this compound was also shown to affect cytoskeleton and cause the apoptosis in primary human melanocytes (Krammer et al, 2005). The above data could relate malassezin to PV alba lesions (Krammer et al, 2005).

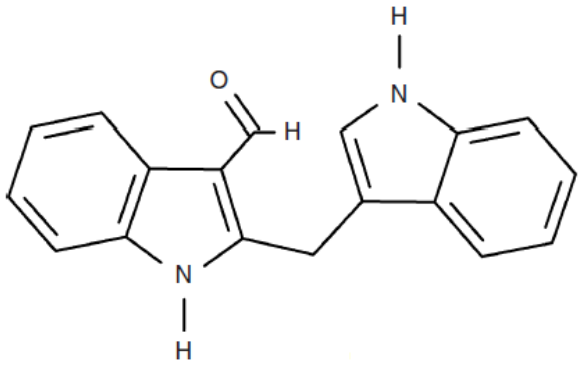


Figure 8: Molecular structure of **Malassezin.** The structural symmetry of the molecule is a common characteristic of indolic compounds

Indolo[3,2-b]carbazole (ICZ) is a colourless compound, a known potent AhR agonist, able to inhibit specific 2,3,7,8-Tetrachlorodibenzodioxin binding in rat liver cytosol (Gillner et al, 1993) (Figure 9). Mallasezin can be easily converted to ICZ (Wille et al, 2001). It has been demonstrated that the amount of ICZ produced by seborrheic dermatitis (SD) *M. furfur* isolates *in vitro* is higher than that produced from strains isolated from healthy skin (Gaitanis et al, 2008). Finally, it has been also shown that ICZ causes significant upregulation to the xenobiotic enzymes in HaCaT cell line (Magiatis et al, 2013).

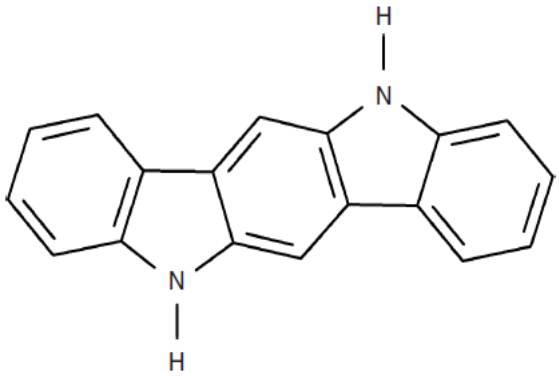


Figure 9: Molecular structure of **Indolo[3,2-b]carbazole (ICZ)**

Indirubin is the active compound of indigo (Hoessel et al, 1999), and responsible for the respective color (Figure 10). Indirubin is known as a protein kinase inhibitor (Damiens et al, 2000) and anti-cancer agent (Aggarwal et al, 2006). Moreover, it has been proposed as an endogenous ligand for AhR (Prochazkova et al, 2011). Recently indirubin was shown to be produced from *Malassezia* strains (Gaitanis et al, 2011) and to upregulate P450 activity in HaCaT cells (Magiatis et al, 2011).

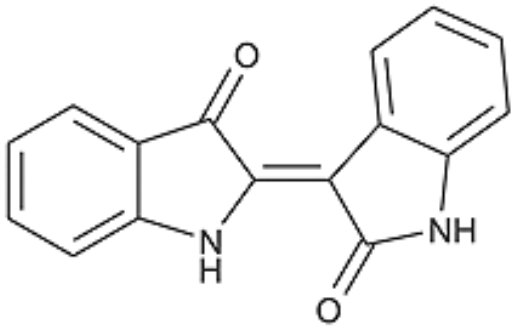


Figure 10: Molecular structure of **Indirubin.** The planar configuration of the molecule facilitates the interaction with cyclin depended kinases.

Formylo-indolo[3,2b]carbazole (FICZ) is known as a photoproduct of tryptophan (Wei et al, 1998) and a potent AhR ligand and Xenobiotic responsive element (XRE) inducer (Wincet et al, 2009) (Figure 11). The ability of this compound to induce xenobiotic metabolism enzymes transcription has been exhibited in various cell lines such as keratinocytes and hepatocytes (Wei et al, 1998; Diani-Moore et al, 2006). Moreover, it has also been proposed as the principal endogenous ligand of AhR (Wincet et al, 2009). It was recently demonstrated that this compound can be also synthesized by *Malassezia* strains (Magiatis et al, 2013). FICZ has been also shown to have functional and phenotypic effects on inflammatory dendritic cells (Bankoti et al, 2010) and to mediate DNA adduct formation in UVR exposed keratinocytes (Nair et al, 2009).

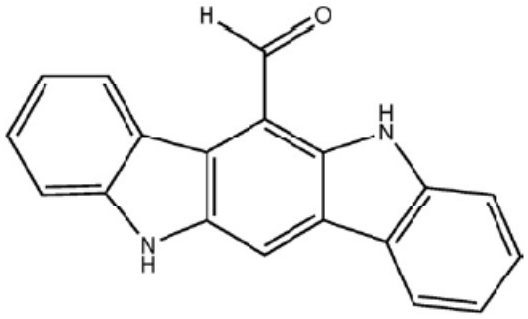


Figure 11: Molecular structure **of Formylo-indolo[3,2b]carbazole (FICZ).**

Identification methods

Conventional methods

Although *Malassezia* species can, to some degree, be distinguished through microscopic observation, the different biochemical properties and metabolic abilities of the strains provide a much more useful tool for identification and taxonomy.

The first successful culture of this fungus was performed from Panja in 1927 (Panja, 1927). Culture in 'selective' media has been used for *Malassezia* identification and species discrimination. Due to the lipophilic characteristics of this genus (Benham, 1939; Guillot, 2008), an array of culture media supplemented with various lipid sources has been employed (Table 2). Growth on Sabouraud's agar is used for the detection of *M. pachydermatis*, while supplementation of this medium with sterile olive oil was used for the cultivation of other *Malassezia* species (Nyirjesy et al, 1994). Other selective media, such as Dixon's (Gueho et al , 1986), Leeming Notman agar (Leeming et al, 1987) and Ushijima's medium (Ushijima et al 1981), were also developed and employed successfully, increasing the growth rate, the viability and the overall isolation rate of this yeast. Kaneko (Kaneko et al, 2005) developed a methodology for the differentiation of seven *Malassezia* species (*M. furfur, M. sympodialis, M. globosa, M. obtusa, M. slooffiae, M. restricta* and *M. pachydermatis)* according to the morphological characteristics of the colonies using a modified CHROM-agar medium, originally applied for *Candida* cultivation. The same research group further revised this identification system and succeeded in the differentiation of nine *Malassezia* species (the aforementioned seven plus *M. dermatis* and *M. japonica*). The new method was based on an algorithm that included different 'parameters' as the utilization of *Malassezia* CHROMagar, Tween 40 precipitate production, catalase reaction and absence/presence of growth in Sabouraud dextrose agar, Cremophor EL agar, Tween 60-esculin agar (Kaneko et al , 2006; Kaneko et al, 2007).

The ability of certain species to produce indole pigments, when cultured in a new minimal medium containing only tryptophan as the sole nitrogen source, was suggested as a method for the identification of *M. furfur*, as at that time only this species was thought to have this capacity *(*Mayser et al, 1998*).* However, more recent data (Magiatis et al, 2013) demonstrates that this metabolic trait is present in additional *Malassezia* species.

Despite the indisputable contribution of these methods the constantly expanding number of species, 14 so far, underlines the need for a molecular approach in yeast species identification.

Table 2: Selective media used for the cultivation of *Malassezia* yeast (Gueho et al, 1996; Leeming et al, 1987; Ushijima et al, 1985; Cafarchia et al, 2011)

***Malassezia* culture media**	
Medium	Composition(gr or ml/l of distilled water)
Dixon's agar	36 gr malt extract, 6 gr peptone, 20 gr bile, 10 ml Tween 40, 2 ml Glycerol, 2ml oleic acid, 12 gr agar noble
Leeming-Notman agar	10 gr peptone, 5 gr glucose, 0,1 gr yeast extract, 4 gr bile, 1 ml glycerol, 0,5 ml glycerol monostearate, 0,5 ml Tween 60, 10 ml milk, 12 gr agar
Ushijima's agar	10 gr trypticase petone , 5 gr yeast extract, 3 gr glucose, 2 gr NaCl, 12 gr KH2 PO4 (anhydrous), 15 gr agar, 0.1 gr ampicillin, 0,25 gr cyclohexamide, ph 5,5
Modified CHROMagar *Candida*	47,5 gr CHROMagar *Candida,* 8 gr ox-bile, 1 ml glycerol monosterate, 0,5 ml Tween 60
Modified CHROMagar	56,3 gr CHROMagar basal medium, 10 ml Tween 40

MOLECULAR METHODS

In the late 90s conventional methods for *Malassezia* identification were complemented by relevant molecular methods that significantly improved initially identification and further typing of *Malassezia* species. The main approaches in the molecular identification and typing can be distinguished in two categories: a) methods that detect sequencing variations (targeted PCR amplification of selected sequences) and b) methods that selectively amplify polymorphic DNA markers (fingerprinting methods i.e. mini-satellites).

DNA sequencing

The efficiency and accuracy of the PCR identification methods depends on the choice of a suitable gene 'target' region. The target locus should differ enough in sequence to allow the differentiation of species but also to display no or minor variation within the same species (unless subtype distinction is intended). The mainly used DNA domains include the ribosomal DNA chitin synthase 2 gene and RNA polymerase subunit 1 genes. The first and second internal transcribed spacers (ITS-1 and ITS-2), the first intergenic spacers (IGS), D domains (D1, D2) and the large subunit of the nuclear ribosomic DNA provide reliable genetic markers for species differentiation. **ITS-1** was used for the identification of seven species (Makimura et al, 2000) and also for subtype differentiation within the same species (Gaitanis et al, 2006). Makimura (Makimura et al, 2000) used sequencing of the ITS 1 region to differentiate among seven *Malassezia* species and to record the existence of subgroups within *M. furfur, M. sympodialis, M. slooffiae* and *M. pachydermatis*. **Sequencing analysis** of multiple rDNA regions (D1, D2 and ITS1) resulted in the identification of novel species such as *M. japonica, M. carpae* or *M. equina* (Cabanes et al, 2007; Sugita et al, 2004). **Sequence analysis** of **IGS-1** has not been broadly engaged in species taxonomy, but was employed to classify *M. globosa* and *M. restricta* variants according to the isolation rate in seborrheic dermatitis, atopic dermatitis or healthy skin (Tajima et al, 2008; Sugita et al, 2001). Similarly, variations within the IGS-1 region of *M. globosa* and *M. restricta* have been associated with atopic dermatitis patients or healthy subjects (Sugita et al, 2003; Sugita et al,

2004). Sequencing analysis of the chs-2 gene has been also used for the identification of seven *Malassezia* species (Aizawa et al, 2001). Moreover the use of chs-2 revealed multiple genotypes of *M. pachydermatis*, some of which were correlated with skin lesions (Cafarchia et al, 2008).

PCR–based restriction fragment length polymorphism **(PCR- RFLP)** in **ITS-1** and **ITS-2** regions was used for the differentiation of *Malassezia* species (Gaitanis et al, 2006). Application of PCR-RFLP in a different region (26s rDNA) with the use of various restrictions enzymes led to differentiation of seven, nine and finally eleven species (Oh et al, 2009).

PCR-based single strand conformation polymorphism **(PCR-SSCP)** analysis can differentiate 3 genotypes in *M. pachydermatis*, when applied in **chs-2** and **ITS 1 regions** (Cafarchia et al, 2007). In the same way, PCR-SSCP differentiated 5 variants of *M. globosa*, one of which was associated with extensive seborrheic dermatitis, in contrast to *M. sympodialis* that displayed a homogenous PCR-SSCP profile.

Real time PCR has been used in the diagnosis of *Malassezia* infections. In that way, it is now possible, to rapidly attest *Malassezia* rRNA gene amplifications products from DNA extracted directly from skin scales (Paulino et al, 2008) improving previously developed techniques (Gaitanis et al, 2002). Nested PCR using primers for the rRNA gene was used to quantify *Malassezia* species from skin samples isolated from healthy students and patients with psoriasis, atopic dermatitis or seborrheic dermatitis (Tajima et al, 2008). These techniques revealed that *M. restricta* and *M. globosa* are the most prevalent skin species in both healthy subjects and patients with psoriasis, atopic dermatitis and pityriasis versicolor (Sugita et al, 2008; Takahata et al, 2007).

The recent whole genome sequencing of *M. globosa* and *M. resticta* revealed many metabolic pathways that concern glycolysis, amino and nucleic synthesis or lipases synthesis that allow them to adapt to the mammalian skin environment (Dwason, 2007).

Amplification of polymorphic DNA markers

Fingerprinting methods have been used for genomic variation screening of *Malassezia* without prior sequence information (Cafarchia et al, 2011). For example PCR random amplification of polymorphic DNA was used from Gandra (Gandra et al, 2006) in order to analyze genotypes of strains that were isolated from healthy, pityriasis versicolor and seborrheic dermatitis patients. Also in a different perspective, Gaitanis (Gaitanis et al, 2009) used this methodology to associate *Malassezia* species with the geographic origin of the host. Amplified fragment length polymorphism (AFLP) was also used for analysis of *Malassezia* subtypes (Gutpa et al, 2007). Denaturing gradient gel electrophoresis (DGGE) and pulsed field gel electrophoresis (PFGE) (Boekhout et al, 1998; Theelen et al, 2001) have been employed for the fingerprinting of *Malassezia* spp. based on the analysis of PCR-amplified small subunit (SSU) of the nuclear ribosomal RNA (rRNA) gene and chromosomal DNA respectively.

As mentioned, molecular typing led to the discrimination of *Malassezia* species subtypes and their association with various clinical states. In that direction, *M. globosa*, which is the most common isolate from pityriasis versicolor, seborrheic dermatitis and healthy skin, has been sufficiently differentiated into subgroups by nested PCR amplification of DNA extracted from clinical material (skin scales) (Sugita et al, 2003). Assessment of the IGS 1 sequences indicated that certain *M. globosa* strains may display a certain degree of preference for atopic skin. Association of certain IGS 1 sequences with virulence was also observed in *M. restricta.*

Skin diseases associated with *Malassezia* species

Pityriasis versicolor

Pityriasis versicolor (PV) is a frequent skin disorder affecting 2-8% of the general population. The disease exhibits significant geographic variation, from 1% in Sweden to almost 40% in tropical climates (Gupta et al, 2004; Krisanty et al, 2009). PV is characterized by round to oval lesions, approximately 3-5 mm, which can become confluent. The macules are hypo- or hyperpigmented and mostly present on the trunk or the central part of the extremities, while they are accompanied by mild scaling (Figures 12-13).

Although the disease has been reported in infants and children (Jena et al, 2005), it is more prevalent in adolescents, concurring with the increased activity of the sebaceous glands and *Malassezia* abundance (Difonzo et al, 2008). Geographic variations are not only observed in the frequency of the disease but also in the severity. In tropical and temperate climates pityriasis versicolor can be more severe, expanding in other areas of the body as the face or the genitals (Kaur et al, 1996; Dominguez-Soto et al, 1994).

The pathophysiological mechanism of PV is related to the fact that *Malassezia*, which is part of the normal microflora, changes from yeast to mycelia form and thus theoretically invades the stratum corneum (Gupta et al, 2003). Accordingly, the diagnosis is based on the microscopical observation of the presence of both states of the fungi. The specimens are stained with Parker-KOH, Albert solution (Gupta et al, 2001) or newer stains (etc. 1% Chicago sky blue 6B and 8% KOH) and the observed microscopical image is described as “spaghetti and meatballs”, i.e. clusters of yeast cells and short hyphae (Lim et al, 2008).

In addition to the microscopic examination, Wood’s light may be used and pityriasis versicolor lesions may fluorescence a distinctive bright yellow or gold color. The characteristic fluorescence color may also aid in the differential diagnosis, as it is unique to the mycelial form of *Malassezia*. However, a positive Wood’s light examination test is seen in only one third of the cases, while in some positive cases the microscopic confirmation of the yeast is negative (Savin et al, 1996). Recently, (Mayser et al, 2003) a connection was made between the aforementioned fluorescence and the metabolic ability of *Malassezia* to produce indoles. This connection may explain why sometimes the fluorescence is observed without detection of the yeast in the lesions.

A causative association between the load of *Malassezia* yeasts and the development of lesions has been proposed. It has been reported by some authors that the number of yeast and hyphae in the lesions of pityriasis versicolor is greater than in normal skin (Faergemman et al, 1979) whereas others have shown that this difference is not significant (Stein et al, 1983).

Most commonly isolated species from PV lessions is *M. globosa* followed by *M. furfur* or *M. sympodialis* (Table 3). The rates of the cultured species varies among researchers, probably due to factors like sampling technique, isolation medium, and geographical variations in the distribution of *Malassezia* species. However, it should be noted that direct microscopy and the observation of the mycelial form of the yeast has diagnostic value. Culture of any *Malassezia* species from superficial lesions can not be considered diagnostic of pityriasis versicolor as this yeast is also found as a normal commensal on the skin.

Table 3: Commonly isolated *Malassezia* species from pityriasis versicolor patients.

Nakabayashi et al, 2000	55% *M. globosa*	
Erchiga et al, 2000	97% *M. globosa*	29% *M.sympodialis*
Gupta et al, 2001	59% *M. sympodialis*	25% *M. globosa*
Aspiroz et al, 2001	90% *M. globosa*	41% *M. sympodialis*
Tarazooie et al, 2004	53% *M. globosa*	25% *M.furfur*
Morishita et al, 2005	94% *M. globosa*	94% *M. restricta*
Erchiga et al, 2006	99% *M. globosa*	
Karakas et al, 2009	47,7% *M. globosa*	36,4% *M.furfur*
Rasi et al, 2010	31,3% *M. globosa*	20,4% *M.furfur*

Various theories have been proposed for the pathogenetic mechanism that underlies the appearance of PV macules. Karaoui et al. concluded from ultrastructural examination of different PV lesions (Karaoui et al, 1981) that hyperpigmented skin has thicker stratum corneum than hypopigmented, but both are thicker than uninvolved skin. In hypopigmented skin the melanosomes were individually dispersed and fewer in number than in the uninvolved skin. In hyperpigmented skin, the melanosomes were sequestered in most cells suggesting that melanosome transfer to keratinocytes was hindered (Karaoui et al, 1981). Additionally, *Malassezia's* lipolytic activity produces metabolites like azelaic acid. This

compound inhibits the action of the enzyme dopa-tyrosinase, which blocks the polymerization of tyrosine precursors to melanin and results in the appearance of hypochromic spots. The histopathologic study of the skin in these areas shows the presence of melanosomes that are smaller than those found in normal skin (Luis et al 2010). Pityriacitrin, an indole metabolite of *Malassezia,* exhibits UV filtering characteristics prohibiting melanocytes' activation (Gampichler et al, 2007; Machowinski et al, 2006), while malassezin induces apoptosis in human melanocytes (Krammer et al, 2005).

Despite the significant fungal load, skin lesions in PV do not demonstrate inflammation, especially in the hypopigmented form of the disease. Thus, in histopathologic sections of PV lesions there was minimal inflammatory infiltrate as measured by the neutrophil granulocyte count despite the impressive fungal load (Wroblewski et al, 2005). A reduced immunogenity is hypothetically accredited partly to the lipid content of the fungal membrane (Thomas et al, 2008) and partly to the indole derivatives that appear to have immunosuppressive properties.

Although PV lesions are not inflammatory, the presence of large quantities of yeasts and their metabolites probably induce fine desquamation of the skin, which is present in all cases (Gupta et al, 2004). Until now, no studies relating metabolites of this fungus to epidermis desquamation rates have been published. We also do not know why *Malassezia* spp stimulate melanin production in the dark patches in the hyperchromic variety of PV (Mendez-Tovar, 2010). The histologic studies of hyperchromic spots have only shown melanosomes with a larger-than-usual diameter and presence more abundant in contrast to healthy skin.

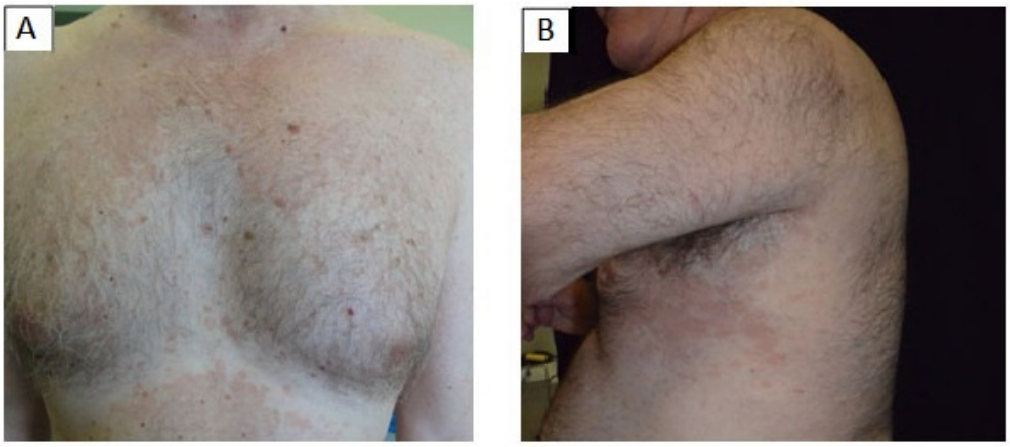


Figure 12: Panel A and B) A patient with the characteristc hyperpigmented lessions of pityriasis versicolor. The absence of clinically evident erythema signifies the minimal inflammatory reaction.

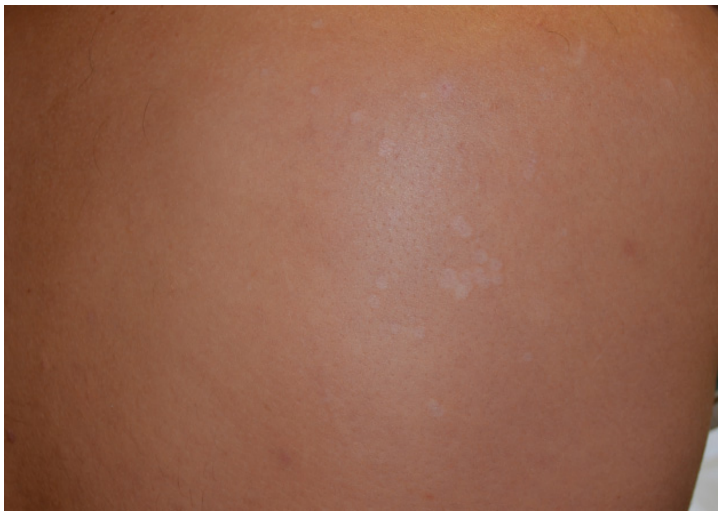


Figure 13: Hypopigmented form of pityriasis versicolor. Hypopigmented lesions are more evident when the neighboring healthy skin darkens after UV exposure.

Seborrheic dermatitis and Dandruff

Seborrheic dermatitis (SD) is a common skin disorder that affects about 1-5% of the general population (Gupta et al, 2004). SD is a characterized by the development of erythematous patches with yellow gray scales (Gaitanis et al, 2012). Appearance is more common on the face, scalp, the upper chest and back. Dandruff is usually considered as a milder variant of SD that is limited in flaking scales on the scalp, although the term has been used to describe all origins of scalp flaking (Pierard et al, 2000). Dandruff is very common affecting up to 45% of the population (Gupta et al, 2004). SD incidence displays two age peaks, one in infancy and a second one around the fourth decade of life. It is more common in men. The condition appears often in infants with prevalence of 70% and usually between the 3rd and 12th month of life (Chatproedprai et al, 2008). On the contrary, in adulthood the disease is characterized by a chronic-relapsing course. SD is also more frequent in patients with AIDS (20-83%) (Schechtman et al, 1995), with Parkinson's disease (Marco-Llorente et al, 2002), patients that receive psychotropic drugs like lithium or chlorpromazine (Binder et al, 1984; Lambert et al, 1984) and also patients with trisomy 21 (Ercis et al, 1996) and amyloidosis (Rocha et al, 2005). Interestingly SD occurrence is often co-incident with other skin diseases, like atopic dermatitis (Gupta et al, 2004).

The development of seborrheic dermatitis is usually gradual but might appear abruptly in AIDS patients. In the face it usually affects the lateral sides of the nose, nasolabial folds, eyebrows and forehead hair boundaries (Figure 14). The disease tends to be influenced by season changes. The lesions worsen during winter, whereas sunlight seems to improve the clinical appearance of the disease (Maietta et al, 1991).

Although the pathogenesis of SD is not fully understood, there is evidence that point towards a causative association with the *Malassezia* yeasts. After few years of ambiguity, the relationship between *Malassezia* and SD was re-established in the 80's, when it was shown that the main common property of all substances used in the treatment of SD was their antifungal activity (Shuster, 1984). *Malassezia* load on diseased skin is not necessarily larger, when compared to healthy skin, as studies on this show contradictory results (Sandstrom et al, 2005; Zaidi et al, 2001). However, with anti-fungal treatment *Malassezia* population size is reduced and the SD also improves (Gupta et al, 2004). DNA typing and metabolomics have co-related *Malassezia* subtypes with SD. The most commonly isolated

species appears to be *M. globosa* (Table 4). Tajima (Tajima et al, 2008) has shown that a SD skin harbors distinct *M. globosa* subtypes as compared to healthy skin.

It was initially observed that *Malassezia* lipases could hydrolyze sebum and trigger SD in susceptible individuals (Dawson et al, 2007). This was recently confirmed by the *in vivo* observation of increased *Malassezia* lipase RNA in SD lesions (Pini et al, 2011). Fatty acids resulting from the lipolysis of sebum triglycerides are considered one of the main causative agents of SD. Oleic acid that results from the hydrolysis of sebum triglycerides by *Malassezia* lipases, can induce desquamation in dandruff subjects, but not in non-dandruff susceptible individuals (Deangelis et al, 2005). However no differences in IgM or IgG levels against yeast surface or extracted proteins between healthy subjects and SD patients could be observed (Parry and Sharpe, 1998), while in lesions of SD an irritant stimulation of the immune system was observed with both Th1 and Th2 cells (Faergemann et al, 2001).

M. furfur strains isolated from SD skin synthesizes significantly higher quantities of bioactive indolic substances as compared to healthy skin isolates (Gaitanis et al, 2008). The in vivo existence of these indoles has been recently demonstrated (Magiatis et al, 2013) and further supports the role of these yeasts in the pathogenesis of SD.

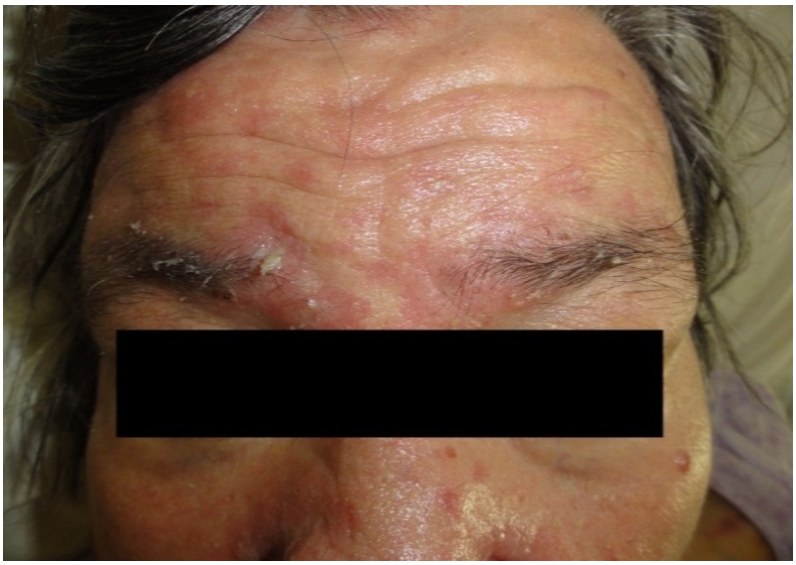
Table 4: Various research groups compared the characteristics (yeast load-yeast species) of the *Malassezia* population between healthy individuals and lesional or healthy skin of SD patients. In some reports the isolation was made through swab application and following cultivation while in others direct counting was applied. In general, as in PV, *M. globosa* appears as the predominate species in SD lesions.

Reference	Patient groups and anatomical areas sampled	Fungal load measurement	Outcome
Heng et al, 1990	Sampling of facial skin of SD patients	Culture in Dixon's agar-measurement of CFU*	Positive correlation between clinical severity of SD and *Malassezia* load
Wikler et al, 1992	4 groups: SD with HIV, SD without HIV, HIV without SD, healthy Sampling of face, chest ,back	Fungal load measurment after the cultivation in Dixon's agar	In HIV seronegative patients a larger load of the yeast was cultivated
Ashbee et al, 1993	Sampling from chest , back, face, from SD patients or healthy controls	Fungal load measurment after the cultivation in Dixon's agar	No significant variations between the load of *M. furfur* at a given site in patients and corresponding site in controls
Schechtman et al, 1995	Sampling from lesional skin of SD patients, HIV seropositive or seronegative	1)Direct counting from Sellotape-stripped skin 2) measurement after culture using contact plates	A trend between numbers of yeasts present on lessional skin, severity of SD and CD4-positive T lymphocytes count in HIV-positive patients was noted. No quantitative differences were observed between HIV-related and non-HIV related SD
Tolleson et al, 1997	Sampling from head and chest of infants with SD before and after the cure of disease	Fungal load measurment after cultivation in Dixon's agar	No difference in *M. furfur* load
Pachere et al, 1999	Forehead samples of patients grouped by SD state and HIV positivity	Fungal load measurment after cultivation in Dixon's agar	SD and HIV positive patients exhibited a higher fungal load
Nakabayashi et al, 2000	Sampling form lessional or non lessional skin of atopic dermatitis, SD and PV patients or healthy controls	Fungal load measurment after cultivation in Dixon's agar	*M. furfur* (35%) and *M. globosa* (22%) were isolated from lessional skin on the face at significantly higher rates in co-mparison to non lessional skin

Reference	Patient groups and anatomical areas sampled	Fungal load measurement	Outcome
Nakabayashi et al, 2001	Samples from the face of SD and healthy control infants	Direct microscopic counting	Yeast cells were more numerous in patients with infant SD (ISD). *M. furfur* and *M. globosa* were isolated from ISD patients at significantly higher rates
Zaidi et al, 2002	Scalp flakes from Dandruff patients and healthy controls	Microscopicall examination	*Malassezia* load increases in proportion with the severity of seborrheic dermatitis
Rendic et al, 2003	Scrapings from face lessional skin of SD patients and healthy controls	Microscopical exanimation for load estimation and culture for species distinction)	*Malassezia* was not more common in SD patients than in healthy controls, however there was a correlation of yeasts load with the clinical severity
Sandstrom et al, 2005	Sampling from lessional and non lessional skin of SD, AD patients and healthy controls	Fungal load measurment after cultivation in Dixon's agar	No significant differences in *Malassezia* load between SD lessions and healthy skin
Rincon et al, 2005	Samples were isolated from SD , AD and PV patients, from lessional and non lessional skin	Fungal Load measurment and species identification after cultivation in Dixon's agar	*Malassezia globosa* was involved at higher frequency in patients with dermatological pathologies, suggesting a higher pathogenicity of this species
Prohic-Kasumagik, 2009	Sampling included skin scrapings from SD, PV, PS and healthy individuals in order to compare *M. pachydermatis* prevalence	Cultures were made in Sabouraud agar and modified Dixon's agar	*M. pachydermatis* was recovered only from one SD patient

Reference	Patient groups and anatomical areas sampled	Fungal load measurement	Outcome
Oh Bh et al, 2010	Foreheads, cheeks and chests of SD patients and healthy individuals were sampled	Fungal load measurment after cultivation in Dixon's	None significant difference in *Malassezia* load between SD and healthy skin was found
Lee et al, 2011	Scalp scales from SD patients and healthy individuals were sampled	Direct analysis of the scales with 26 r DNA PCR-RLFP	*M. restricta* was more commonly identified in SD patients compared to *M. globosa* in control group

A



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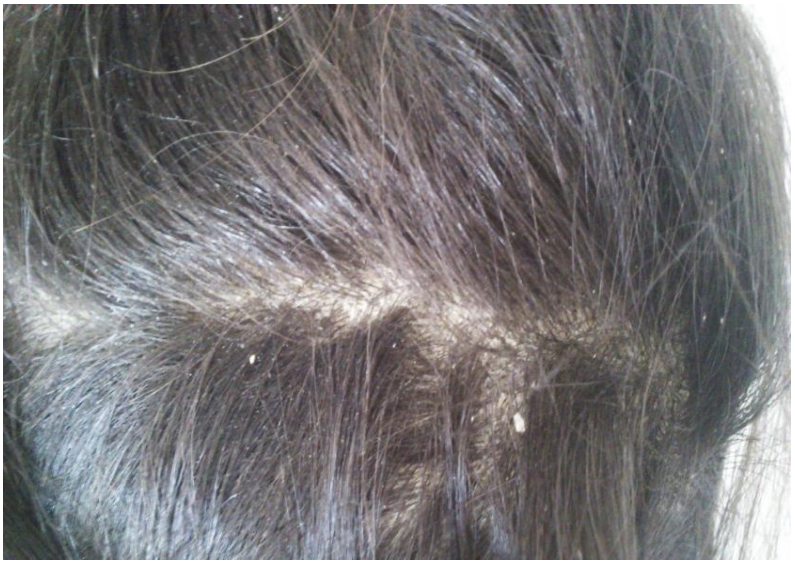


Figure 14: Panel A) Patient with severe seborrheic dermatitis. Predilection for certain skin areas rich in sebaceous glands. Panel B) Typical scalp scaling in a patient with dandruff.

Folliculitis

Malassezia folliculitis is a skin disorder characterized by erythematous papules and sometimes pustules (Figure 15), which may be accompanied by pruritus and local skin irritation (Gaitanis et al, 2012). Moreover, in many cases nodules and cysts have been described (Gupta et al, 2004). The diagnosis is based on the clinical picture in combination with direct microscopy, culture and histopathological findings and the effect of antimycotic treatment (Difonzo et al, 2008). The disorder is more common in immunosuppressed individuals like transplanted, diabetes mellitus patients or in patients receiving antibiotics (Archer-Dubon et al, 1999; Tragiannidis et al, 2010). At histological level, a typical dilated follicle containing abundant round budding yeast cells and sometimes hyphae is the typical finding. Other micro-organisms are usually absent (Back et al, 1995).

Like PV and SD, folliculitis is usually present oin the back, chest and the upper part of the arms. In tropical countries the disease is sometimes described as more severe with involvement of the face (Gupta et al, 2004).

At histological level, the inflammatory infiltrate in *Malassezia* folliculitis consists of lymphocytes (anti leu-3 reactive), histiocytes, and neutrophils (Faergemann et al, 1986; Sina et al, 1995). The infiltration appears in hair follicles and is accompanied by focal rupture of the follicular epithelium (Sina et al, 1995). The inflammatory cell infiltrate is more dense in folliculitis than in PV, however the type of the cells remains the same. The distribution of the cell infiltrate is primarily perifollicular and a high number of yeasts are often seen within the follicle, even in the deeper parts. Type I hypersensitivity was tested, as a possible mechanism of *Malassezia* folliculitis by application of *Malassezia* extracts in prick test (Faergermann et al, 1986). The negative results for the majority of the patients excluded the aforementioned hypothesis. With the use of indirect immunofluorescence a higher titer of circulating antibodies were found in *Malassezia* folliculitis patients, when compared to PV patients or healthy individuals (Faergemann et al, 1983). A possible explanation could be the infiltration of *Malassezia* in deeper skin structures, as the hair follicles, in the case of folliculitis. Interestingly it has been shown that folliculitis can develop in the skin of patients with positive folliculitis history when *Malassezia* yeasts are applied under occlusion. However this does not happen in SD patients or healthy individuals (Goodfield et al, 1987). This observation underlines the importance of the host factors in the pathogenesis of this condition.

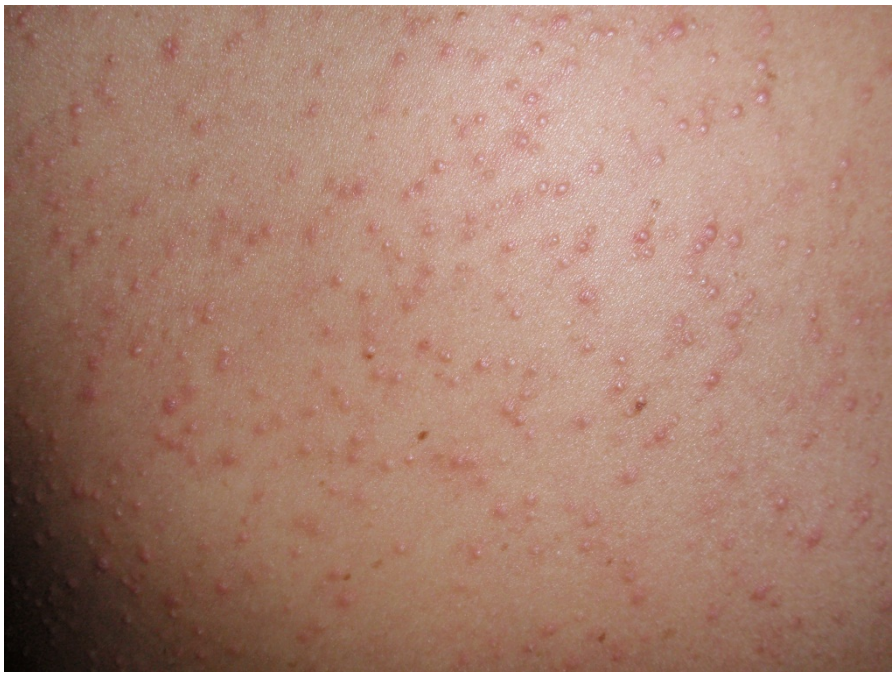


Figure 15: *Malassezia* folliculitis. The differentiation from other clinical entities that cause similar acneiform eruptions should be based on microscopy and culture of the infectious agent.

Atopic dermatitis

Atopic dermatitis (AD) is a common skin disease that has a wide spectrum of clinical manifestations. Different criteria-instruments have been employed for AD diagnosis, however the most accepted and validated are those proposed by Hanifin and Rajka (Hanifin and Rajka, 1980) and from the UK AD working group (Brenninkmeijer et al, 2008). For the diagnosis of AD according to the first instrument at least one major and three minor criteria are required: (A) Major: characteristic distribution, pruritus, chronic progression with recurrences and family history of atopy. (B) Minor: signs of skin dryness, facial pallor–pityriasis alba, nipple eczema, tendency to infections, wool intolerance, sudation pruritus, ophthalmological manifestations, food intolerance, IgE elevation. The UK working group's diagnostic criteria include an itchy skin condition plus three or more of the following: history of flexural involvement, history of asthma/hay fever, history of generalized dry skin, onset of rash under the age of 2 years, flexural dermatitis. In a comparative study Hanifin and Rajka's criteria displayed a statistical advantage in sensitivity (De et al, 2006) (Figure 16).

Prevalence in Japan was measured up to 5% (Kusunoki et al, 2009), while in western countries was also 5.6% (Olesen et al, 1997). The prevalence of the disease displays an increase in comparison to the past that could be at least partially attributed to more effective diagnosis (Williams et al, 1992).

AD is established as a condition of infancy that gradually decreases through childhood and significantly drops in teenage years (Williams, 2000). It is estimated that 60% of the typical and well defined AD cases resolve and clear of symptoms in early adolescence. Regarding the predominance of the disease in sexes, although it seemed to be more frequent among females in the 90s, a rise of the incidence in males balances the previous difference (Kusunoki et al, 2009). Although seasonal variation of the severity of skin symptoms is considered as a basic feature of atopic dermatitis, recent studies suggest a decrease in the seasonal dependence of the dermatitis in European countries over the last decades (Uenishi, 2001).

The role of genetic predisposition in AD had been early hypothesized, but was strongly proved with an epidemiologic study in twins by Larsen (Larsen et al, 1986). Application of linkage studies, candidate genes studies or microarrays in AD patients revealed numerous genes that are related to the genetics of AD (Morar et al, 2006).

Malassezia yeasts have been implicated in AD, especially in the cases that the disease is localized in the head and neck (Waersted et al, 1985). The yeast is isolated by skin scales of atopic dermatitis AD adults and children in 100% (Takahata et al, 2007). *M. restricta* is the predominant species in children and *M. globosa* and *M. restricta* in adults (Takahata et al, 2007). However, several culture based studies described *M. sympodialis* as the predominant species in AD patients (Yim et al, 2010; Gupta et al, 2001), while others associated the severe cases of the disease with *M. globosa* and *M. restricta* (Kaga et al, 2011). More recently it was shown that atopic dermatitis patients, sensitized to *Malassezia sympodialis* had a more severe disease course than the rest (Soneson et al, 2012).

The main hypothesis of *Malassezia* implication in AD is that of an allergen in susceptible individuals (Darabi et al, 2009). This is supported by positive patch test results to extracts of the yeast and by elevated *Malassezia* specific IgE serum levels in AD patients (Darabi et al, 2009; Tengvall et al, 2000). Certain allergens have been identified in various *Malassezia* species. For example MGp42 appears to be a major allergen cleaved from the *M. globosa* hsp70 protein in the serum of AD patients (Ishibashi et al, 2009). *M. sympodialis* Mala s1 (Vilhelmsson et al, 2007) and *M. furfur* f1 (Zargari et al, 2001) can also act as allergens that elicite specific IgE and T-cell reactivity in patients with AD. A total of 13 *Malassezia*-derived allergens are currently included in the official allergen nomenclature catalogue (www.allergen.org) (Table 5).

Table 5: Established *Malassezia* allergens. (www.allergens.org)

Species	Size (kd)	Function	Origin
Malassezia furfur			
Mala f 2	20	Peroxisomal protein	TIMM 2782
Mala f 3	21	Peroxisomal protein	TIMM 2782
Mala f 4	35	Malate dehydrogenase	TIMM 2782
Malassezia sympodialis			
Mala s 1ₒ	36	Unknown	ATTCC 42132
Mala s 5ₒ	18	Peroxisomal protein	ATTCC 42132
Mala s 6ₒ	17	Cyclophilin	ATTCC 42132
Mala s 7ₒ	16	Unknown	ATTCC 42132
Mala s 8ₒ	18	Unknown	ATTCC 42132
Mala s 9ₒ	14	Unknown	ATTCC 42132
Mala s 10	86	Heat shock protein	ATTCC 42132
Mala s 11	22	MnSOD	ATTCC 42132
Mala s 12	67	GMC oxidoreductase	ATTCC 42132
Mala s 13	12	Thioredoxin	ATTCC 42132

Several reports in the literature document that patients with AD have increased levels of IgE antibodies (Savolainen et al, 2001). In these studies, specific *Malassezia* IgE antibodies were found in 20% to 100% of the patients with AD. Among them approximately 40% to 65% have IgE antibodies and/or skin reactivity (positive patch test) against *M. furfur*. The same patients display also higher T-cell response rates against this yeast, when compared with healthy individuals (Rasool et al, 2000). Zargari (Zargari et al, 2003) evaluated the presence of IgE antibodies to different *Malassezia* species in patients with AD, and concluded that the use of only one *Malassezia* species is not sufficient to detect all patients sensitized to *Malassezia*. Finally, Kato (Kato et al, 2006) quantified serum titers of IgE antibodies specific against eight *Malassezia* species and concluded that the species-specific antibody titers were highest against *M. restricta*. Nevertheless, it is worth noting that broad disease-aggravating allergic sensitization to commensal and pathogenic organisms is a universal phenomenon in AD patients, as it has been observed for bacteria, especially *Staphylococcus aureus*, as well as other yeasts and filamentous fungi, such as *Candida* species and *Trichophyton rubrum* (Ong and Leung, 2010).

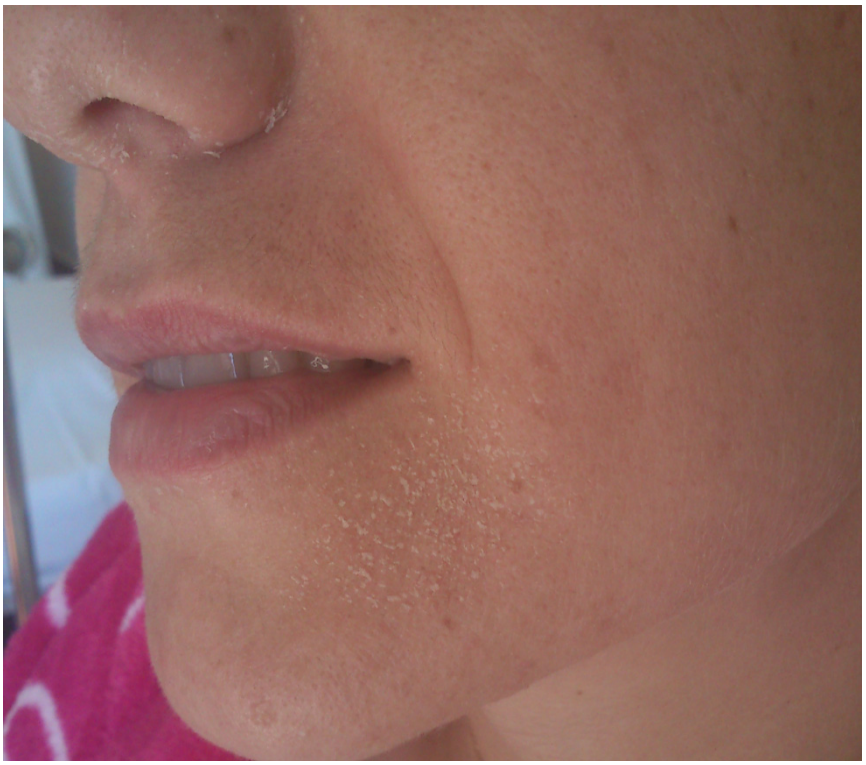


Figure 16: Dry skin and itching usually complement the clinical picture of atopic dermatitis.

Psoriasis

Psoriasis is a chronic skin disease, affecting approximately 3% of the general population (Baroni et al, 2004). Histologically it is characterized by hyperkeratosis and hyperproliferation of the epidermis, alteration of the skin capillary network with increased tortuosity and dilatation of dermal papillary blood vessels and inflammation of both dermis and epidermis (Bongiovanni et al, 2011).

Regarding the pathogenesis of psoriasis theories that implicated the immune system and the skin itself have been proposed. (Wolf et al, 2012). Nikoloff (Nikoloff et al, 1999; 2000) supported the immune basis of psoriasis by proving evidence that injected immunocytes from a psoriatic patient into engrafted allogeneic normal human skin induced a psoriatic plaque. This experiment supported the theory that abnormal T-cells were the leading cause of psoriasis. On the other hand Zenz (Zenz et al, 2005) showed that deletion of *JunB* and *C-Ju*n in animal models led to an epidermal phenotype that resembled psoriasis. Moreover, these skin lesions were still apparent in mice devote of T-cells (Rag2 mice). The orchestrating role of keratinocytes, underlined by the aforementioned observations also supported by additional findings, led to the formulation of the so-called “epidermal barrier hypothesis”. According to this postulation, the disruption of the epidermal barrier from various causes, including transgutaminase inhibition (Harisson et al, 2007), reduced ceramide epidermal content (Hong et al, 2007) or infective agents (Travers et al, 2000) heralds epidermal hyperproliferation that could trigger psoriasis in genetic predisposed individuals (Sun et al, 2010; Wolf et al, 2012). Despite the above argument, it is accepted that the induction and the maintenance of skin inflammation in psoriasis requires a genotypic predisposition and an interaction between the environment (stress, trauma, microorganisms, drugs) and host’s skin and immune system (Nestle et al, 2009).

The possibility of a relationship between *Malassezia* and psoriasis was set in 1982 when Lober and colleagues (Lober et al, 1982) applied heat killed and sonicated suspension of this yeast on the lesion-free forearms of psoriatic patients and were able to reproduce psoriatic lesions, in contrast to the control group and in a higher rate than with the use of S. *epidermidis*. The aforementioned observation was further supported by case reports of *Malassezia* folliculitis that evolved to psoriatic lesions (Elewski, 1990). Fry (Fry et al, 2007) claimed that guttate disease exacerbation is linked to colonization of the skin by certain microorganisms, including *Malassezia*, *Staphylococcus aureus* and *Candida albicans.* Of the

five main clinical subtypes of psoriasis recognized to date (plaque, guttate, inverse, pustular and erythrodermic) plaque psoriasis (Figure 17) is the most common form (80-90%) and that one that has been more consistently associated with *Malassezia*, followed by guttate psoriasis (Sandhu et al, 2003). As in AD the association usually refers to psoriasis that involves the scalp (Van de Kerkhof, 2001; Rosenberg et al, 1989). Improvement of scalp psoriasis (Farr et al, 1985) or chronic plaque psoriasis (Narang et al, 2004) with the use of antifungal agents further supports this association.

Pathogenetic mechanisms through which *Mallasezia* could trigger psoriasis include direct interaction between the yeast and keratinocytes or immune cells as well as their inbetween communication. More specifically, *Malassezia* cells have been shown to act as chemoattractants for the polumorphonuclear cells (PMNCs) of psoriasis patients in contrast to PMNCs isolated from healthy controls and AD patients (Bunse and Mahrle, 1996). Mathov (Mathov et al, 1996) reported two circulating antibodies that recognized the n-acetyloglycosamine terminals of *Malassezia* glycoproteins, in the blood of psoriatic patients. These antibodies were not detected in the sera of healthy subjects or patients with *Malassezia* related diseases. Regarding keratinocytes, Baroni (Baroni et al, 1996) reproduced the upregulated tgf-b1 and hsp70 that characterized psoriatic keratinocytes, after exposing keratinocytes from psoriasis patients to *M. furfur in vitro*.

Keratinocytes express TLR 2 and TLR 4 and these receptors are modulated by microbial components as those of *Candida albicans* (Pivarsci, 2003). These receptors modify the immune response of keratinocytes. Accordingly it has been shown that stimulation of cultured keratinocytes by *M. furfur* results in the increased expression of TLR2, MyD88, HBD-2, HBD-3 and IL-8 mRNA (Baroni et al, 2006).

The species that is mainly isolated from psoriasis lesions is *M. restricta*, followed by *M. globosa* (Takahata et al, 2007), however these are the predominant species also in the lesion-free skin of patients or the healthy controls, pointing towards a more complex interaction between *Malassezia* yeasts and the pathogenesis of psoriasis. Along these lines, using PCR analysis of skin scales it has been shown that the predominant fungal microorganism of psoriatic patients was *Malassezia,* exhibiting however differences in the isolated *Mallasezia* microbiota between the psoriasis lesions and the skin of healthy individuals (Paulino et al, 2006). Moreover, Prohic (Prohic et al, 2003), using Dixons culture mediated methods, claimed to have found significant differences in the distribution of the predominant species between psoriatic and healthy skin and in the distribution of the species according to the severity of the psoriatic lesions.

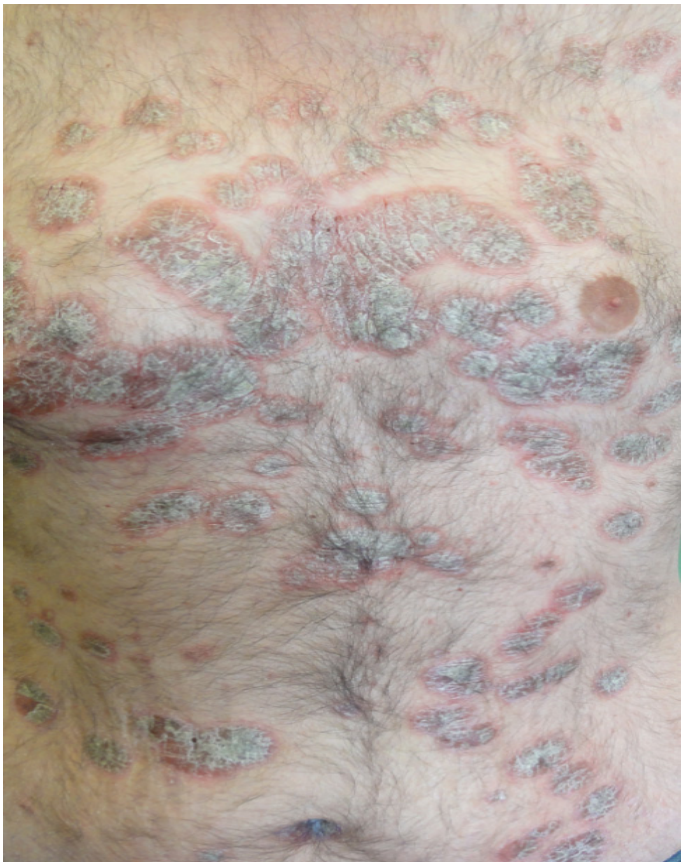


Figure 17: A patient with typical plaque psoriasis.

Aryl hydrocarbon receptor (AhR)

Physiology and function

AhR is a ligand activated transcription factor, member of the basic loop-helix-loop family Per-Arnt-Sim (PAS) of transcriptional regulators. Common characteristic of this family of receptors is the PAS domain that consists of 260-310 amino acids (Crews et al, 1988) and includes two highly preserved hydrophobic repeats named PAS-A and PAS-B. These domains are separated by a variable preserved spacer. Facilitating partner selection during formation of bHLH-PAS heterodimers (Huang et al, 1993), binding small molecules (Dolwick et al, 1993), and conferring target gene specificity of bHLH-PAS heterodimers are important functions of these PAS-domains (Zelzer et al, 1997).

Under steady state conditions AhR remains in the cytoplasm in a complex with hsp90, X-associated protein 2 and p23 (Meyer et al, 1998). These proteins stabilize AhR and prevent its degradation. Upon ligand binding, AhR complex is transformed and hsp90 leaves the complex resulting in the exposure of the nuclear localization signal (NLS) (Pollenz et al. 1994). Within the nucleus the AhR-ligand complex is dimerised with aryl nuclear traslocator (ARNT) and together they form a high affinity transcription factor (Reyes et al, 1992). The N terminals are dimerised while the C terminals exhibit the transactivation domains. A series of transcription co-activators like p300, SRC1, pCIP, sp1 and others assist and regulate gene promotion (Kobayashi et al, 1997; Nguyen et al, 1999). If AhR fail to dimerise with ARNT or to bind to DNA then a nuclear export sequence is exposed (NES). NES assists the cytoplasmic transport of AhR, which is followed by ubiquitination and degradation (Roberts et al, 1999).

The dimer binds, among other targets, to the enhancer of the genes responsible for the xenobiotics metabolism. These include the innate and delayed xenobiotic response genes like *Cyp1A1*, *Cyp1A2*, *Cyp1B1* and *Gstp1, Gsta1*, *Ugt1a2.* However, the role of AhR is much more wide than simply xenobiotic processing, as it can modify basic cell functions, like cell differentiation or apoptosis (Bock et al, 2009) and consequently more complex, integrating physiological processes like organogenesis (Wells et al, 2010), estrogen receptors regulation (Shanle et al, 2011) or the circadian rhythm (Shimba et al, 2009).

AhR ligands

The typical AhR ligand is a planar, aromatic, hydrophobic molecule with maximal dimensions of 14x12x5 A^{o} (Backlund et al, 2004). However, a variety of compounds with characteristics that diverse significantly from the aforementioned have been described to bind and activate AhR. This fact is easily exhibited by the different structures of ligands such as carbaryl, primaquine, brevotoxin and bilirubin (Backlund et al, 2004; Henseler et al, 2009). Alternative theories regarding the AhR-ligand interaction include (a) the existence of various ligation positions within the binding pocket of AhR, (b) the ability to activate AhR without interacting directly with its binding pocket and (c) the ability to activate AhR without binding at all (Backlund et al, 2003; Fontane et al, 1999).

Exogenous ligands: The majority of these compounds belong to the families of aromatic hydrocarbons, like polyhalogenated dibenzo-p-dioxins (Figure 18), dibenzofurans and biphenyls, or in the family of polycyclic aromatic hydrocarbons like 3-methylcholanthrene, benzo(a)pyrene, benzanthracenes and benzoflavones. Aforementioned substances are planar, hydrophobic compounds that represent potent agonists for AhR, with binding affinity in the pM to nM scale (McKinney et al, 1985; Waller et al, 1995).

Recently, a large number of exogenous compounds with impressively different structures (omeprazole, thiabendazole, guanabenz) from the aforementioned 'classical' compounds were described to activate AhR (Denison et al, 2003). The latterly described compounds, that in some cases contained only a single unsaturated ring, are usually weak AhR activators.

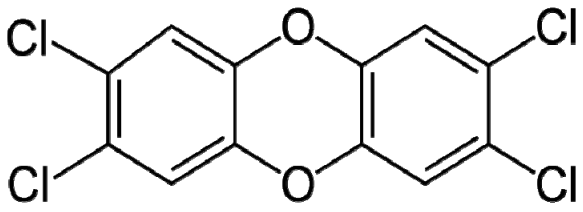


Figure 18: **2,3,7,8-Tetrachlorodibenzo-*p*-dioxin,** a polychlorinated dibenzo-*p*-dioxin

Endogenous or naturally occurring ligands: The main source of natural AhR ligands in humans comes from their diet (Denison et al, 2003). It had been early observed that some vegetables, fruits or tea could cause induction of *Cyp1a1* (Herzog et al, 1992). Later on, the ability of dietary compounds, such as dibenzoylmethanes (Mac Donald et al, 2001), curcumin (Ciolino et al, 1998) and carotenoids (Gradelet et al, 1996), to induce DRE genes expression was confirmed.

Indoles: indolic ligands that have AhR agonist characteristics are tryptophan products that occur either through biological metabolism or through physicochemical processes (Figure 19). Tryptophan has been reported to be metabolized to indirubin, indigo, tryptamin, indoleacetic acid as well as other indoles under the function of plant and mammalian enzymes (Lee et al ,2010). Moreover, yeasts, like *Malassezia,* and bacteria can also produce compounds like malassezin or tryptathrin (Patten et al, 1996; Wille et al, 2001; Gaitanis et al, 2008). Additionally, it is known that UV radiation can transform tryptophan to FICZ and that acidic conditions can convert tryptophan to ICZ (Wei et al, 1999).

Indigo and indirubin, important ingredients of the traditional Chinese medication Danggui Longhui Wanwith antileukemic activity (Eisenbrand et al, 2004) are potent AhR activators in mammalian cells. Later indolic compounds have been also found in human urine and in the concentration of 0,07nM in the fetal bovine serum (Adachi et al 2001). FICZ is currently considered as a candidate intracellular chemical messenger of the cellular light effects (Wei et al, 1999) and is thought to mediate the *Cyp1a1* upregulation in UV exposed keratinocytes, Hepa cells and lymphocytes.

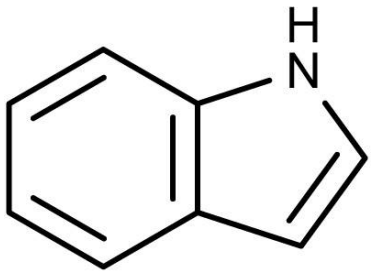


Figure 19: **Indole** is compiled of a benzene ring fused to a pyrrole ring. The molecular formula for indole is C_8H_7N.

Tetrapyroles are compounds that contain four pyrole rings. These rings are connected with carbon bridges in linear or cyclic way. Due to their ability to form metal complexes they are particularly important in biological systems. Vitamin B12, chlorophyll and heme are widespread tetrapyroles. Among the heme breakdown products bilirubin (Figure 20) and bilverdin were shown to activate AhR (Sinal et al, 1997). Adachi (2001) questioned bilirubin and other heme products as possible endogenous ligands of the AhR based on their low blood concentration levels. However, bilrubin, bilverdin and possibly other tetrappyroles are gaining a role in AhR-mediated antioxidative, atheroprotective (Bock and Kohle, 2010) or cell signaling functions (Maines, 2003). Bilirubin metabolism occurs through enzymes UGT1 and CYP1A1/2. Both of these enzymes are upregulated through the AhR pathway. Based on the above, an internal feedback mechanism may be suggested for the AhR activation by bilirubin (Phelan et al, 1998).

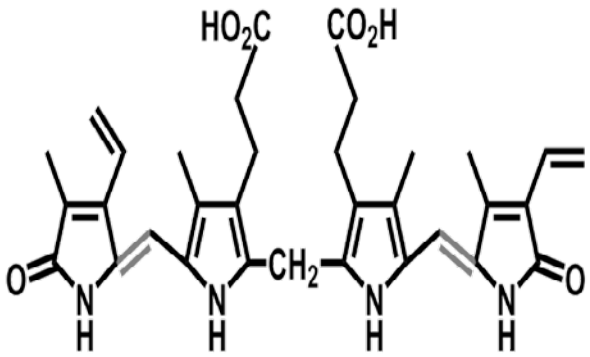


Figure 20: Bilrubin, a metabolic product of the terapyrole heme, is a known AhR agonist

Flavonoids are a family of naturally occurring polyphenolic ingredients of different vegetables, fruits and beverages as coffee, red wine and tea (Holmann and Katan, 1997). Different flavonoids have diverse effect on the phase 1 and phase 2 enzymes of xenobiotic metabolism. Quercetin (Ciolino et al, 1999) upregulates Cyp1a1 expression and activity as well as does diosmin, diosmetin and chysin (Ciolino et al, 1998). On the contrary, flavone (Figure 21) (Zhai et al, 1998) acacetin (Dostdar et al, 2000) and galangin (Ciolino et al, 1999) have a downregulative effect. Corresponding effects have been observed for Cyp1b1, Cyp3a4, Cyp2e1 but also for phase II enzymes as are Ugt, Gst (Moon et al, 2006). Activation of above enzymes might represent an anticarcinogenic flavonoids effect through xenobiotic

detoxification. Additional anticargenogenic effects might happen from inhibition of enzymes like aromatase (Cyp19) which dowregulates estrogen biosynthesis and acts as a sulfotransferase 1A 1nhibitor (Moon et al, 2005). It must however be mentioned that following oral intake the bioavailability of flavonoids is relatively low (Scalbert et al, 2000).

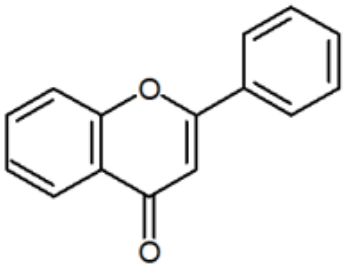


Figure 21: Molecular structure **of flavones** central skeleton.

Arachidonic acid metabolites: The ability of lipoxinA4, a lipoxygenase product of arachidonic acid and several prostaglandins [most notably prostaglandin E2 (Figure 22) (Seidel et al, 2001)] to induce AhR-dependent gene expression supports the hypothesis that the aforementioned metabolites are AhR ligands. Except from PG2 several other prostaglandins (PGA3, PGB3, PGD3, PGE3, PGF3a, PGG2, PGH1, PGH2, PGK2) can activate the AhR pathway and induce AhR dependent genes transcription in mouse hepatome cell lines (Seidel et al, 2001; Denisson and Nagy, 2003). Nevertheless, much higher molar concentrations were needed to achieve results comparable to TCDD.

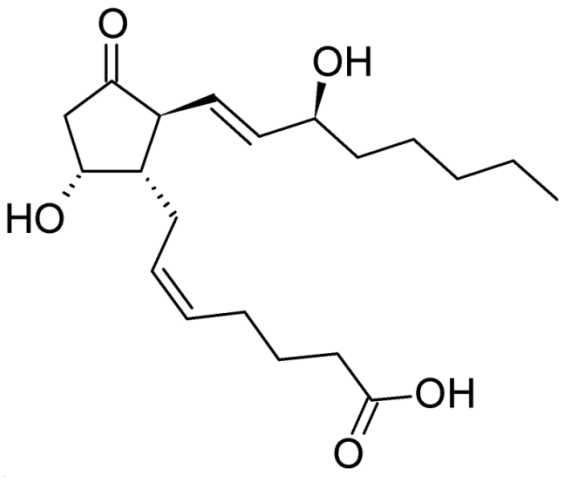
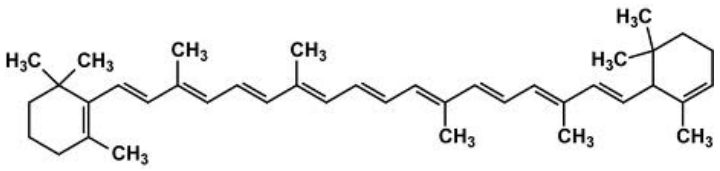


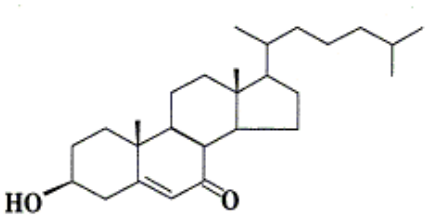
Figure 22: Prostaglandin E2 is a weak AhR agonist

Additionally AhR activation has been proved for other dietary compounds such as certain **carotenoids** (canthxanthin, astaxanthin, apo-8-carotinal) (Figure 23) but not all since others (lutein, b caroten, vitamin A) did not induce *Cyp1a1* or *Cyp1a2* expression (Astorg et al, 1994). In 1995 (Vecchiniet al, 1995) proved that phase I and phase II enzymes are induced in cultured human keratinocytes after exposure to retinoic acid. Additionally he proved the existence of retinoic responsive elements in the promoter of *Cyp1a1*. On the other hand, AhR activation has established effects on retinoic acid synthesis, catabolism, transportation and excretion (Murphy et al, 2007). In the organism carotenoids are cleaved in retinol and retinoids, with the last possessing proven interactions with the AhR signaling pathway.

AhR pathway has an emerging role in the pathogenesis of cardiovascular diseases (Puga, 2011). Most of the data so far that implicates AhR in atherosclerosis and foam cells formation has been achieved with the use of exogenous ligands. (Vogel et al, 2004). Recently various **oxysterols** were tested for their AhR activation capacity. Among the tested steroles, 7-ketocholesterol was able to inhibit TCDD binding in AhR (figure 24). The aforementioned effect was observed in concentrations of 7 ketosterol that could occur *in vivo* (Savouret et al, 2001).



Canthanxanthin



7-ketocholesterol

Figure 23: Molecular types of canthanxanthin and 7-ketocholesteroll, potential AhR endogenous activators.

Skin immune system

Skin is the primary barrier between the environment and the human organism, providing the first defense line against pathogens and physical insults. Due to its exposed localization and its relatively large size, skin immunosurveillance can be really challenging for the organism.
The human skin is divided in three compartments: the hypodermis, the dermis and the epidermis. Epidermis provides a barrier against dehydration and toxic agents and is composed from stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The aforementioned layers consist of keratinocytes that differentiate as they move in the upper layers until they become corneocytes. Additional specialized 'symbiotic' cells in the epidermis are melanocytes, Langerhans cells (LCs) and T cells. Dermis on the other hand consists mainly of connective tissue and provides tensile strength and elasticity. The major cell types in the dermis are fibroblasts, adipocytes and macrophages.

Keratinocytes (KCs) are equipped with receptors that recognize pathogen-associated molecular patterns. These receptors are located either in the cell membrane (like most Toll like receptors) or in the cytoplasm, like Nod-like receptors. TLR 1-6 and 9, known to be expressed in KCs, (Kawai et al 2002) can be activated by ligands like lipopolysacharites (LPS), double stranded RNA etc. (Kawai et al, 2002; Dai et al, 2006; Miller et al, 2005). Likewise, Nod like receptors sense non-microbial insults resulting in proinflammatory signaling through inflammasome activation (Martinon, 2009).
Additionally, keratinocytes are able to produce antimicrobial peptides such as b-defencin and cathelicidins (Schroder et al, 2010) acting as innate immune mediators. In addition to antimicrobial peptides keratinocytes secrete, either steadily or after induction, numerous cytokines, including IL-1, IL-6, IL-10, IL-18, tumor necrosis factor (TNF) (Nozaki et al, 1992) and chemokines, like CXCL1 and CXCL8. Deregulation in the expression of these cytokines is considered, at least partly, responsible for the pathogenesis of psoriasis (Sabat et al, 2007). Finally KCs are known to express major histocompability complex (MHC) antigens, which are upregulated in many skin disorders characterized by T cell infiltration (Nickoloff, 1993), a fact indicating that they can also function as non-professional antigen presenting cells.
Langerhans cells (LCs) are the main dendritic cells of the epidermis and among the first to come into contact with microbial antigens that enter the epidermis. These cells take in and

process antigens that flowingly present to T cells (Hunger et al, 2004), inducing their differentiation into Th2 cells (Klechevsky et al, 2008). However, in spite of their almost invariable presence in healthy and also diseased skin LCs are not the exclusive antigen-presenting cell type. For example, antigen presentation and initiation of immunological responses against cytolytic viruses is primarily executed in the skin by langerin^{+}cd103^{+} DCs (Zhao et al, 2003). On the other hand, additional data show that LCs can act as mediators of tolerance in the skin since their absence resulted in enhancement of contact hypersensitivity (Kaplan et al 2008).

An additional immune cell population of the epidermis is the **inflammatory dendritic epidermal cells** (iDECs). Their main characteristic is the presence of CD206 receptor, an indication of monocytic origin. Although they are found in the epidermis, it has been suggested that they can populate both dermis and epidermis (Guttman et al, 2007). These cells overexpress Fc receptor which enables them to bind IgE allergens (Bieber, 2007).

Dermal DCs (DDCs) are characterized by the expression of the surface MHC-like antigen presenting molecules **B**lood **DC a**ntigen–**1** (BDCA-1) as well as the expression of C-type lectins, such as the pathogen sensor DC-specific ICAM-grabbing nonintegrin (DC-SIGN or CD209) and DEC205 (also known as CD205) (Nestle and Nickoloff, 2007). In the past dermal DCs were divided into three subsets: BDCA-1^{+}CD1a^{+}CD14^{-} and BDCA-1^{+}CD1a^{-}CD14^{-} DDCs that have strong antigen-presenting ability and BDCA-1^{+}CD1a^{-}CD14^{+} DDCs that have limited antigen-presenting capacity but could, under certain conditions, develop into CD1a^{+} and Langerin^{+} LC receptors (Nestle et al, 1993). To these categories a subset of proinflammatory DCs, Tnf–iNos producing (Tip DCs), was added later (Serbina, 2003). Another addition is the type I interferon producing plasmatoyid DCs (pDCs) that accumulate in the skin under chronic inflammation conditions such as psoriasis and lupus erythematosus (Wollenberg et al, 2002).

Skin macrophages are characterized by the presence of the factor XIIIa, a factor better known from the coagulation process. However, it is also a tissue transglutaminase participating in wound healing (Nickoloff, 1993). Moreover, they are expressing CD163 receptor, a scavenger receptor that is uniformly co-expressed with XIIIa (Zaba et al, 2007). These cells cannot stimulate T-cells but they demonstrate high phagocytosis capacity (Zampa et al, 2003).

The number of **T cells** in the skin of healthy individuals is twice the number of T cells in blood (Clark et al, 2006). Epidermal T cells are mostly of the CD8^{+} ab memory cell phenotype (Bos et al, 1987) and are usually located in the basal layers in rather close spatial relationship to

the LCs. In the dermis $CD4^+$ and $CD8^+$ T cells exist equally, usually around post-capillary venules (Bos et al, 1993). During inflammatory skin conditions $CD4^+$ cells may be of Th1, Th2 or Th17 phenotypes, while also Th22 cells have been recently identified in the skin of patients with atopic dermatitis (Nograles et al, 2009). γδ T cells and NK T cells are the less conventional T cells subpopulation of the skin since they constitute no more than 2-9 % of skin's T cells (Nestle, 2009). It is lately believed that resident T cells, rather than recruited ones, play the main role in the skin's immune homeostasis and pathology (Boyman et al, 2007).

Malassezia interactions with the skin immune system

The dual role of *Malassezia*, as a commensal and a pathogen supports the hypothesis of a complicate interaction with the skin's immune system. Consequently, it is speculated that *Malassezia* yeasts can upregulate or downregulate the immune response through not yet fully clarified mechanisms. One of the first attempts to demonstrate *Malassezia*'s capacity to modulate the function of the immune system was undertaken by Takahashi (Takahashi et al, 1984; Takahashi et al, 1986). In the above studies injection of *Malassezia* in mice resulted in the protection against *Salmonella enterica* infection or against challenge with tumor cells lines.

Malassezia is capable of activating both the classical and the alternative pathways of the complement system (Sohnle et al, 1983). Complement activation has been related to various dermatoses including psoriasis (Tagami et al, 1992). The molecule responsible for this activation has not yet been clarified although it is speculated that b-glycan in the cell wall may be the one involved (Suzuki, 1998).

Apart from the complement several antimicrobial peptides like LL-37 are present in the skin. LL-37, which is elevated in skin diseases like pityriasis vercicolor (PV), was found to be effective against *Malassezia* (Lopez et al, 2006). HBD -22 is another antimicrobial peptide that was secreted from keratinocytes that were treated with *Malassezia furfur* (Donnarumma et al, 2004). Finally, transferrin secreted in the human skin has also demonstrable inhibitory effects in the growth of *Malassezia* yeasts (Bond et al, 2005).

Regarding the cellular response against these yeasts, it was shown that although they are rapidly phagocytized from neutrophils, intracellular killing is quite inefficient (Richardson et al, 1991). The binding of *Malassezia* and monocytic cells is mediated from the mannose receptors, b-glycan receptors and complement receptor 3 (Bunse et al, 1996). The main inflammatory cells found in lesions of PV are T cells positive for the antileu 3a, i.e. of a helper inducer phenotype, while cells expressing the suppressor/cytotoxic phenotype are more rare (Scheynious et al, 1984). Moreover, Brasch (Brash et al, 1993) demonstrated that accumulation of Langerhans cells in pityriasis versicolor lesions is common, yet usually without CD4 expression.

In the case of seborrheic dermatitis the predominant infiltrating cell species are $CD4^+$ T cells. Faegermann (Faegermann et al, 2001), who also compared the local tissue inflammatory cell populations between SD lesions and healthy skin, described a dense infiltrate of

lymphocytes, macrophages, monocytes, Langerhans cells and a few granulocytes in the biopsies from the lesions. With the exception of CD68 all cellular markers examined (CD4, CD8, HLA-DR, NK1, CD16, CD54) showed an increased cellular staining in seborrheic dermatitis when compared with healthy controls. A high number of HLA-DR1, NK11 and CD161 cells were also found.

The lesion inflammatory cell infiltrate has been also characterized in *Malassezia*-folliculitis. When inflammation is contained, in the absence of follicular rupture lymphocytes are the main cell type in these lesions. Once the follicle ruptures, the infiltrate is more extensive and also includes macrophages, eosinophils and plasma cells (Potter et al, 1973).

Co-culture of *Malassezia* species with keratinocytes demonstrated significant changes in the KCs cytokine production profile. The results varied between researchers depending on the studied *Malassezia* species, the ratio between the number of microorganisms and KCs and further conditions of the co-culture. For example Baroni et al (2001) found that *M. furfur* may have significant immunological effects on the skin by concurrently limiting the secretion of IL1a, TNFa and IL6 and increasing the secretion of IL10 and TGFb1 by KCs. On the other hand, Watanabe (Watanabe et al, 2001), found that *M. furfur* did not stimulated the production of the studied cytokines (IL1b, IL6, IL8, TNFa and MCP). In contrast the rest tested *Malassezia* species (*M. pachydermatis, M. slooffiae, M. sympodialis*) increased the production of IL1b, IL6, IL8 and TNFa. When cellular immune responses against *M. furfur* were measured using the lymphocyte transformation assay (LT) and the leucocyte migration inhibition assay (LMI), no significant differences between seborrheic dermatitis patients and healthy controls were found (Bergbrant et al, 1999). In contrast the same research revealed a significantly lower response against *M. furfur* in patients with pitysiasis vercicolor. However, since SD is very common in patients with cellular immunity deficiencies like AIDS (Chatzikokkinou et al, 2008) or transplanted patients (Odom, 1994) and despite Bergbrant's observations other researchers anticipate that the impaired cellular immunity facilitates the fungi survival on the skin (Prohic et al, 2010).

Introduction and Aim of the Study

Aim of the present Thesis was to investigate the mechanisms at the level of yeast-skin interaction through which the genus *Malassezia* acquires its clinical significance. Although the association of *Malassezia* to pityriasis versicolor (PV) and to a lesser degree to seborrheic dermatitis (SD) is well established in the literature and the pathobiological link between the growth of these fungi on the skin and the pathogenesis of the corresponding diseases is of great scientific interest, the details of the aforementioned interactions at the tissue level remain still quite obscure. Thus, in order to contribute in the aforementioned details exposing we experimentally assessed the following: a) Identification and typing of *Malassezia* species isolated from SD patients and their family members b) The **production of lipolytitic enzymes** by *Malassezia* strains isolated from SD patients and healthy volunteers c) The *in vitro* **effect of indolic compounds produced by SD associated *Malassezia* strains** on dendritic cells (DC).

Family distribution of *Malassezia* strains

Different epidemiological studies resulted in conflicting data regarding the existence of a predominant *Malassezia* species on human skin surface, depending on the isolation methods and the host population. Interestingly host's geographical origins as well as the underlying skin's condition were related to isolated *Malassezias (M. furfur) genome.* More specifically, when *M. furfur* strains were isolated from Scandinavians permanently residing in Greece, Greek controls, Bulgarians and Chinese, all either healthy or suffering from SD or pityriasis vercicolor, it was shown that Scandinavian, Greek and Bulgarian strains formed distinct genetic clusters (Gaitanis et al, 2009). Above data provided initial evidence for an association with the host's geographic origin and the underlying skin disease. This geographic association was also shown for an *M. furfur* strain isolated from a male Dutch SD patient who had been living in China for 5 years. The internal transcribed spacer sequences of the isolated *M. furfur* strain were identical to the reference CBS7982 strain isolated from a European healthy individual (Ran et al, 2008).

In a more recent study from China (He et al, 2008) 21% of the patients with pityriasis versicolor (PV) had a positive family history for this disease. Moreover, the prevalence of this skin disease among the first degree relatives of the patients was 5.3% in contrast to the 1.23% of the control group. Heritability, according to Falconers method (Emery, 1986), in the first degree relatives was estimated at 48%. However, none of the spouses of the 191 cases studied had PV.

Recently Paulino (Paulino et al, 2008) compared the *Malassezia* microbiota between psoriatic lesions and healthy skin. Although no significant differences were substantiated they found that the strains correlated more with the host than with disease state.

Family members usually share a common living space and also a genetic background. One scope of this study was to examine the distribution of *Malassezia* species within families with at least one member being a seborrheic dermatitis patient.

Phospholipase production and correlation to epidemiological data

As mentioned above, *Malassezia* yeast (with the exception of *M. pachydermatis*) require lipids in their culture medium in order to by-pass their inability to synthesize long-chain fatty acids (Porro et al, 1976; Juntachai et al, 2009). The yeasts acquire *in vivo* their prerequisite lipids through the production of an array of lipolytic enzymes that hydrolyze lipids of sebum (Juntachai et al, 2009). Among these, also enzymes with phospholipase activity have been characterized (Riciputo et al, 1996; Juntachai et al, 2009). Lipase production is a well-established biological trait both in yeasts and molds like *Candida albicans* (Meyser et al, 1996; Ibrahim et al, 1995), *Cryptococcus neoformans* (Chen et al, 1997), *Aspergilus fumigatus* (Ghanoum et al, 1998) and *Rhodotorula rubra* (Mayser et al, 1996). To date extensive studies have been performed, regarding the significance of *Candida's* phospholipase for human disease pathogenesis. Dagdeviren (Dagdeviren et al, 2005) verified phospholipase production only in *Candida* strains that caused bloodstream infections in contrast to non-blood isolates. Barrett-Bee (Barrett-Bee et al, 1985) found a positive correlation between the phospholipase activity of *Candida albicans*, its ability to adhere to buccal epithelial cells and its pathogenicity in mice (increased mortality after IV injection). When virulence of phospholipase lb deficient *Candida albicans* strains was compared to wild type, employing their respective ability to penetrate endothelia and epithelial cell

monolayers *in vitro* (Leidich et al, 1998), a significantly attenuated penetrating ability of the former was recorded. Accordingly, supernatant from wild-type *C. albicans* culture was twice more efficient in causing cell damage to epithelial cells (Ghannoum, 2000) than that of the phospholipase Ib deficient cultures. Finally, Mukherjee (Mukherjee et al, 2001) provided data from an *in vivo* mouse model according to which phospholipase producing *Candida albicans* strains penetrated deeper into the gastric mucosa when compared to phospholipase-deficient strains.

To date in the genus *Malassezia* the production of lipolytic enzymes has been studied for the species *M. pachydermatis,* which is mostly isolated from animals and it is not strictly lipophilic (Cafarchia et al, 2004). *M. pachydermatis* strains, isolated from skin lesions of *Malassezia* dermatitis in dogs, were secreting higher amounts of phospholipase than strains that were isolated from healthy canine skin (Cafarchia et al, 2008). Interestingly, two different genotypes were correlated with the aforementioned skin disease and the phospholipase production ability. Recent data implies a relationship between endogenous opioid peptides (endorphins) and lipolytic enzyme production by *M. pachydermatis* (Cafarchia et al, 2010). Moreover, the same data suggests that the presence of μ-opioid receptor on the cell membrane of *M. pachydermatis,* isolated from dogs, is linked to the phospholipase production of the yeast. Concerning the anthropophilic *Malassezia* species, phospholipase production has been observed in *M. furfur* (Riciputo et al, 1996), but the involvement of this enzyme in the development of human skin disease is still under investigation. However, the extracellular location of these enzymes in *M. globosa* has been confirmed with proteomics approach (Xu et al, 2007).

Human skin has the ability to express pro-opiomelanocortin, the precursor of b-endorphin (Milington, 2006). Given that increased concentration of b-endorphin found in skin lesions of different diseases, such as psoriasis (Glinksi et al, 1994) and atopic dermatitis (Glinski et al, 1995), an interaction between *Malassezia* yeasts and endorphin, as suggested from animal studies (Cafarchia et al, 2007; 2010), could be possible. More specifically, Cafarchia proved that b endorphin stimulation could affect in vitro the number of phospholipase producing *Malassezia pachydermatis* strains and the amount of the produced enzyme. In accordance, phospholipase production by *M. pachydermatis* isolates has been proposed as a candidate virulence factor in animals but relevant data is missing in humans. **Thus, an additional aim of the present Thesis was to investigate the ability of the isolated *Malassezia* strains in the epidemiological part of the study to produce *in vitro* phospholipase and also to evaluate the modification of the lipolytic activity after b- endorphin stimulation.**

Indolic compounds and their role in inflammation

Gaitanis (Gaitanis et al 2008) recognized that *Malassezia furfur* isolates that originated from SD patients (SD lesional skin) were characterized by their capacity to produce increased quantities of the potent AhR ligands indole-3,2β-carbazole (ICZ) and malassezin. Meanwhile, it has been shown that almost all currently known *Malassezia* species can synthesize *in vitro* variable quantities of potent indolic AhR ligands such as indirubin, formyl-indole-3,2β-carbazole and tryptanthrin (Giakoumaki et al, 2008; Magiatis et al, 2010), when their culture medium contains L-tryptophan as the exclusive nitrogen source. Importantly, the aforementioned *Malassezia* derived indolic AhR ligands have been associated with diverse clinical phenomena observed in the course of *Malassezia*-associated skin diseases: the absence of inflammation in PV or, on the contrary, the induction of distinct inflammation and epidermal hyperproliferation in seborrheic dermatitis (Gaitanis et al, 2008; Mayser and Gaitanis, 2010). The above facts underscore the significance of this biosynthetic pathway as a candidate virulence factor of this yeast species.

AhR is a cytosolic transcription factor (Bock et al, 1994). Under steady state conditions it remains in the cytoplasm in an inactive state in association with molecular chaperones as are hsp90, AIP and p23 (Esser C, 2009; Ikuta et al, 2009). AhR ligand binding is followed by AhR translocation into the nucleus and a dimer formation with Aryl hydrocarbon nuclear translocator (ARNT). Here upon the dimer binds to xenobiotic responsive elements flanking the 5' end of genes and modifies their transcription (Ikuta et al, 2009). AhR activation, DNA binding, gene modulation and the subsequent down-stream effects are known to be ligand, cell type and tissue specific (Prochazkova et al, 2011, Esser C, 2009). AhR has multiple roles in skin physiology participating in wound healing (Ikuta et al, 2009), stress response to UVB (Fritche et al, 2007) cell cycling (Ray and Swanson, 2009; Knockaert et al, 2004), modification of the inflammatory response to immune signals (Kerkvliet, 2009; Bankoti et al, 2010) and melanogenesis (Luecke et al, 2010). Currently, the assessment of the expression of *Cyp1a1* and *Cyp1b1* is broadly used for the quantification of AhR activation state (Schmidt and Bradfield, 1996).

Dendritic cells (DCs) are crucial immune sentinels in all body organs, including skin as they connect innate and adaptive immunity. In the skin they appear both in the epidermis and in the dermis and their function consist of recognition, phagocytosis and subsequent presentation of antigens to T cells, which results to their activation (Kaplan, 2010). In the

epidermis DCs present either as inflammatory epidermal DCs or Langerhans cells (Toebak et al, 2009; Nestle et al, 2009). Dermal inflammatory DCs are of monocytic origin while emerging evidence supports the same derivation for steady state dermal and inflammatory epidermal DCs (Nestle et al, 2009; Lopez-Bravo et al, 2008; Leon and Ardavin, 2008). When "danger" is sensed via DCs pattern recognition receptors (PRRs) they start a cellular process called maturation. During maturation they start to produce pro-inflammatory cytokines and upregulate costimulatory molecules such as CD80, CD86, CD83 and MHC class II. Afterwards, maturing DCs can migrate to secondary lymphoid organs where they activate (naïve) T cells. Recently, it was shown that activation of AhR modifies the differentiation and the response to inflammatory stimuli of Langerhans cells and DCs (Bankoti et al, 2010; Simones and Shepherd, 2011; Platzer et al, 2009). In the majority of these studies the synthetic exogenous AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been employed (Procházková et al, 2011; Kerkvliet N, 2009; Bankoti et al, 2010) and due to that, the downstream cellular effects noted might diverge from physiological responses, since TCDD is metabolized extremely slowly. On the contrary, *Malassezia*-synthesized indoles are naturally occurring ligands and indirubin has even been suggested as an endogenous AhR ligand (Adachi et al, 2004; Procházková et al, 2011). The effect of these ligands on DCs has not been studied up to now**. Based to the aforementioned data, in the present Thesis we also screened selected AhR ligands that are produced by *Malassezia* yeasts for their capacity to induce the AhR pathway in monocyte derived dendritic cells (moDCs) as measured by *Cyp1a1* and *Cyp1b1* activation. Moreover we examined the modifying effect of these ligands in the phenotype of the dendritic cells, in their capacity to produce and secrete mediators of inflammation and in their ability to stimulate T cell proliferation.**

Materials and methods

Isolation and cultivation of *Malassezia* species.

Hospital Ethics Committee permission was granted and from August 2008 to July 2009 a total of 84 volunteers (mean age 43) either with SD (N= 43) or healthy controls (N=41) were included in this study. The SD patients were consecutive patients recruited in the outpatient clinic of the Dermatology Department of the University Hospital of Ioannina, while the healthy controls included first degree relatives (27 families) who consented to participate in the study as well as patients that visited the outpatients clinics with conditions unrelated to SD or other inflammatory dermatoses (n=3). Eleven reference strains (CBS 6001 *M. furfur*, CBS 9558 *M. nana*, CBS 7876 *M. obtusa*, CBS 1879 *M. pachydermatis*, CBS 7966 *M. globosa*, CBS 9726 *M. yametoensis*, CBS 10434 *M. caprae*, CBS 9170 *M. dermatis*, CBS 9432 *M. japonica*, CBS 7956 *M. slooffiae*, CBS 7272 *M. sympodialis*) were also included in the study. According to clinical presentation SD lesions were categorized as mild, moderate and severe and skin scales were sampled employing a sterile blade and transferring the scales in the laboratory into Petri dishes, as previously described (Gaitanis et al, 2006). The sampled area was determined by the predilection localization of the lesions and in most cases was scalp, face or sternum, anatomical areas that are favored by SD. In lesional fields, where scales were available, they were preferably removed with a scraper for *Malassezia* isolation. In addition, part of the scales was stained with Parker's ink in order to directly confirm the presence of the yeast-like form of the fungus under the microscope (magnification x400). In healthy controls, samples were taken with a sterile swab moistened in phosphate buffered saline, which was rubbed in an area delineated by a 2cm diameter sterile ring for 30sec. Both the swabs and sampled skin scales were inoculated in 9cm Petri dishes in modified Dixon's agar (6% yeast extract, 0.6% mycological peptone, 1% agar noble, 1. 2% bile salts, 1% Tween 40, 0.2% glycerol all from Sigma-Aldrich, Germany) and were incubated for two weeks at 32^{o}C degrees. The dishes were examined every 2 days for growth of *Malassezia* colonies. As sampling was not quantitative, no colony-forming units were counted. Stereoscopic evaluation of cultures was performed and phenotypically different colonies were subcultured for 1 week in modified Dixon's medium without antibiotics in order to maximize

growth. Accordingly, the morphologic characteristics of the growing colonies and conventional taxonomic techniques (Gueho et al, 1996) were used in order to differentiate between species.

Identification of *Malassezia* species

Both conventional (Gueho et al, 1996; Boekhout et al, 2010) and molecular techniques (Gaitanis et al 2002; Gaitanis et al, 2006) were combined to optimize the identification of the isolated strains at species level. Initial identification was made with conventional methods and the results were subsequently confirmed with molecular methods.

a) **Confirmation of lipid dependency**: The strains were transferred in Sabouraud dextrose agar (peptone 10g/L, dextrose 20g/L, agar 17g/L Fluca 84086, Germany), a medium lacking the essential for the growth of the lipophilic species oleic acid. This step discriminates *M. pachydermatis,* which is able to grow on a non-lipid medium, but is also helpful for distinguishing between fungi of the genus *Malassezia* and other, non-lipophilic yeasts that may present with similar macro- and micromorphological characteristics.

b**) Catalase reaction**: Catalase is a tetrameric protein of four polypeptide chains, each over 500 amino acids long. It contains four heme (iron) groups that allow the enzyme to react with hydrogen peroxide, and split it into water and oxygen (Chelikani et al, 2004) (Figure 24). A small quantity of yeast biomass was transferred onto a glass slide and was exposed to a drop of H_2O_2 30% (Singma-Aldrich H3410, Germany). If the strain was able to produce catalase, hydrogen peroxide was hydrolyzed to water and oxygen, reaction that was evidenced as formation of bubbles. Catalase reaction is positive for all *Malassezia* species except *M. restricta.*

Figure 24: Catalase hydrolyses H_2O_2 to water and oxygen. Oxygen is readily liberated and this is evidenced as sample effervescence.

c) **Tween assimilation**: Gueho (Gueho et al, 1996) introduced an identification technique for *Malassezia* species based on their ability to assimilate different lipid sources. A simplified modification of this technique was used for the conventional identification of *Malassezia* species (Guillot et al, 1996. Identification of Malassezia species: A practical approach). A loopful of the strain to be identified was inoculated in 5ml of distilled and sterile water and the clumps of the yeast were dispersed by short vortexing. 2 ml of the above suspension were mixed with 16ml of Sabouraud dextrose agar and were plated on a 9cm diameter petri dish. After the medium had solidified, 4 wells were opened symmetrically with a 5mm cork borer and in these wells 5µl of either Tween 20, 40, 80 (P2287, P1504, P4780 all Sigma, Germany) or Cremophor-el (Sigma S5135, Germany) was injected. This step was followed by 7 days incubation at 32 ^{o}C.

M. furfur can grow assimilating all 4 lipids. *M. slooffiae and M. dermatis* can assimilate all Tweens but not Cremophor-El, while *M. globosa* and *M. obtusa* can use Tween 40 and to some degree Tween 20 displaying a faint ring of growth after a few days. *M. sympodialis* can assimilate Tween 40 and 80 and partially Tween 20. Species identification is based on above phenotypic results (Figure 25).

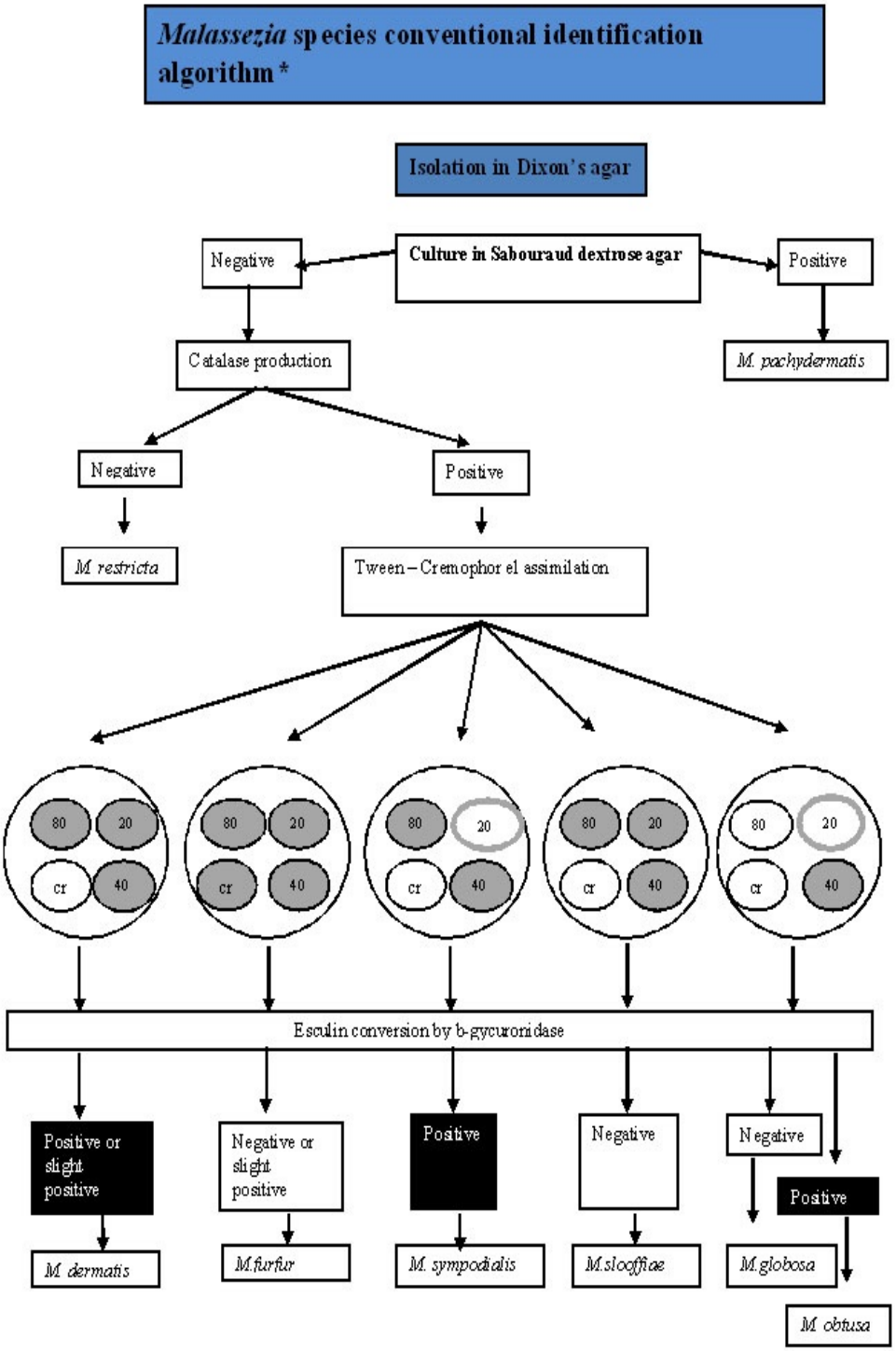


Figure 25: Algorithm of *Malassezia* species by conventional microbiological methodology (Gueho et al, 1996; Meyser et al , 1997) (80: Tween 80, 40: Tween 40, 20: Tween 20, cr-l: Cremophor-l)

d) Esculin test: This test complements Tween assimilation in order to facilitate the distinction between *M. dermatis* and *M. slooffiae* and between *M. globosa* and *M. obtusa* that provide the same phenotype in the Tween assessment.

The test is based on the production of b glucuronidase by some species, which can convert esculin to esculetin and glucose, releasing in this way dissolvable ferum ions that convert the color of the growth medium to black (Kerr and Levy, 1947; Hale et al, 1911) (Figure 26). The strain under investigation is inoculated and incubated at 25 ^{o}C for 2-5 days. *M. furfur* and *M. globosa* do not split esculin to esculetin in contrast to *M. obtusa, M. dermatis* and *M. sympodialis*.

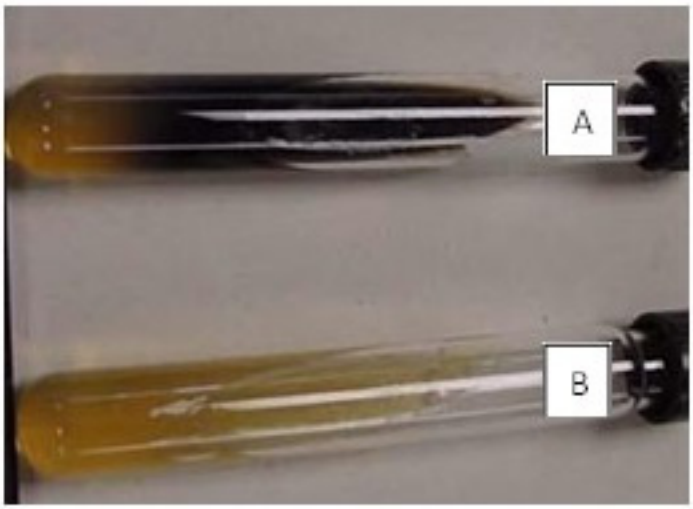


Figure 26: The release of iron ions by *Malassezia* species that possess b glucuronidase turns the medium's color to black. Vial A: Positive esculin test. The medium has turned into black after inoculation of a loopful of a *M. sympodialis* strain. Vial B: Negative esculin test. The medium has not changed in color after inoculation of a loopful of *M. globosa* culture. Both tubes were incubated at 32^{o}C for 5 days.

DNA Isolation- PCR RFLP

DNA identification was performed as previously described (Gaitanis et al, 2002; Gaitanis et al, 2006) employing automated DNA extraction (Maxwell 16, Promega, Madison, WI, USA) (Gaitanis et al, 2009). The sample was subsequently spectrophotometrically adjusted at 250μl/ml concentration of and was used in the PCR reaction.

The 50 μl of the PCR master mix contained 6 μl $MgCl_2$ (25 μM), 10 μl Mg^{2+}-free buffer (100mM Tris–HCl, 500mM KCl and 1% Triton-X), 2.5U Taq Polymerase (Promega, Madison, WI, USA), 0.04 mM of each dNTP (CLONTECH, Palo Alto, CA, USA), and 5μl of template DNA. The PCR reactions were performed in a PCR thermocycler (Robocycler, Gradient 40, Stratagene, USA) at 30 repetitions of the cycle: 1min at 95 ^{o}C, 1min at 55 ^{o}C, and 90 s at 72 ^{o}C. The final cycle comprised of 1 min at 95 ^{o}C, 1min at 55 ^{o}C and 5 min at 72 ^{o}C.

The universal fungal ITS 3 (5'- GCATCGATGAAGAACG CAGC-3') and ITS 4 (5'- TCCTCCGCTTATTGAT ATGC-3') (INTERACTIVA Biotechnologie GmbH, Ulm, Germany) primers (Interactiva, GmbH, Germany) were used for the amplification of *Malassezia* species sequences (White et al, 1990) (Figure 27).

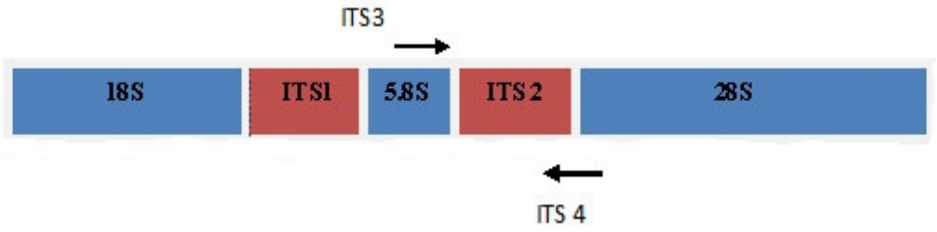


Figure 27: Structure of the fungal rRNA gene. Arrows schematically represent the binding positions of the ITS 3 and ITS 4 primers. ITS: Internal Transcribed Spacer

When the primer set ITS3/ITS4 was used, the amplified ITS 2 region differentiated three groups of *Malassezia* species within the 11 species. *M. obtusa, M. pachydermatis, M. japonica* and *M. furfur* comprised the first group, *M. globosa, M. restricta, M. slooffiae* and *M.yamatoensis* comprised the second group and *M. nana, M. sympodialis and M. dermatis* the third. However, as was previously described by Gaitanis (Gaitanis et al, 2006), with the use of three restriction enzymes AluI, BanI and MspAI (New England Biolabs, Beverly, MA, USA) different restriction profiles were recognized for each of the 11 species (Gaitanis et al, 2006) (Table 6).

The PCR products and the corresponding restriction fragments were submitted to electrophoresis in 6% 29:1 acrylamide:bis-acrylamide non-denaturating gel at 180 V for 3 h and 30 min or, alternatively, in 10 cm long 4% standard agarose gels at 100 V for 4 h. The gels were stained with ethidium bromide (2,5-3%) and visualized under UV illumination (Herolab,EASY, Weisloch, Germany).

Table 6: Identification of *Malassezia* species based in the RFLP analysis of the ITS 2 region of the ribosomal DNA of *Malassezia* yeasts (Gaitanis et al, 2006)

Group	*Malassezia* species (total, *n* = 120)	ITS 3/4 PCR product (bp)	*Alu*I	*Ban*I	*Msp*AI
I	*M. furfur* (*n* = 35)	557	306, 251	389, 168 NRS[b]	525, 32
I	*M. obtusa* (*n* = 2)	554	NRS[b]	396, 158	NRS
I	*M. japonica* (*n* = 2)	528	394, 134	183, 199, 146	498, 30
I	*M. pachydermatis* (*n* = 10)	529	412, 117	NRS	499, 30
II	*M. slooffiae* (*n* = 4)	505	385, 120	NRS	472, 33
II	*M. globosa* (*n* = 15)	477	221, 16, 240	NRS	447, 30
II	*M. restricta* (*n* = 3)	463	NRS	186, 277	432, 31
II	*M. yamatoensis* (*n* = 1)	470	NRS	NRS	NRS
III	*M. sympodialis* (*n* = 44)	420	NRS	NRS	281, 109, 30
III	*M. dermatis* (*n* = 3)	416	NRS	186, 230	192, 85, 109, 30
III	*M. nana* (*n* = 1)	428	NRS	188, 240	286, 110, 32

Malassezia type, reference and clinical strains of 11 *Malassezia* species tested for actual nucleotide polymorphisms in the ITS 2 amplified region by RFLP analysis The lengths reported (in base pairs) are approximate as small intraspecies variations exist.

b: NRS-no restriction site.

PCR fingerprinting (STR-PCR)

M. furfur further was genotyped to subtypes with the use of PCR fingerprinting methods and more specifically short tandem repeat (STR) – PCR. The mini satellite – specific core sequence of the wild type phage M-13 (5-GAGGGTGGCGGTTCT-3) was used as primer. The technique was performed as described previously (Gaitanis et al, 2009). PCR master mix comprised from 2 ml MgCl2 (25mM), 2.5 ml MgCl2-freestorage buffer B (Promega), 1.5 μM Taq Polymerase (Promega), 0.015mM of each dNTP (HT Biotechnology, Cambridge, UK), 50 pmol of the M13primer and 200–250 ng of template DNA. The PCR reaction consisted of 1 initial denaturation cycle (5 min; 94 ^{0}C) and 45 amplification cycles (1 min; 94 ^{0}C, 1 min; 42 ^{0}C and 2 min; 72 ^{0}C) and was performed in a Techgene Technethermal cycler (Stone Staffordshire, UK). PCR products were electrophoresed in 4–6% 29:1 acrylimide/bis-acrylimidestacking gels for 3.5 h at 180V, stained with ethidium bromide and visualized with UV (Herolab, E.A.S.Y., Weisloch, Germany).

The dendrogram was created with Bionumerics Software Version 4 (Bio-Maths, Bilthoven, Belgium; Figure 19) using the Dice Coefficient of similarity and cluster analysis with the unweighted pair-group method, the arithmetic averages (UPGMA), 1.00% position tolerance and no optimization in order to obtain the greatest variation in similarity.

Assessment of phospholipase production

Phospholipase production by the Type, reference and clinical strains was assessed using the egg-yolk plate method (Samarayanake et al, 1984; Mayser et al, 1996; Riciputo et al, 1996). Before inoculation into the egg-yolk agar, all strains were initially incubated in Dixon's agar for 1 week at 32^{o}C and subsequently a loopfull of the strains was inoculated into Dixon's agar, or b-endorphin (human b-endorphin, Sigma, Saint Louis) containing Dixon's agar at 1nM and 100nM. Dixon's and egg yolk agar were prepared as previously described (Gaitanis et al, 2006; Riciputo et al, 1996.) After 4 days, 3 individual colonies of each strain were picked and transferred to the egg-yolk agar [6.7 g nitrogen yeast base, 5 g glucose, 0.15 g asparagine, 0.1 g yeast extract, 3.75 g glycine, 0.55 g $CaCl_2$, 58 g NaCl, 0.1 g KNO_3, 0.06 g $FeSO_4$, 12 g % agar noble and 80 ml egg yolk emulsion, all diluted in 1 l of distilled water (Sigma Aldrich, Germany)] and were incubated for 9 days (best time point after titration regarding the amount of secreted enzymes) at 32^{o} C. Due to the poor growth in the egg-yolk medium, it was not possible to inoculate an equivalent amount of *Malassezia* cells in the egg-yolk medium, as described for E-test evaluation (Cafarchia et al, 2004) and equivalent yeast-biomass was transferred by picking 2 mm diameter colonies. In each egg-yolk plate 4 colonies of the strains tested were inoculated and experiments were individually performed 3 times. In order to attest if phospholipase activity was restricted within the boundaries of the cultures and no visible precipitate extending outside the borders of the colony could be visualized, the colonies were removed and when precipitate was observed by naked eye underneath the colony phospholipase production was recorded as positive. For the semi-quantitative evaluation of the phospholipase activity of individual colonies the ratio of the colony to the precipitate diameter was used**. In that way the greater the phospholipase production the smaller the absolute value of the ratio** (Figure 28). The impact of endorphin exposure on phospholipase activity in dependence to (a) the different *Malassezia* species and (b) the origin of the isolates from healthy or SD lesional skin was inferred employing the Wilcoxon sign test for paired data, the χ^2- test and multinomial regression analysis, all calculated using the SPSS for Windows 17.0 statistical package.

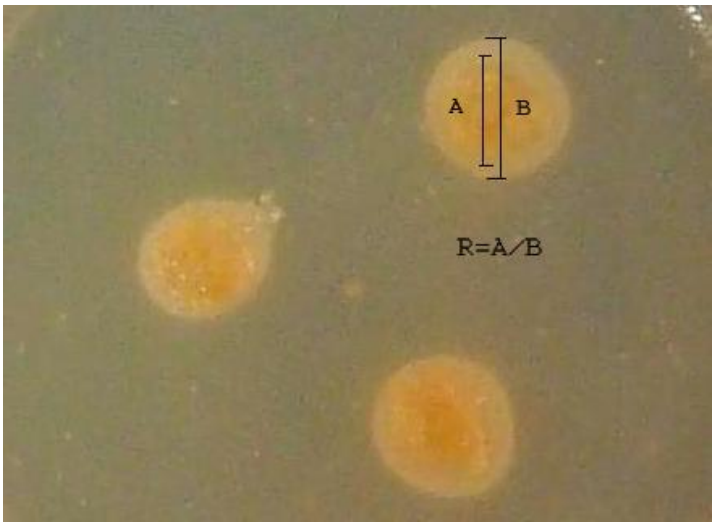


Figure 28: Precipitation zone around colonies of the *M. furfur* isolate UOI 35 after growth in egg-yolk agar for 9 days at 32 ^{o}C. The ratio (R) of the colony diameter (A) to the precipitation diameter (B) was considered as indicative of phospholipase activity. In that way the greater the phospholipase production the smaller the absolute value of the ratio.

Dendritic cells culture and exposure to TLR and AhR ligands

Malassezia produced ligands (indirubin, ICZ, malassezin, tryptanthrin) were synthesized as described previously (Gaitanis et al, 2008, Giakoumaki et al, 2008; Magiatis et al, 2010), dissolved in DMSO (10 mM) and used for titration experiments at final concentrations 100pM, 1nM, 10nM, 100nM and 1μM. TCDD (AccuStandard Connecticut, USA) was dissolved in DMSO and used, initially for the titration experiments and subsequently at 100 nM concentration. TLR-ligands used were: LPS (100ng/ml, Sigma) ligand of TLR4, R848 (4μg/ml, Axxora) ligand of TLR 7/8, poly (I:C) (20 μg/ml, Sigma) ligand of the TLR 3. The AhR inhibitor CH223191 (Tocrisbiosience, Minneapolis, USA) was also dissolved in DMSO and used in concentration of 10 μM. CH223191 was added in the cell medium 1 hour prior to the AhR and TLR ligands. Final DMSO concentration in all experiments was <0.1% and use of according controls displayed no effect on DCs viability or maturation.

Buffy coats were obtained from blood of healthy volunteers according to the Helsinki Declaration. Monocyte-derived DCs (moDCs) were generated as described previously (Kramer et al, 2007). Immature moDCs were harvested on day 6 (day 6 moDCs) using cold PBS and afterwards were plated (0,5 * 10^6 cells/well) in 1ml of RPMI-1640 medium, enriched with 10% FCS, 5nM L-glutamine and 1% antimycotic/antibiotic (Gibco). In order to investigate the effect of the indolic compounds on AhR pathway activation, day 6 moDCs were exposed to indirubin, ICZ, malassezin, tryptanthrin and TCDD in 100 pM, 1nM, 10nM, 100nM and 1 μM for 6h or 24h.

Day 6 moDCs were exposed to TLR (LPS, R848, poly (I:C) and AhR ligands tested either as single agents or in combination for 6 or 24h in the aforementioned concentrations for the TLR ligands and at 100nM for the AhR ligands . Mature moDCs were acquired by exposing day 6 moDCs to the TLR ligands LPS or R848 for 24 hours. Subsequently, the effect of indirubin or ICZ was assessed by adding these indoles for 6 more hours.

Myeloid $BDCA1^+$ DCs (myDCs) were isolated from peripheral blood monocytes by positive selection using BDCA-1 beads and negative selection using CD19 beads, according to the manufacturer's instructions (Miltenyi Biotec). Purity achieved was consistently up to 95% as assessed by double staining with BDCA-1-PE and CD11c-APC monoclonal antibodies (Miltonic Biotic and BD Pharming, respectively). Myeloid $BDCA1^+$ DCs were cultured and exposed to the TLR ligands (LPS, R848) with or without the AhR-ligands (indirubin, ICZ). The

medium of exposure was X-vivo 15 medium (Lonza), enriched with 2% human serum and GM-CSF (450 U/ml). All cell cultures were performed in a humidified atmosphere at 5% CO_2 (v/v) and 37°C.

RNA isolation and quantitative PCR (qPCR)

RNA isolation was performed according to the manufacturer's instructions with the Quick-RNA Mini prep isolation kit (Zymo Research). RNA concentration and purity was calculated by Nanodrop 2000 (Thermo scientific, USA). RNA was treated with DNase I (Amplification Grade-Invitrogen) and reverse-transcribed into cDNA by using random hexamers and Moloney murine leukemia virus reverse transcriptase (RT) (Invitrogen) as suggested by the manufacturer. Genomic DNA contamination was excluded by inclusion of control samples without RT. cDNA was stored at -20°C until further use. mRNA levels for target genes were determined by quantitative PCR (qPCR) in a Bio-Rad CFX (Bio-Rad) employing SYBR Green (Applied Biosystems) and corresponding software (Bio-Rad CFX-1.6). PBGD/HMBS amplification was employed for relative quantification. Primer sets were acquired from the Primer Bank database (Wang and Seed, 2003).

Cell fixation and immunofluorescence

Following exposure to indirubin or ICZ, cells were allowed to adhere on poly-l-lysine (PLL) coated coverslips in serum-free medium and were fixed with 1% paraformaldehyde. Fixed cells were washed in PBS and permeabilized using 0,1% Triton X-100 in PBS followed by incubation in CLSM buffer (PBS with 3% BSA and 10 mM glycine buffer) that contained 1% human serum to block Fc–receptors. Consequently cells were stained with 5µg/ml mouse monoclonal AhR antibody (Abcam). After 2 washes, secondary staining was executed using Alexa 488-conjugated goat-anti mouse IgG (Invitrogen). Nuclear staining was performed using DAPI, and sealed using mowiol. Imaging was performed with an Olympus FV-1000 confocal microscope and corresponding Olympus FluoView software version 1.6a (Olympus).

Western blotting

Equal amounts of protein, extracted from $1.5*10^6$ cells, were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted into nitrocellulose membranes (Bio-Rad) and followed by probing with the indicated antibodies. CYP1A1 rabbit polyclonal (Abcam) and AhR mouse monoclonal antibodies (Abnova GmbH, Germany) were used in 1:50 (CYP1A1) and 1:200 (AhR) ratio. After washing, membranes were incubated with IRDye anti-mouse or anti-rabbit IgG (Li-Cor Biosciences). Relative quantification was determined with the use of actin (Sigma). All images were taken with the Odyssey Imaging System (Li-cor, USA).

Flow cytometry

Cells were harvested with cold PBS and washed twice in PBA (PBS containing 0.5% bovine serum albumin and 0.01% sodium azide) supplemented with 2% human serum. Cells were incubated with primary antibodies CD86, CD80 (both BD Pharmingen), CD83 (Immunotech), CD14 (Beckman Coulter), anti-HLA-DR/DP (Q5/13, BD Pharmingen) and the appropriate isotype for 20 minutes on ice followed by 2 washing steps in PBA. Subsequently, cells were incubated with Alexa488-labeled goat anti-mouse IgG antibody (Invitrogen Viability was measured by propidium Iodide (5µg/ml) staining.

Flow cytometry was executed on a CyAn Flow cytometer (Beckman Coulter, USA) and the results were analyzed using Summit v4.3 software (Dako, USA).

ELISA

Cells were cultured for 24h at $0,5*10^6$/ml in 12 well plates before supernatant collection. TNF-α, IL-10 and IL-6 production were analyzed using standard ELISA kits (TNF-α and IL-10 from BD and IL-6 from Sanquin, respectively) according to manufacturer's instructions. IL-12p70 production was analyzed with murine monoclonal capture and HRP-conjugated anti–IL12 antibodies (Pierce Endogen, USA), using standard ELISA procedure.

Mixed lymphocyte reaction (MLR)

MoDCs were stimulated with LPS and R848 in the presence or absence of indirubin and ICZ (100nM) and their allostimulatory capacity was assessed with the use of MLR. Allogeneic peripheral blood leukocytes (PBLs) were co-cultured with the activated moDCs in a 96-well round bottom plate (moDC:PBL ratio 1:20 with $1*10^5$ PBLs). After 4 days of culture, 1 μCi/well of tritiated thymidine (GE Healthcare, Eindhoven, Netherlands) was added for 9 h and incorporation was measured in a beta-counter.

Results

Distribution of *Malassezia* species within family members and phylogenetic analysis of the isolated *M. furfur* strains.

Out of the 84 patients included in the study 63 had positive cultures and 65 strains, belonging to five species, were isolated: *M. furfur* (N=30)*, M. sympodialis* (N=20)*, M. globosa* (N=8)*, M. restricta* (N=6)*,* and *M. slooffiae* (N=1) (figure 17). From 2 patients more than one species were isolated: *M. globosa* with *M. resticta* and *M. restricta* with *M. sympodialis*. *M. furfur* was the most frequently isolated species (Figure 29). None of the isolated *Malassezia* species clustered within families in a statistical significant way. In 10 out of the 18 families a single *Malassezia* species was isolated from all the members of the family, irrespective of presence or absence of skin disease. This included 7/10 families with *M. furfur* as the single isolated species, 2/10 with *M. sympodialis* and 1/10 with *M. globosa*. The *Malassezia* species isolation rates did not correlate with gender, sampling site or disease origin.

Phylogenetic associations of the *M. furfur* isolates were assessed by PCR-fingerprinting (Figure 30). Genotypic analysis of the banding pattern within the polyacrylamide gels was performed with the use of appropriate software (Bionumerics version 5.1). All *M. furfur* strains studied clustered in a group with ~40% genomic similarity (Figure 31). The genomic similarity of *M. furfur* strains isolated from a certain family ranged from 95% to 40%. Nevertheless, the present experimental data does not support the dominance of a particular *M. furfur* subtype within a family.

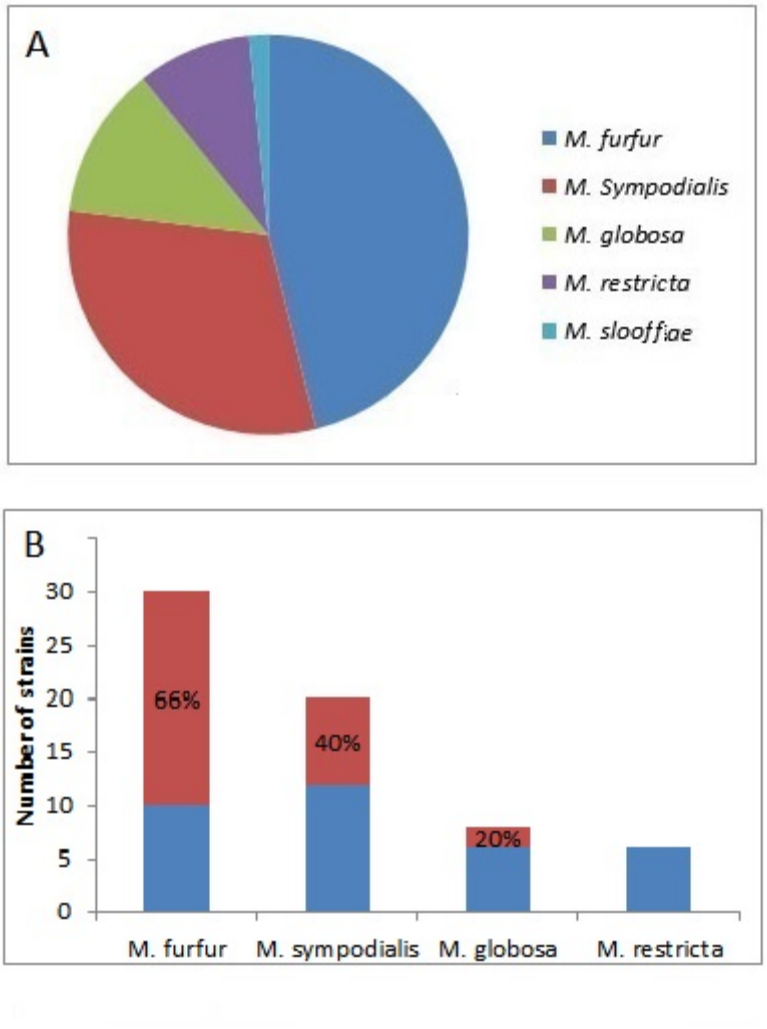


Figure 29: A) Distribution of the isolated species. *M. furfur* and *M. sympodialis* were the most frequently isolated species B) The number of *Malassezia* species strains isolated from at least 2 members of the same family (red) in comparison to the total number (red and blue).

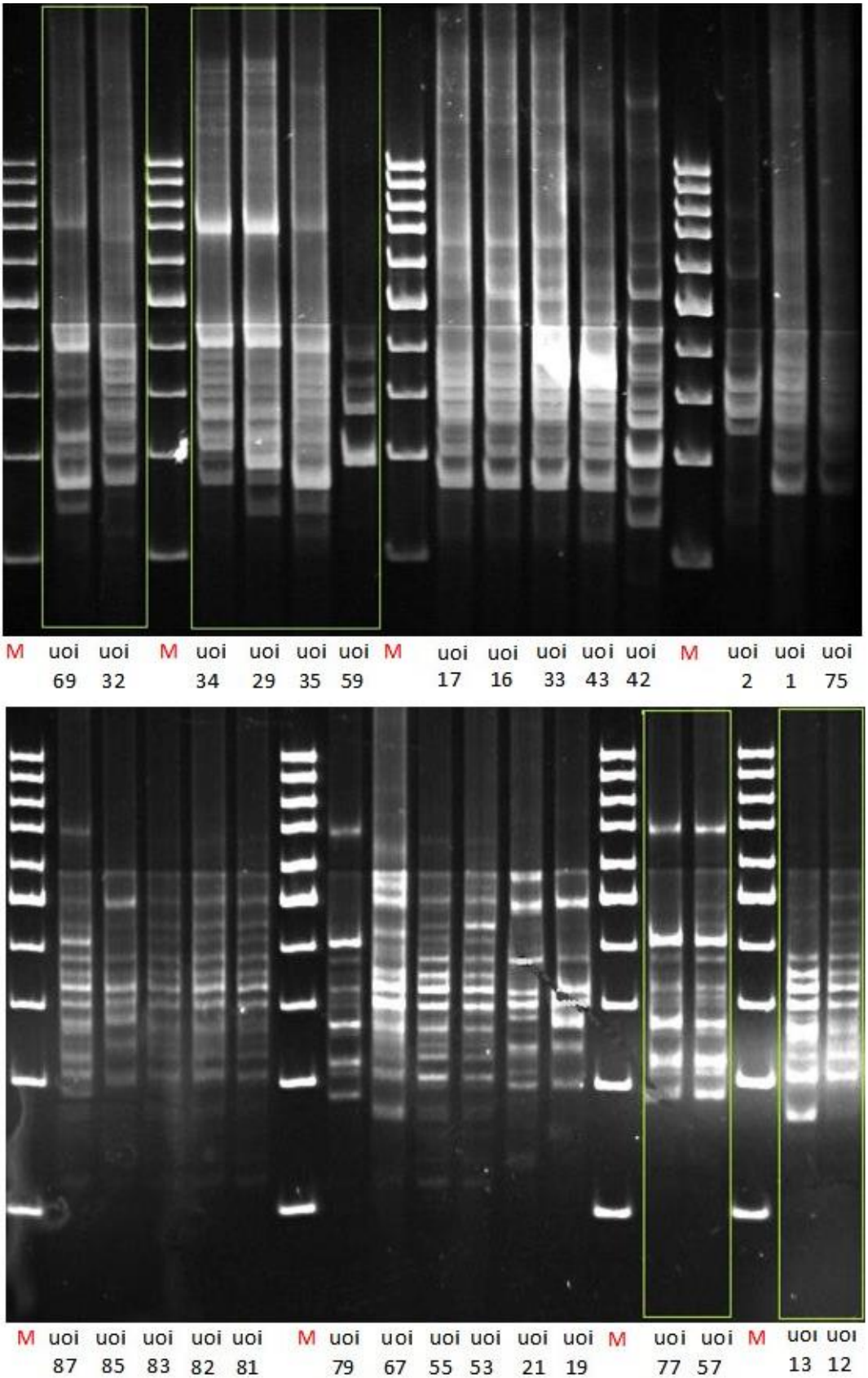


Figure 30: Genotypic analysis of the *M. furfur* subtypes with the use of PCR fingerprinting. In the representative polyacrylamide gels within each of the green boxes are strains isolated from members of the same family. (M: marker, uoi xx: taxonomy code of *Malassezia* species-University of Ioannina)

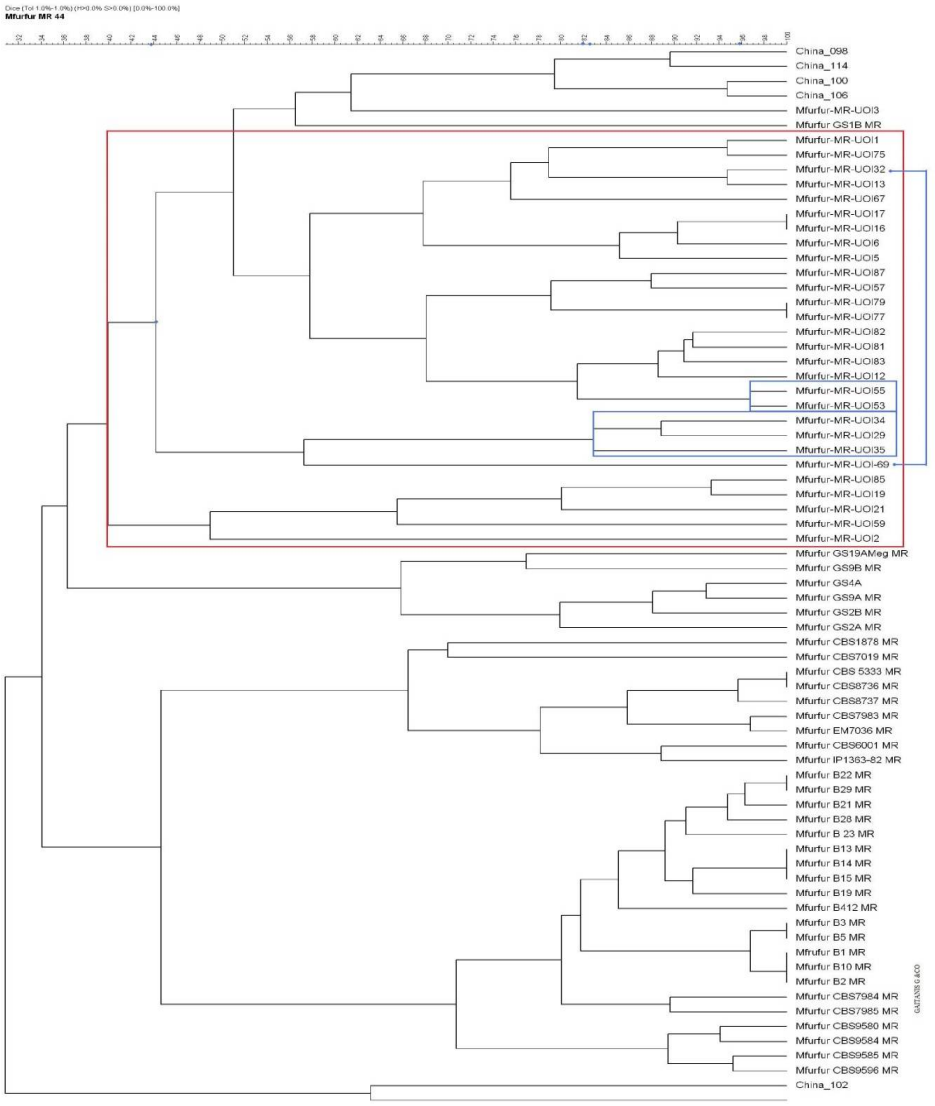


Figure 31: *M. furfur* phylogenetic tree. *M. furfur* strains isolated during this study (red rectangle) are compared with *M. furfur* isolates from a previous study (Gaitanis et al, 2008). A trend towards geographic clustering can be observed. However, strains isolated from the same family cluster either closely (blue rectangles) or with distinct phylogenetic distance (blue double arrow). The dendrogramm was generated employing Bionumerics software (version 4) using as an input the PCR fingerprinting results. (Origin of strains Gs: Swedish residing in Greece, B: Bulgaria, Cbs: fungal biodiversity centre - Netherlands, Ch: China, Uoi: University of Ioannina)

Phospholipase production by *Malassezia* strains in relation to yeast species, health status of the host and b-endorphin exposure of the relevant strains.

Phospholipase production was confirmed in the following Type and reference strains: *M. furfur* CBS 6001*, M. caprae* CBS10434*, M. pachydermatis* CBS1879*, M. dermatis* CBS 9170*, M. slooffiae* CBS7956*, M. globosa* CBS7966. The limited growth rate of certain reference strains of species such as *M. nana* (CBS 9558) might have affected their ability to produce a visible hydrolysis of lipids in the egg yolk medium.

Regarding the clinical strains phospholipase was not produced by 9% of the strains pre-incubated in the control medium and 13% of the strains pre-incubated in the b-endorphin (100nM) supplemented medium. An additional 3% of the isolated strains did not produce phospholipase in egg yolk medium irrespective of the preincubation medium. All the non-phospholipase producing strains were almost equally distributed among patients and healthy subjects. No association was observed between phospholipase production and gender, sampling site or family distribution. Additionally no significant difference was observed between the basic phospholipase production (without b-endorphin pre-exposure) of strains of different species (p=0.683).

Regarding the effect of b-endorphin in phospholipase activity, growth in 1 nM b-endorphin containing Dixon's medium had no significant effect in phospholipase activity. However, the 100 nM b-endorphin demonstrated a trend towards increased b-phospholipase activity in strains of the species *M. furfur* (p=0,063) and *M. restricta* (p=0,157) in contrast to *M. globosa* (0.655) and *M. sympodialis* (p=0.533) (Figure 32). For *M. furfur* the mean phospholipase activity index was altered from 0,93 (prior to exposure) to 0,88 and for *M. restricta* from 0,94 to 0,88. When compared to strains isolated from healthy skin, strains from seborrheic dermatitis lesional skin tend to have decreased phospholipase activity before (p=0.057) and increased after (p=0.061) exposure to b-endorphin (Figure 33). Phospholipase inducibility by b-endorphin per strain was measured as the ratio of phospholipase activity after b-endorphin exposure to basal enzyme activity. This inducibility ratio did not correlate with *Malassezia* species (p=0.652), however, it was found higher in strains isolated from SD lesional skin as compared to healthy skin (p=0.036) (Table 8).

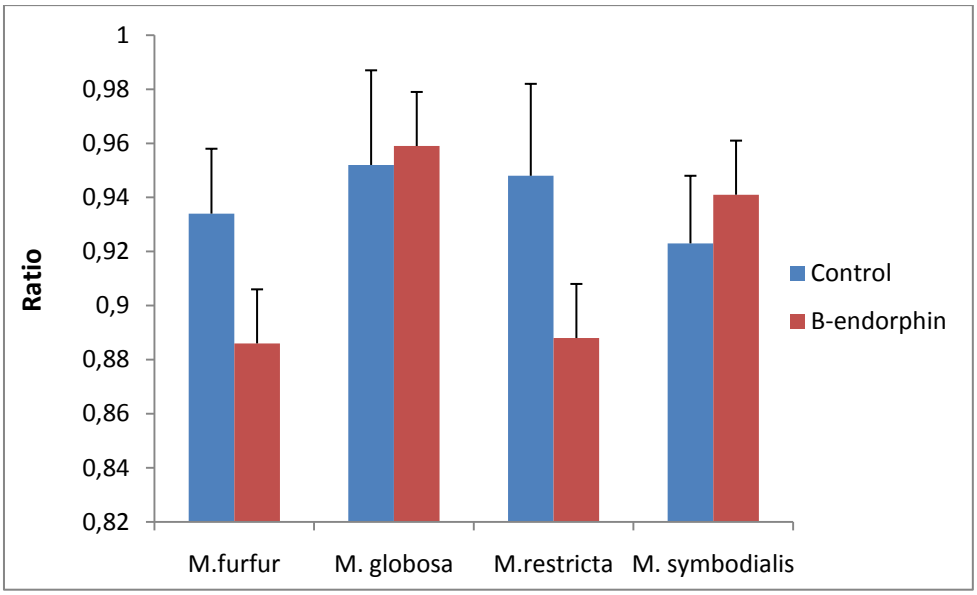


Figure 32: The recorded phospholipase activity was increased in *M. furfur* and *M. restricta* after b-endorphin (100 nM) exposure. This was not observed for *M. globosa* and *M. sympodialis*.

Table 8: Inducibility of phospholipase activity by b-endorphin is not depended on the particular species of the tested strain but rather on the skin disease it was isolated from. This strongly suggests an association of phospholipase inducibility and skin disease such as seborrheic dermatitis.

Malassezia species	*M. furfur*	*M. globosa*	*M. restricta*	*M. sympodialis*	All
No strains tested	30	8	6	20	64
Control	0.934±0.024	0.952±0.035	0.948±0.034	0.923±0.025	0.934±0.015
B-endorphin	0.886±0.032	0.959±0.041	0.888±0.071	0.941±0.018	0.912±0.018
P (Wilcoxon paired)	0.063	0.655	0.157	0.533	0.166
Ratio b-endorphin / control	0.950±0.027	1.01±0.017	0.929±0.045	1.034±0.034	0.981±0.018

Likelihood Ratio Test P-values

Phospholipase activity	Effect of *Malassezia*. species	SD vs. healthy
Control culture	0.683	0.057
Endorphin	0.606	0.061
Endorphin/control	0.652	0.036

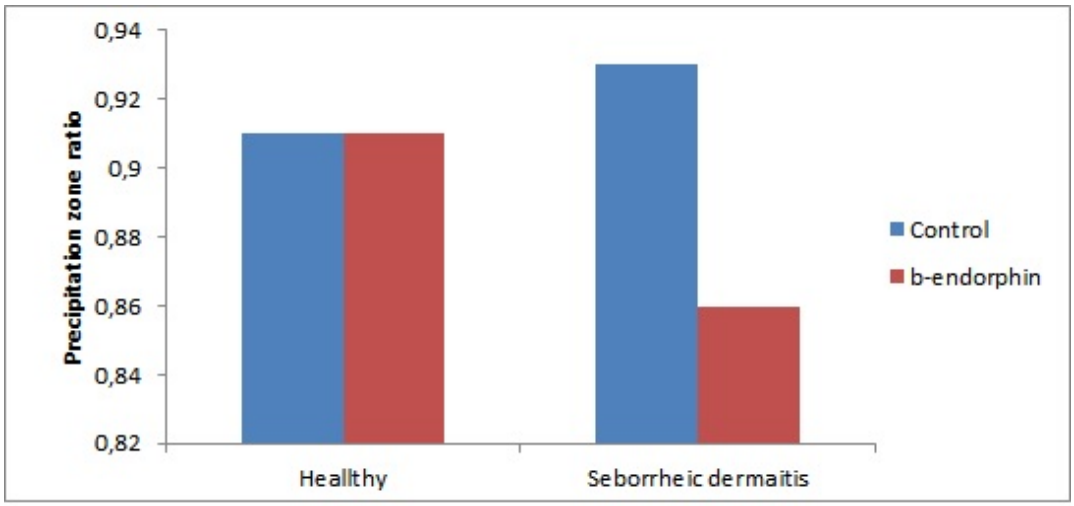


Figure 33: B-endorphin exposure does not alter the mean ratio of phospholipase activity in strains isolated from healthy individuals (p=0,65). Seborrheic dermatitis isolates tend to produce more phospholipase prior and after b endorphin exposure (p=0,057 and p=0,051 respectively) when compared to strains isolated from healthy skin. Also b-endorphin exposure (100 nM) significantly augments the lipolytic enzyme activity in those strains (p=0,037).

Discussion on *Malassezia* phospholipase production and species distribution among family members.

Proopiomelanocortin (POMC) is the precursor molecule of melanocyte stimulating hormones (a-MSH, b-MSH and c-MSH), corticotrophin (ACTH), lipotropins and b-endorphin and these active peptides are produced in a tissue specific manner (Milington et al, 2006). POMC is mainly synthesized in the central nervous system (pituitary gland and hypothalamus) but also in the skin by melanocytes and keratinocytes in a precursor form or as active peptide (Milington et al, 2006). B-endorphin can also be synthesized in unmyelinated nerve fibers that are found in the epidermis just beneath the stratum corneum (Biglardi et al, 2004). The presence and action of melanocortins in the skin is further indicated by the identification of the melanocortin receptor in human sebaceous glands and the control of lipid metabolism in rodent sebum glands by a-MSH (Thiboutot et al, 2000). B-endorphin binds to μ-opiate receptors, which are found in the basal cell layer of the epidermis and in the epithelial cells of skin adnexal structures (Bigliardi et al, 1998). The above data provide evidence that b-endorphin is present and has a physiological role in the epidermis (Biglardi et al, 2004; Kauser et al, 2004). Thus, it could modify the tissue effects of *Malassezia* on the adjacent skin.

Phospholipase activity is an established virulence factor in yeast species like *Candida albicans* (Mayser et al, 1996), *Cryptococcus neoformans* (Chen et al, 1997) and *Rhodotorula rubra* (Mayser et al, 1996) and also *M. pachydermatis* and *M. sympodialis* (Pini et al, 2011). Recently, with the use of immunoblotting and immunofluorescence, two potential micro-opioid receptor-like proteins were described on the surface of *M. pachydermatis* strains isolated from both healthy and dermatitis suffering dogs (Cafarchia et al, 2009). The functionality of these ‘’potential opioid receptors’’, regarding their effects on the yeast phospholipase activity, was tested using both agonistic and antagonistic ligands (b-endorphin and naloxone) (Cafarchia et al, 2009, Cafarchia et al, 2008). Phospholipase activity was modulated in *M. pachydermatis* strains isolated from dogs with dermatitis, in a dose depended way. On the contrary, strains originating from healthy dogs did not produce lipases (89,5%) (Cafarchia et al, 2004; Cafarchia et al, 2010).

The herein presented results strongly support the concept that phospholipase enzymatic activity and especially its inducibility after b-endorphin exposure is a metabolic feature of the yeast that correlates with *Malassezia* strains isolated from SD lesions. Our current

findings suggest that phospholipase activity is present *in vitro* in all clinically significant *Malassezia* species and could represent a candidate virulence factor in accordance to the previous results shown for *M. pachydermatis* (Cafarchia et al, 2004;Cafarchia et al, 2010). This is further in line with the recent demonstration of increased *Malassezia* lipase and phospholipase mRNA expression in SD lesional skin (Patino-Uzcategui et al, 2011). Furthermore, the production of secreted lipases by *M. globosa* has been associated with the development of dandruff (Dawson et al, 2007) which is considered by some investigators as a mild form of SD.

A stimulating novel finding in this study is the induction of phospholipase enzymatic activity of *Malassezia* SD isolates after incubation with 100nM b-endorphin. This supports the existence of *Malassezia* strains with diverse pathogenic capacity within the same species of this genus, a finding that has been observed in the past exclusively for *M. furfur* (Gaitanis et al, 2012; Kaneko et al, 2011). It has been confirmed that SD is not associated with seborrhea (Burton et al, 1983) but rather with phases of altered production of sebum in the skin. An indication of this relationship is provided by the decrease in sebum production in autumn and the simultaneous exacerbation of SD (Youn et al, 2005). Sebum is the primary source of energy for *Malassezia* yeasts (Gaitanis et al, 2012). Thus, it is possible that strains with enhanced phospholipase activity might have a survival advantage in a dynamically varying skin microenvironment, like that of SD skin lesions. This is further supported by the observation that *Malassezia* blood isolates from patients with catheter associated fungemia have increased *in vitro* phospholipase activity when compared with strains from the same patients' skin or other healthy individuals (Kaneko et al, 2011). Blood circulation is a hostile environment for *Malassezia* yeasts and the extreme incapacitating conditions due to host's immune reactions could be coupled by increased survival ability of the infecting strains by the increased phospholipase activity. Likewise, in inflammatory skin diseases immune responses can be coupled by the modified *Malassezia* phospholipase activity.

Conclusively, inducibility of secreted phospholipase activity by b-endorphin is a candidate virulence factor for *Malassezia* yeasts. The correlation with skin diseases, particularly SD, could be the target for further research evaluating enzymatic expression characteristics of this yeast. Nevertheless, the expression of functional opioid-like receptors by anthropophilic *Malassezia* yeasts will have to be verified and more accurately quantified with molecular methods.

Regarding the familial distribution of *Malassezia* species and its relation to disease state or severity, as presented above, none statistical significance appeared. The presence of more

than one species per host, in some cases, underlines the complexity in the relationship between the species and the epidemiological characteristics of the examined population. *M. furfur* appears to be more likely isolated and distributed into families than the rest species, but this result is biased by the constitution of the examined population (the number of family members is greater than the control population) and the higher M. furfur isolation rate. The high growth rate of the specific species (*M. furfur-M. sympodialis*) that increases their chance of isolation should also be taken under account. The genotypic analysis of the *M. furfur* strains did not support the dominance of a particular subtype within a family, nor did collate a subtype with seborrheic dermatitis. This fact further indicates that the appearance of seborrheic dermatitis is not clearly based on the genomic characteristics of the yeast but on a more complicated yeast-host interaction that has also a metabolomics perspective. Moreover, this genotypic analysis made evident that a close association between hosts, as in family, is not enough to ensure a strain or species dominance on the host's skin.

In vitro effects of indolic compounds produced in *vitro* by *Malassezia* yeasts on human dendritic cells.

Cyp1a1 and *Cyp1b1* gene transcription depends on ligand structure and concentration.

Day 6 immature moDCs were exposed for 6h to 100pM, 1nM, 10nM, 100nM and 1μM of indirubin, ICZ, malassezin, tryptanthrin and TCDD. As shown in Figure 34A, no increase in the expression of the AhR-target *Cyp1a1* was observed at concentrations of 100 pM or 1nM. Addition of 10nM indirubin resulted in significant ($p<0.05$) induction of *Cyp1a1*, whereas all other ligands including TCDD did not induce any *Cyp1a1* expression. At the higher concentrations of 100nM and 1μM, all ligands tested significantly upregulated *Cyp1a1* transcription with indirubin being still the most potent ligand (>40-fold at 100 nM concentration compared to unstimulated cells; Figure 34A). ICZ was the second most potent ligand, and induced *Cyp1a1* to similar extent as indirubin at 1μM. Notably, stimulation with indirubin showed 4-fold increase of *Cyp1a1* at 6 hours compared to the well-studied exogenous ligand TCDD (Figure 34B). 24h after stimulation, CYP1A1 mRNA was reduced for indirubin, when compared to the 6h time point. A similar trend was also observed for the remaining of the naturally occurring ligand. TCDD induced activation was also decreased, yet to a lesser degree (Figure 34B).

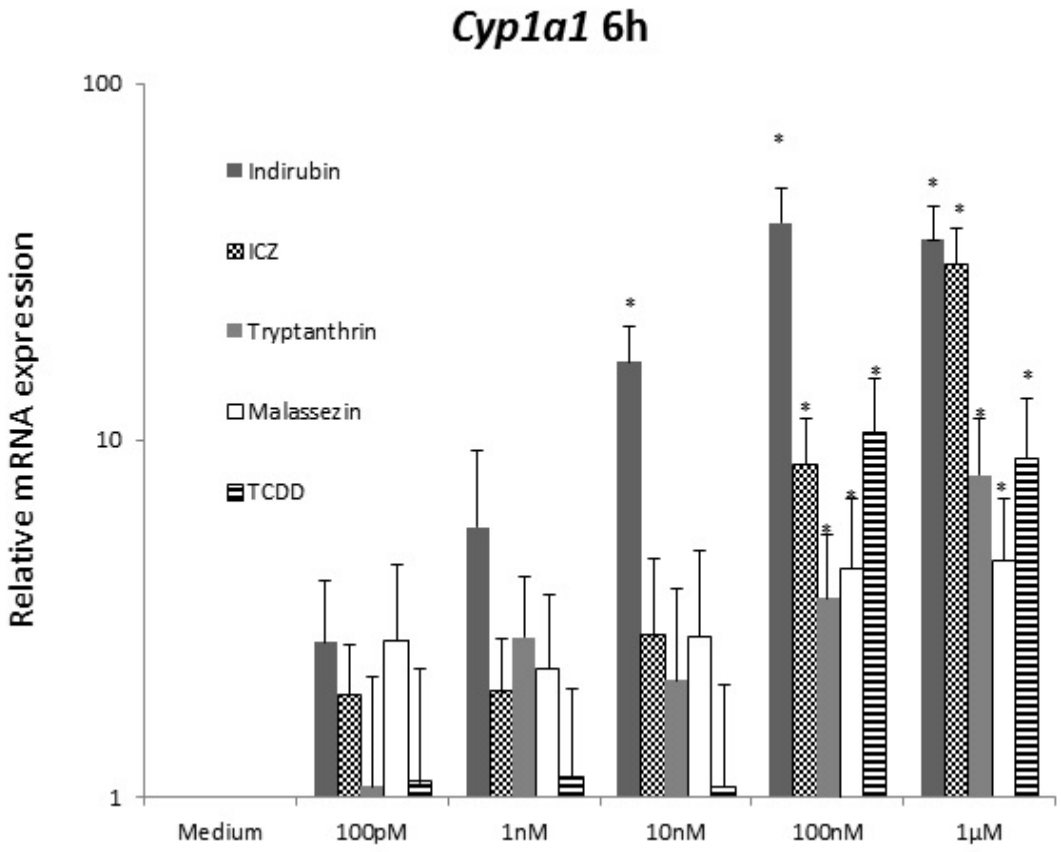


Figure 34A: Induction of *Cyp1a1* in monocyte derived dendritic cells exposed to increasing concentrations of indirubin, ICZ, tryptanthrin, malassezin and TCDD for 6 hours. 6 hours after exposure of monocyte derived dendritic cells to the selected concentrations of AhR-ligands mRNA was isolated and CYP1A1 mRNA was determined. Relative CYP1A1 mRNA concentrations are displayed as quotients to unstimulated cells (medium only groups). All tested compounds significantly upregulated *Cyp1a1* expression. Indirubin and ICZ seem to be the most potent ones. (*Statistical significance at $p<0.05$ compared to medium; axons demonstrate standard deviation, all results are averages of at least three independent experiments)

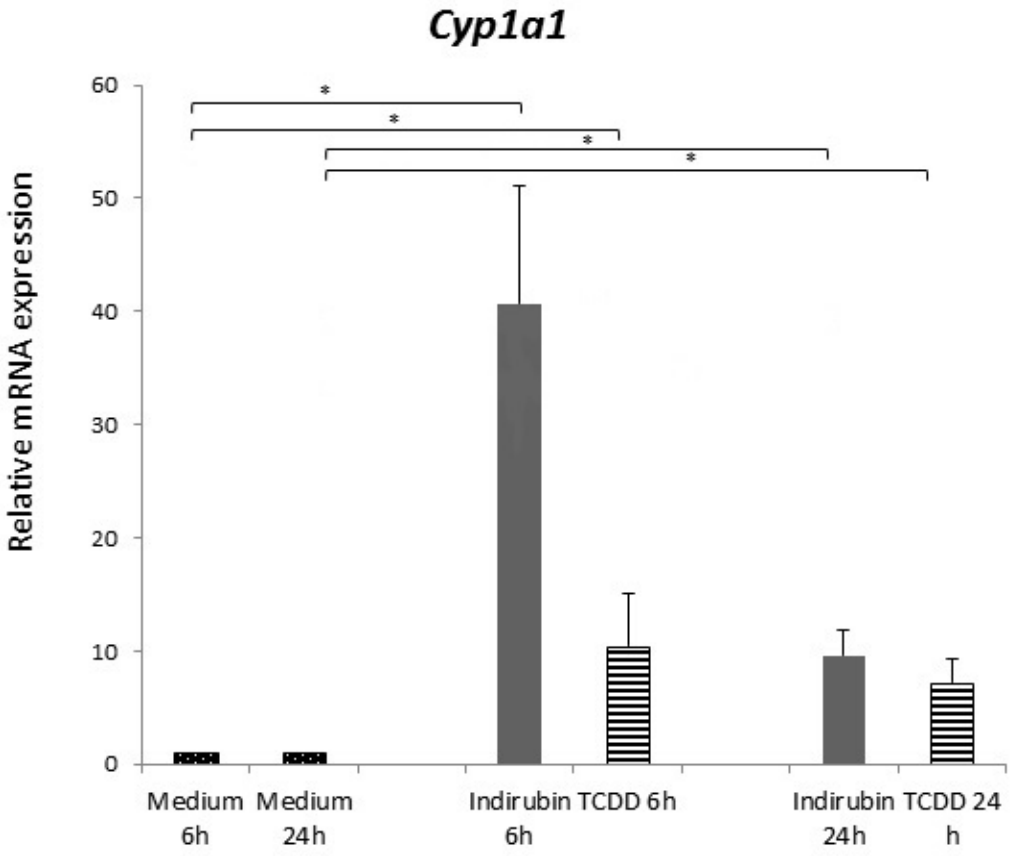


Figure 34B: AhR pathway activation by indirubin in the 24h time point is reduced in comparison to the 6 h time point. Monocyte derived dendritic cells were stimulated with 100 nM of indicated ligands and *Cyp1a1* relative expression was determined at 6 and 24 hours. TCDD caused induction of *CYP1A1* mRNA displayed a mild decrease at 24 hours while the mRNA levels of these enzyme achieved by indirubin stimulation at 6h, were reduced up to 70% at 24 hours.
*Statistical significance at $p<0.05$ compared to medium; axons demonstrate standard deviation, all results are averages of at least three independent experiments

Indirubin and ICZ also significantly induced the expression of other genes, members of the AhR gene battery, like *Cyp1b1 or Ahrr* in moDCs ($p<0.05$; Figures 35A, 35B). $BDCA1^+$ myeloid DCs are a subpopulation of DCs which are also present in the skin (myDCs) (Zaba et al, 2007). We tested whether AhR-dependent gene expression is also modified in these cells upon exposure to the tested indolic ligands. Indirubin and to a lesser extent ICZ induced *Cyp1a1* in $BDCA1^+$ myDCs directly isolated from blood ($p<0.05$; Figure 35C). The AhR dependency of all the aforementioned results was confirmed by partial inhibition of the observed induction during co-exposure with the AhR inhibitor CH223191 (Figure 35D).

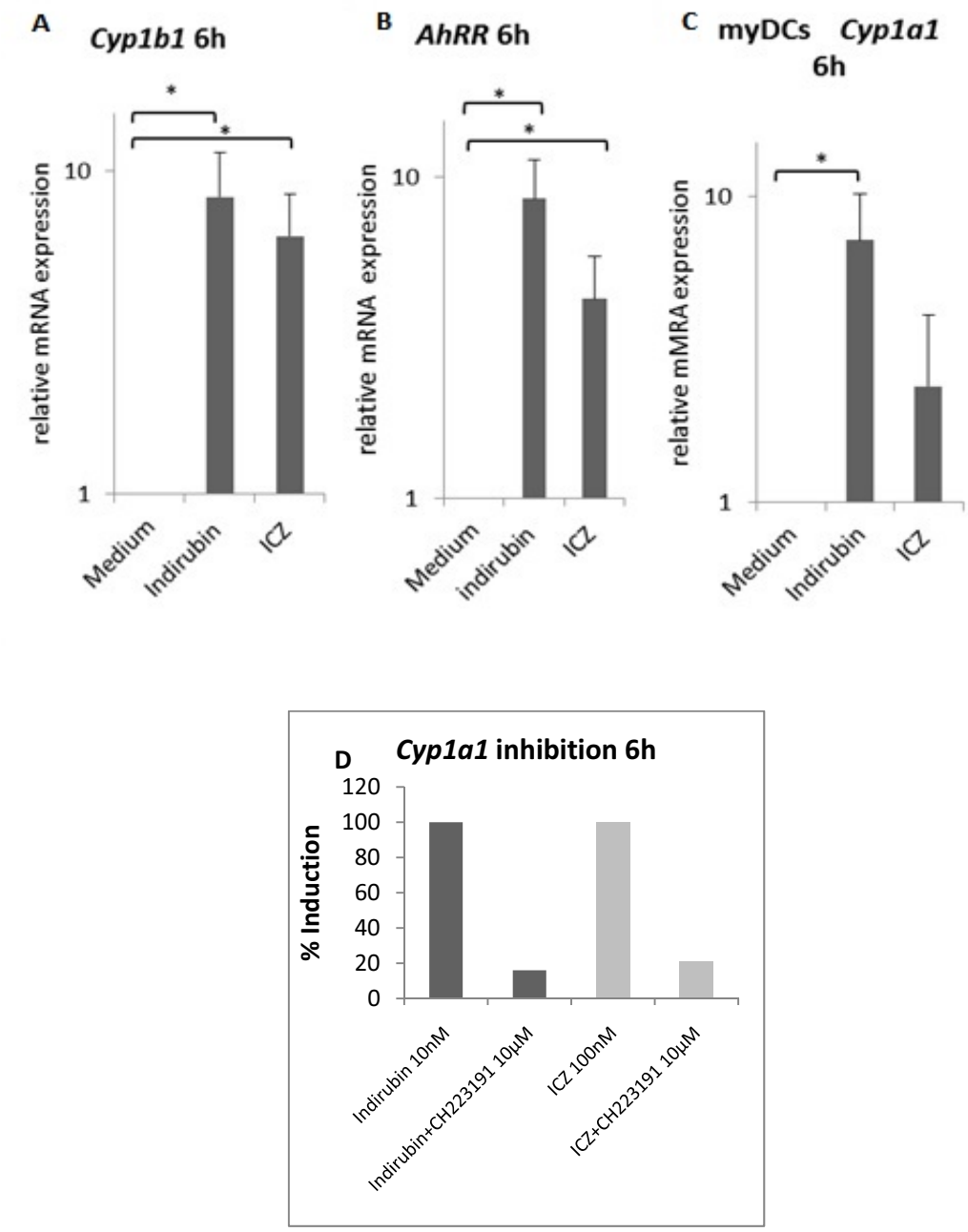


Figure 35: Upregulation of the expression of *Cyp1b1* and *AHRR* in monocyte derived dendritic cells and of *Cyp1a1* in myeloid derived dendritic cells by indirubin and ICZ in an AhR depended way. Panel A: *Cyp1b1* expression was determined as in Figure 34 upon stimulation of monocyte derived dendritic cells for 6 hours with 100nM of the indicated ligands. Panel B: AhRR expression was determined as for (a). Panel C: $BDCA1^{+}$ myeloid DCs (mDCs) were stimulated with indirubin and ICZ and mRNA expression was determined as for Panel A. Panel D: Monocyte derived dendritic cells were co-exposed for 6 hours to 10µM of CH223291 and 10nM of indirubin or 100nM ICZ (*Statistical significance at $p<0.05$ compared to medium; Bars demonstrate standard deviation, all results are averages of at least three independent experiments).

The ability of the tested indolic compounds to induce AhR activation was further verified by demonstrating increased expression of additional target genes of the xeniobiotic responsive element, as is *Gstp1* (Figure 36A).

Exposure of moDCs for 6 or 24h to the selected indolic compounds at concentrations of 100nM (Figure 36B) or 1μM, had no significant effect (despite and incremental trend) on AhR and ARNT mRNA levels.

The above titration experiments showed that indirubin and ICZ were the most active ligands and thus they were selected for subsequent experiments.

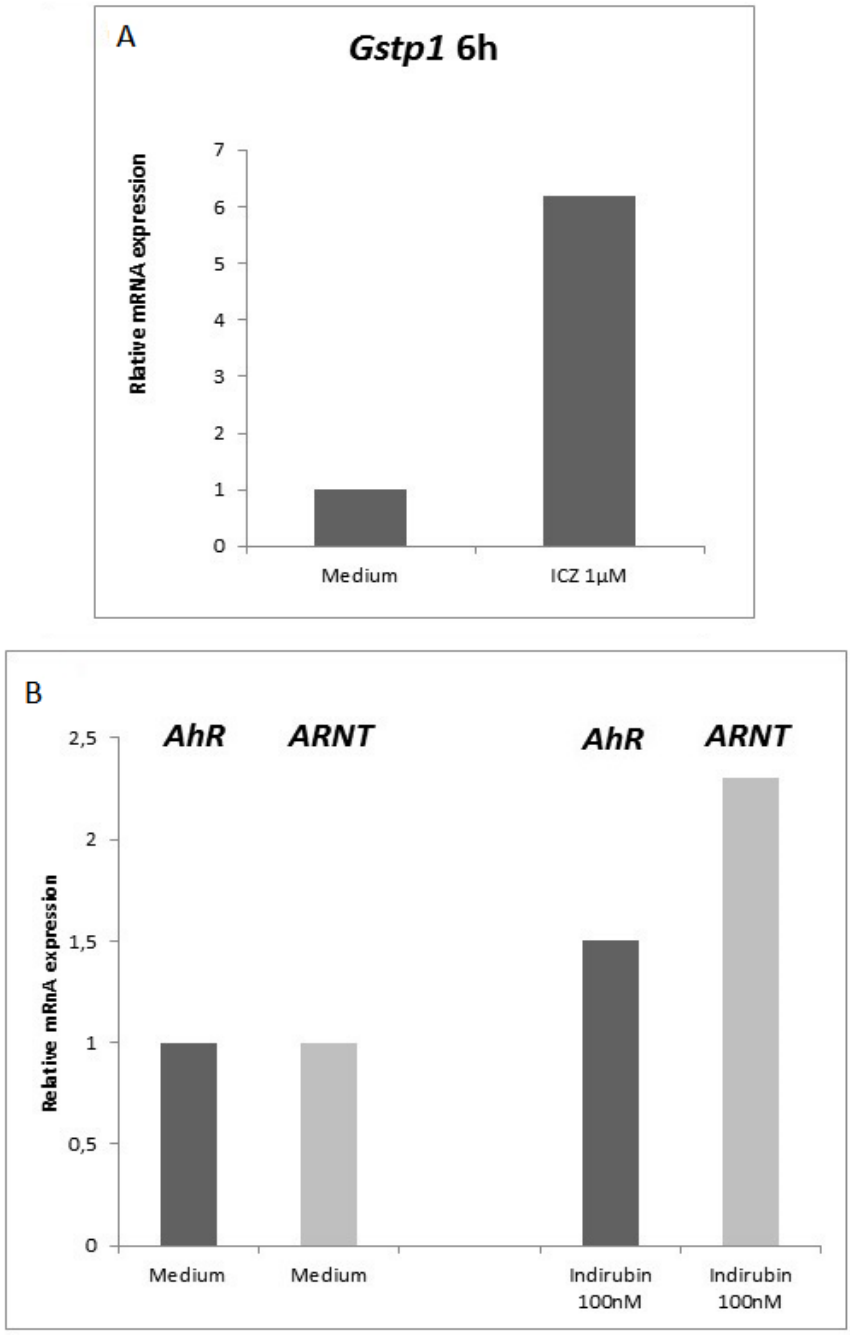


Figure 36: Upregulation of *Gstp 1* in monocyte derived dendritic cells after exposure to 100nM ICZ for 6h (panel A) (representative experiment). *AhR* and *ARNT* are not significantly upregulated in monocyte derived dendritic cells after exposure to 100nM indirubin for 24h (panel B).

Exposure to indirubin and ICZ causes AhR nuclear translocation in moDCs.

Nuclear translocation of AhR in moDCs exposed to 100nM of indirubin, ICZ or TCDD was confirmed by confocal microscopy. In accordance with previous experiments using human and rodent cell lines (Garside et al, 2008) translocation of AhR to the nucleus was evident already at 30 min and was highest at 60 min after the onset of stimulation (Figure 37, 38). After 6h of continuous stimulation, nuclear concentration of AhR decreased. AhR translocation to the nucleus was equivalent in immature moDCs and in moDCs that besides indirubin were concurrently exposed to 100ng/ml of LPS (Figure 39).

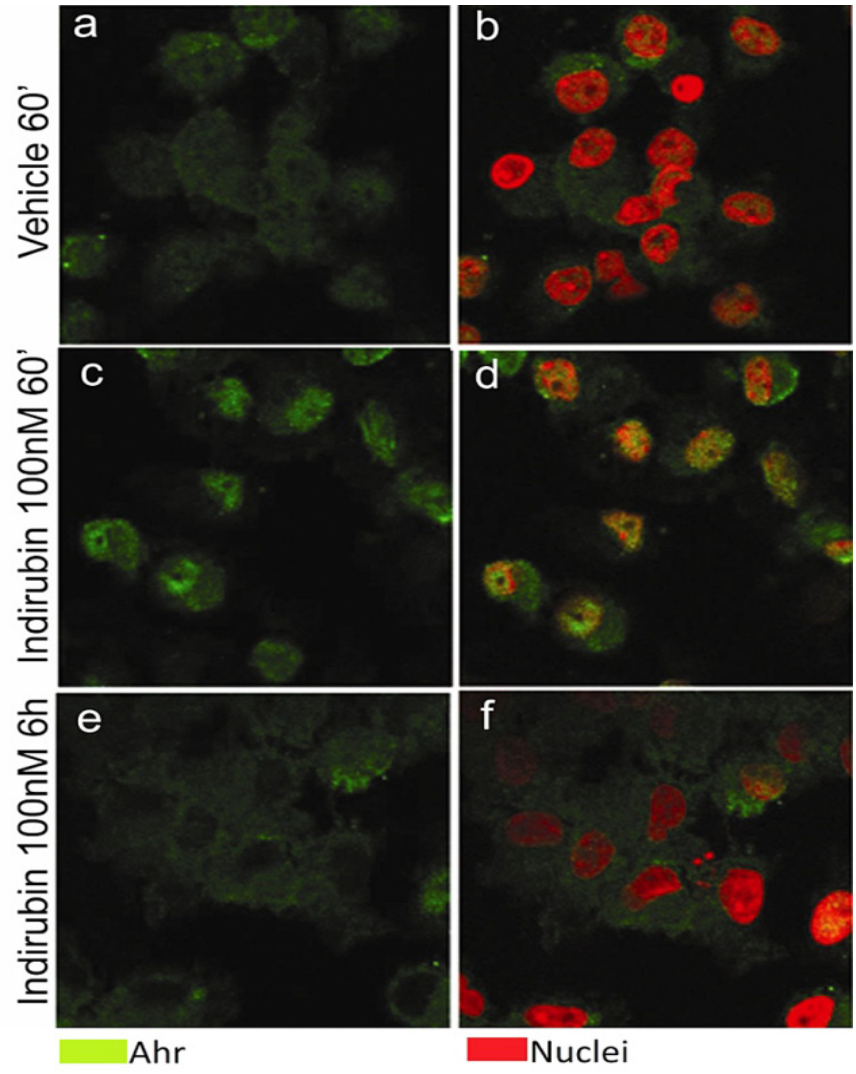
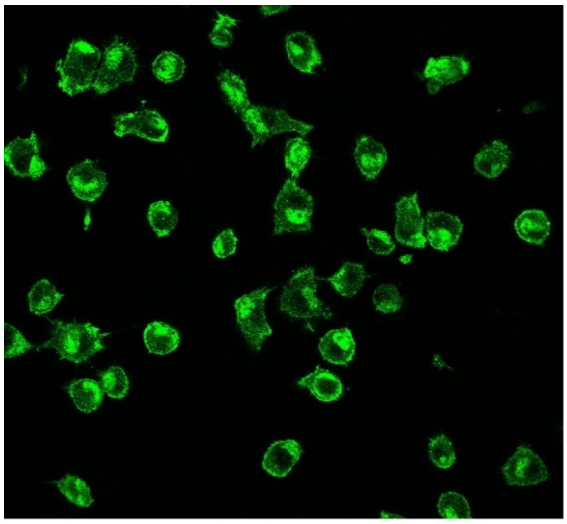


Figure 37: AhR translocates to the nucleus of monocyte derived dendritic cells (moDCs) upon stimulation with indirubin. moDCs were exposed to indirubin or vehicle and subsequently AhR localization was determined by confocal microscopy at the indicated time points. Panels a, b: Vehicle; Panels c, d: exposure to 100nM indirubin for 60 minutes; Panels e, f: exposure to 100nM indirubin for 6 hours. AhR is shown in green, the nuclei in red. The nuclear/cytoplasmic ratio peaks at 60minutes and gradually decreases again in the following hours.



AhR

Figure 38: Monocyte derived dendritic cells were exposed to indirubin (100nM) for 1 hour and subsequently were stained with the AhR antibody. Perforation of the cells with methanol instead of Triton X-100, in order to achieve intracellular and intranuclear staining, results in accumulation of AhR in the cell membrane, besides nuclear accumulation. Presence of AhR is shown as green color.

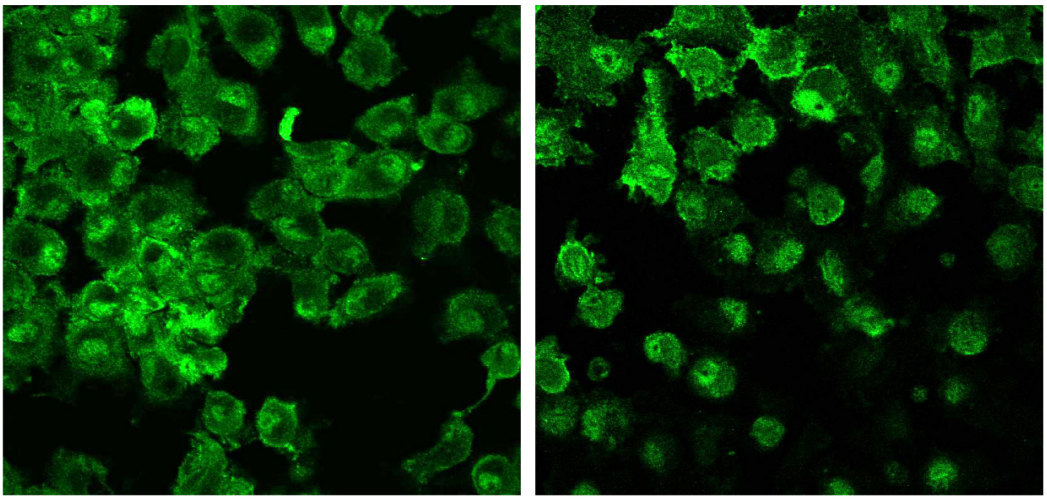
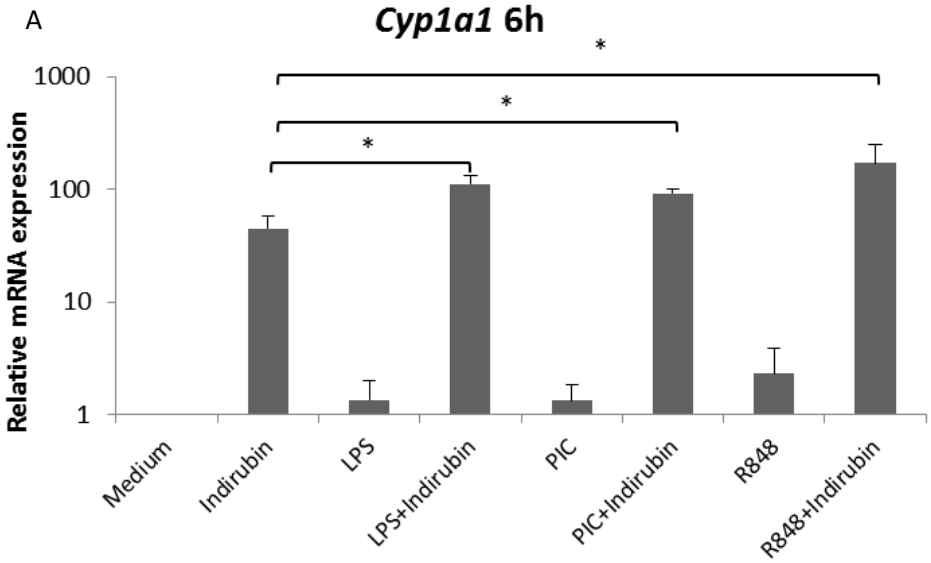
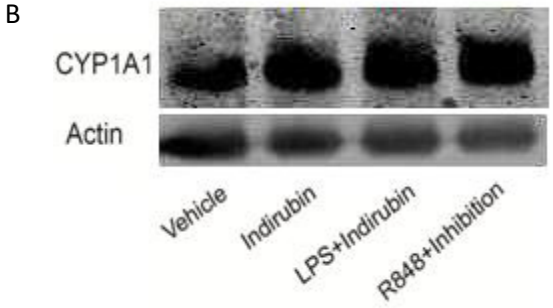


Figure 39: The nuclear concentration of AhR after exposure to indolic compounds is not evidently different between 'steady state' and LPS stimulated dendritic cells. Monocyte derived dendritic cells were simultaneously exposed to indirubin (100nM) alone (right panel) or to either LPS (100 ng/ml) plus indirubin (left panel). Presence of AhR is indicated as green fluorescence.

TLR ligands enhance AhR induced *Cyp1a1* and *Cyp1b1* expression in moDCs.

Both TLR and AhR ligands are present on the surface of the skin as they constitute part of the bacterial or fungal structures (Mempel et al, 2007; Netea et al, 2004) or possibly result from their metabolic activity (Magiatis et al, 2013). To assess possible modifications of AhR signaling on TLR cell stimulation, day 6 moDCs were exposed to the TLR ligands LPS, PIC or R848 in the presence or absence of selected indolic compounds (indirubin or ICZ) for 6 and 24 hours. No upregulation of *CYP1A1* or *CYP1B1* mRNA was found following TLR ligand exposure alone (Figure 40A). However, simultaneous exposure of moDCs to TLR ligands and indirubin for 6h or 24h showed a statistically significant increase in the transcription of the target genes at mRNA level ($p<0.05$; Figure 40A) as compared to indirubin alone. For indirubin/indirubin and LPS or R848 this was also observed and at the protein level (Figure 41B). This increase was also recorded for ICZ and TCDD, yet it was less pronounced (Figure 41C).


A
Cyp1a1 6h
Relative mRNA expression
1000
100
10
1
*
*
*
Medium
Indirubin
LPS
LPS+Indirubin
PIC
PIC+Indirubin
R848
R848+Indirubin


B
CYP1A1
Actin
Vehicle
Indirubin
LPS+Indirubin
R848+Inhibition

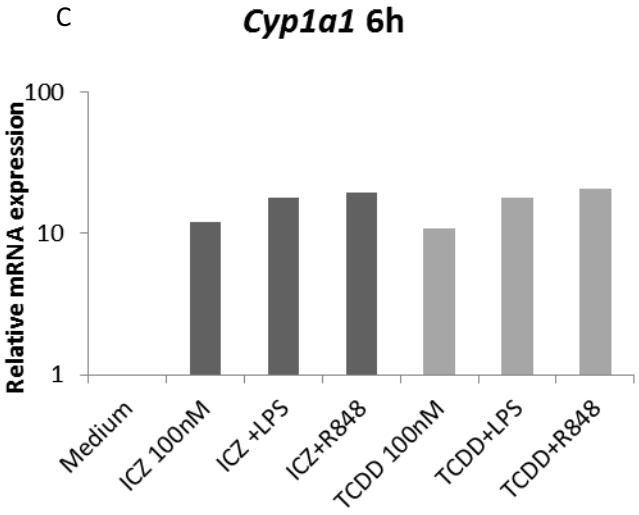

C
Cyp1a1 6h
Relative mRNA expression
100
10
1
Medium
ICZ 100nM
ICZ +LPS
ICZ+R848
TCDD 100nM
TCDD+LPS
TCDD+R848

Figure 41: Co-exposure of moDCs to TLR and AhR ligands increases *Cyp1a1* expression in comparison to single AhR ligand exposure. Panel A: moDCs were stimulated for 6 hours with the indicated TLR ligands (LPS 100ng/ml, R848 4µg/ml or poly(I:C) 20 µg/ml) in the presence or absence of 100nM indirubin. (*Statistical significance at $p<0.05$ compared to indirubin; Bars demonstrate standard deviation, results are averages of at least 3 separate experiments). Panel B: Increased induction of CYP1A1 protein levels upon indirubin stimulation. moDCs were stimulated as described above and CYP1A1 protein levels were determined after 24hrs by Western blotting. Panel c: The increase in Cyp1a1 induction by concurrent exposure to AhR
was also confirmed for ICZ and TCDD.

Next we tested the response of moDCs that were allowed to mature for 24 hours with TLR ligands before AhR stimulation. Interestingly, *Cyp1a1* and *Cyp1b1* expression was increased in mature moDCs exposed to LPS and R848 for 24 hours prior to AhR stimulation for 6 hours (Figure 42), when compared to DCs that were concurrently stimulated with these ligands. This finding indicates a possible link between DCs' maturation state and P450 enzyme's inducibility. When moDCs were exposed to LPS and R848, AhR mRNA levels demonstrated a small increase (~2 fold) (Figure 43A) but these results could not be confirmed at the protein level (Figure 43B). This suggests that induction of *Cyp1a1* and *Cyp1b1* is not simply due to the presence of higher AhR-protein levels in matured moDCs.

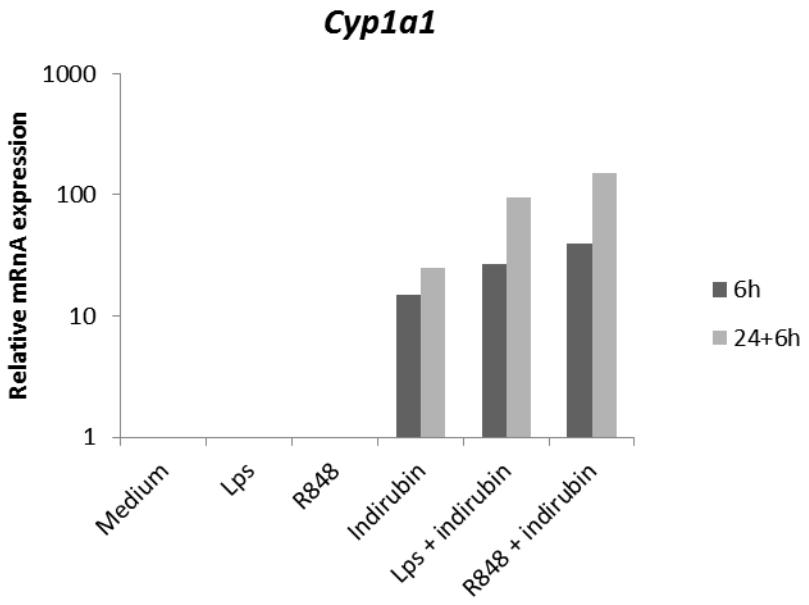


Figure 42: Monocyte derived dendritic cells were either exposed to TLR ligands (LPS 100ng/ml, R848 4μg/ml) and indirubin (100nM) alone or concurrently for 6 hours (indicated as 6h), or were previously matured by exposure to TLR ligands for 24h and subsequently exposed to indirubin for 6 additional hours (indicated 24+ 6h). Exposure to the single TLR ligands (LPS and R848) for 6-24-30 hours did not result in an increase of the *Cyp1a1* mRNA. mRNA expression of *Cyp1a1* was determined as previously described (representative experiment).

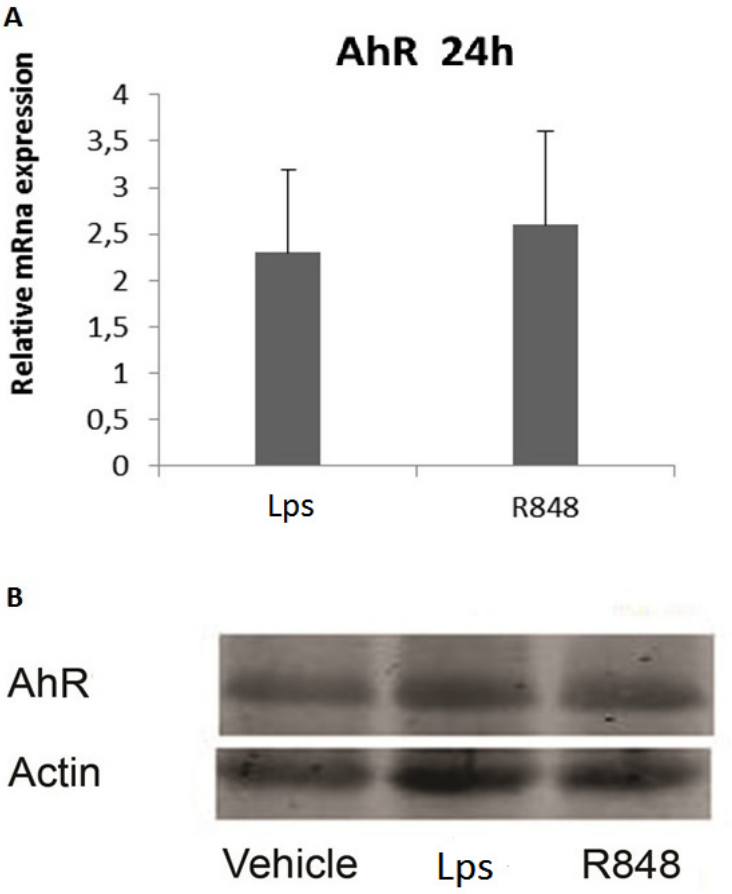


Figure 43: Monocyte derived dendritic cells that were exposed to LPS (100ng/ml) or R848 (4μg/ml) for 24h displayed a mild increase (~2 fold) in AhR mRNA levels (Panel A). However, no significant increase in AhR protein was exhibited, when Western blotting was performed under the same conditions (Panel B). The mild mRNA increase and the non-measurable alteration in the protein levels of AhR indicate that the induction of *Cyp1a1* and *Cyp1b1* could not be solely the result of higher AhR levels in Lps-matured dendritic cells.

Indirubin or ICZ limit the phenotypic maturation of 'inflammatory' moDCs.

The effects of AhR pathway stimulation on certain moDCs phenotypic markers were assessed. When immature moDCs were exposed to indirubin or ICZ for 24 or 48 hours, no significant alterations were found in the expression of the major histocompatibility complex II (MHC) and co-stimulatory molecules (CD80, CD83, CD86) on their cell surface (Figure 44A). On the contrary, stimulation of moDCs with LPS or R848 resulted in upregulation of costimulatory markers and MHC II. However, when moDCs were exposed to indirubin or ICZ during maturation with LPS or R848 (simultaneous exposure), a statistically significant inhibition in the expression of these surface markers was observed ($p<0.05$; Figure 44B). Interestingly, the impact of the indolic compounds on the inhibition of moDC phenotypic maturation was not dose-depended (Figure 45), indicating a complex interaction of AhR downstream with the cellular inflammation pathway.

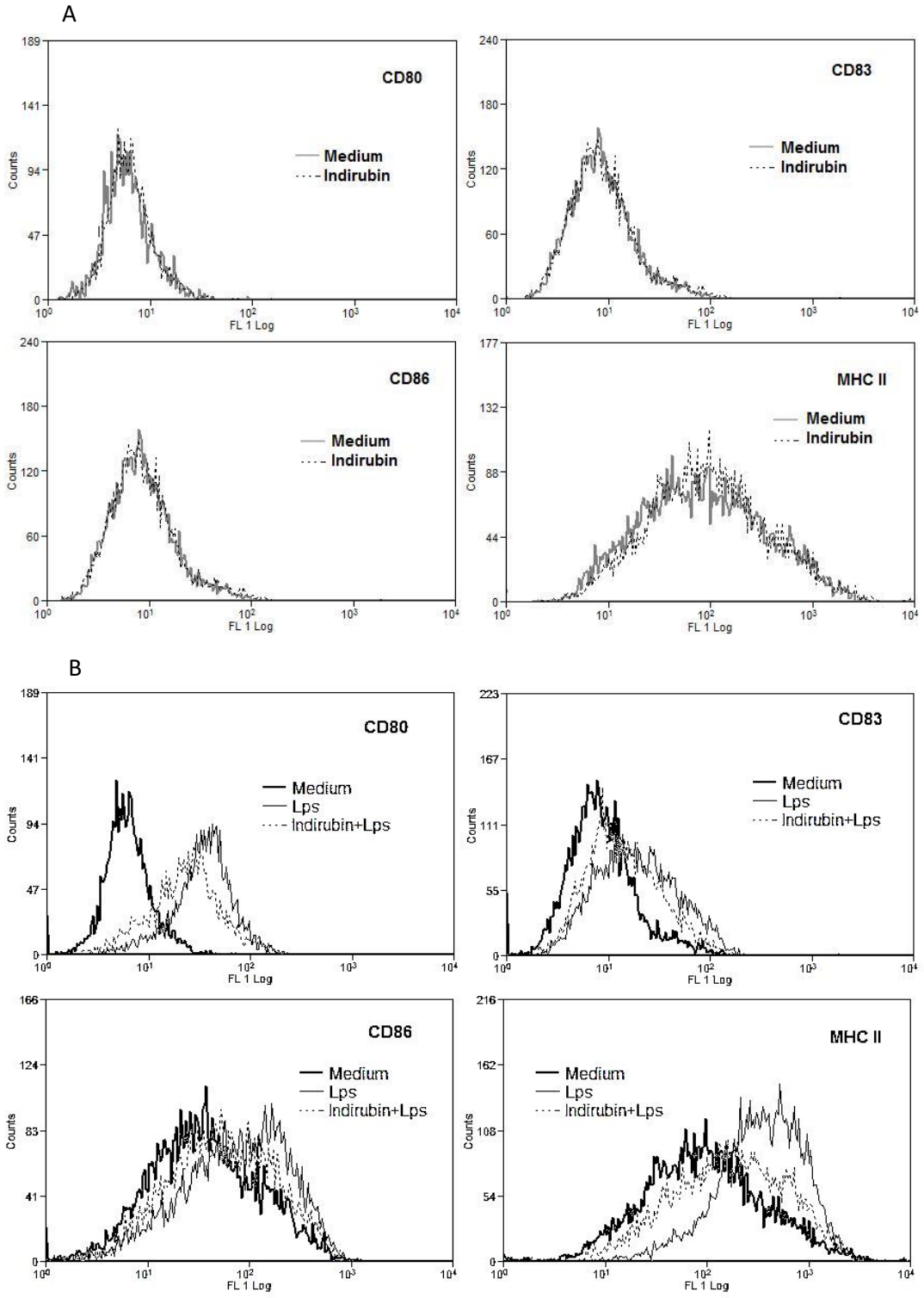
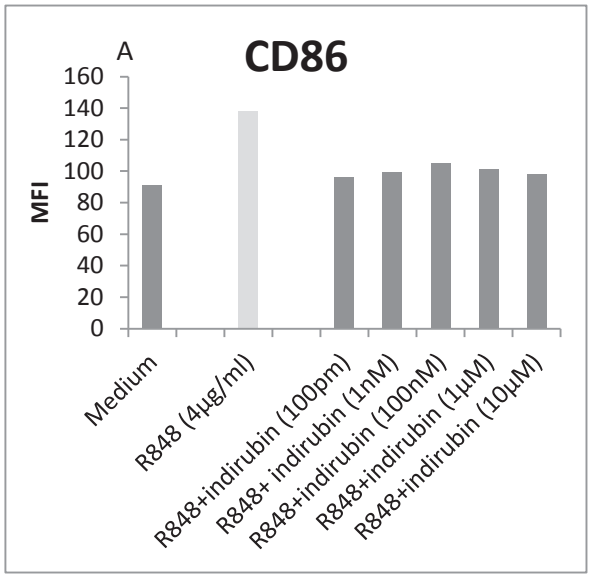


Figure 44: Indirubin or ICZ inhibit the phenotypic maturation of inflammatory monocyte derived dendritic cells. Panel A: Flowcytometry **(**FCM) histograms of surface maturations markers (CD80, CD83, CD86, MHC-II) of immature monocyte derived dendritic cells after exposure to indirubin (100nM) for 24h (dotted line) compared to medium (solid line). No significant alteration regarding the indicated cell surface markers was observed (representative experiment)**.** Panel B: Flowcytometry histograms of surface maturation markers (CD80, CD83, CD86, MHC-II) of immature monocyte derived dendritic cells after

exposure either to LPS (100ng/ml, black line), to LPS and indirubin (Indirubin+LPS, dotted line) or left untreated (medium; grey line) for 24 hours. A partial but significant inhibition in the expression of maturation markers was displayed, with the addition of indirubin (representative experiment).



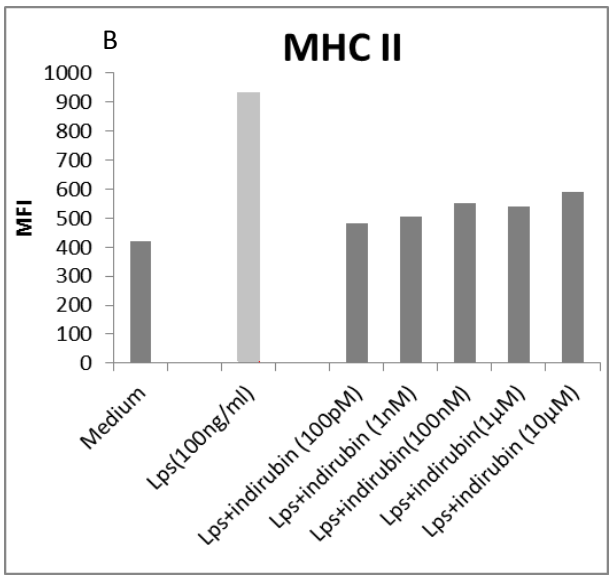
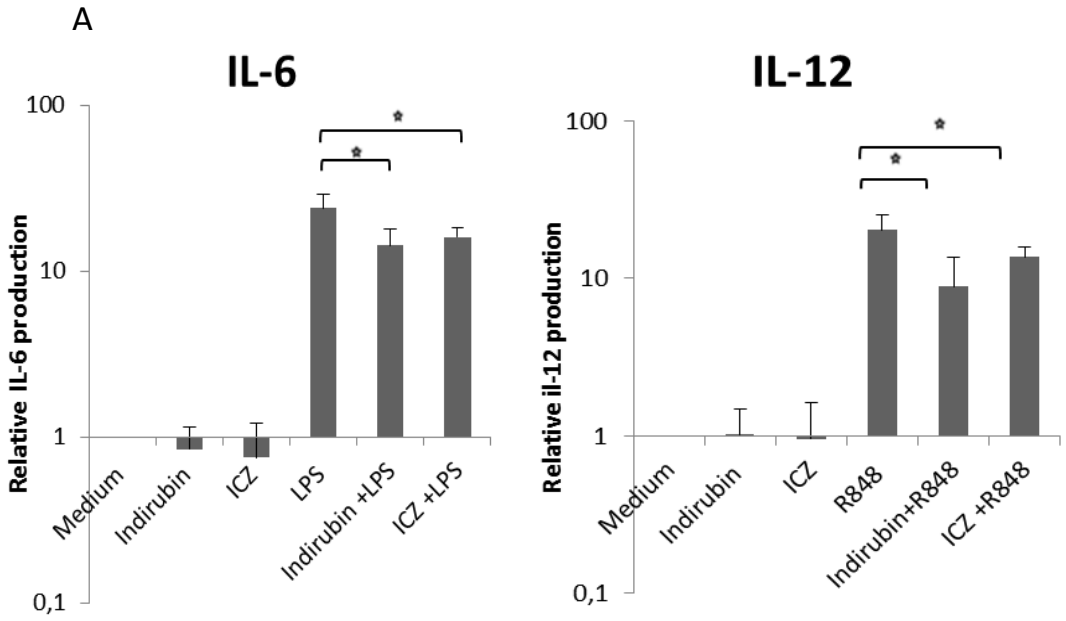
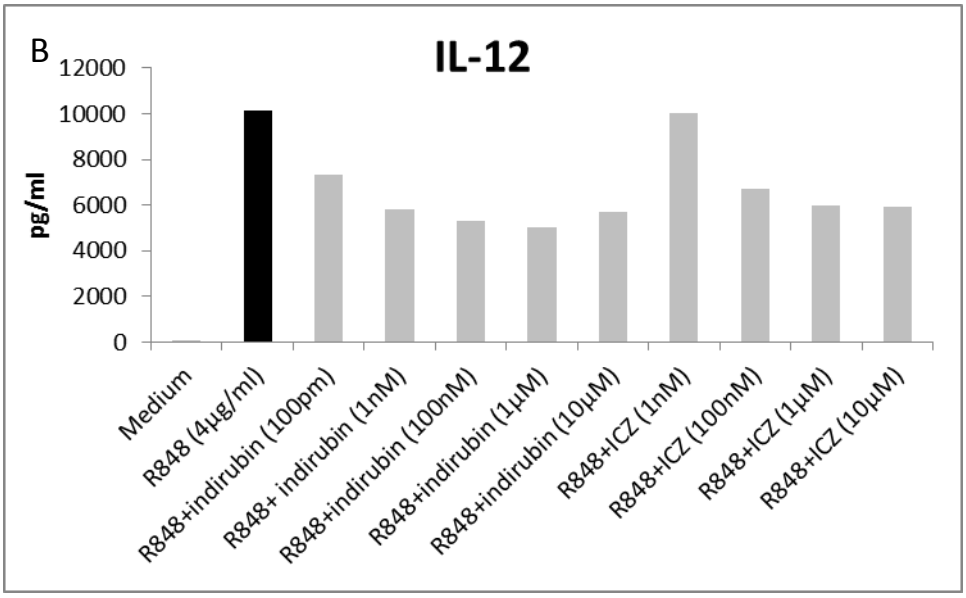


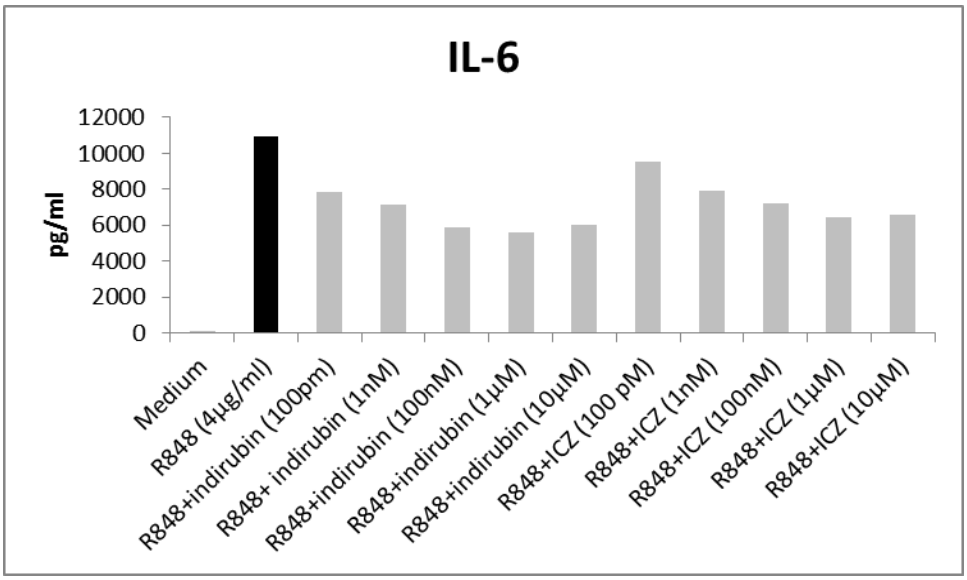
Figure 45: Comparison of basal expression of the maturation surface markers CD86 (Panel A) and MHCII (Panel B) (mean fluorescence intensity per cell; MFI) of monocyte derived dendritic cells, with the expression after TLR stimulation (R848: 4µg/ml, Lps: 100ng/ml) or after TLR and AhR ligand (indirubin in increasing concentrations) combinations exposure. A linear dose dependency was not demonstrated.

The production of IL-6 and IL-12 is significantly reduced in inflammatory moDCs exposed to indirubin and ICZ.

As expected, secretion of the cytokines IL-6, IL-12, TNFα and IL-10 was increased in moDCs that were stimulated with the TLR ligands LPS (100 ng/ml) or R848 (4µg/ml) for 24 hours. The production of IL-6 and IL-12 was reduced when moDCs were concurrently exposed to a TLR ligand and either indirubin or ICZ, in comparison to only TLR exposure ($p<0.05$; Figure 46A). TNFα was also slightly reduced and IL-10 showed a moderate increase in the case of concurrent exposure, however, without reaching statistical significance (Figure 46C, IL10 p=0.1, TNF-a p=0.23). The observed inhibitory effect was more pronounced at 100nM of indirubin and 1µM of ICZ. The effect of indoles on cytokine production was dose-dependent (Figure 46B). Exposure of immature moDCs to indirubin or ICZ alone did not affect the production of the tested cytokines (Figure 46A).




B
IL-12
pg/ml
12000
10000
8000
6000
4000
2000
0
Medium
R848 (4µg/ml)
R848+indirubin (100pm)
R848+ indirubin (1nM)
R848+indirubin (100nM)
R848+indirubin (1µM)
R848+indirubin (10µM)
R848+ICZ (1nM)
R848+ICZ (100nM)
R848+ICZ (1µM)
R848+ICZ (10µM)


IL-6
pg/ml
12000
10000
8000
6000
4000
2000
0
Medium
R848 (4µg/ml)
R848+indirubin (100pm)
R848+ indirubin (1nM)
R848+indirubin (100nM)
R848+indirubin (1µM)
R848+indirubin (10µM)
R848+ICZ (100 pM)
R848+ICZ (1nM)
R848+ICZ (100nM)
R848+ICZ (1µM)
R848+ICZ (10µM)

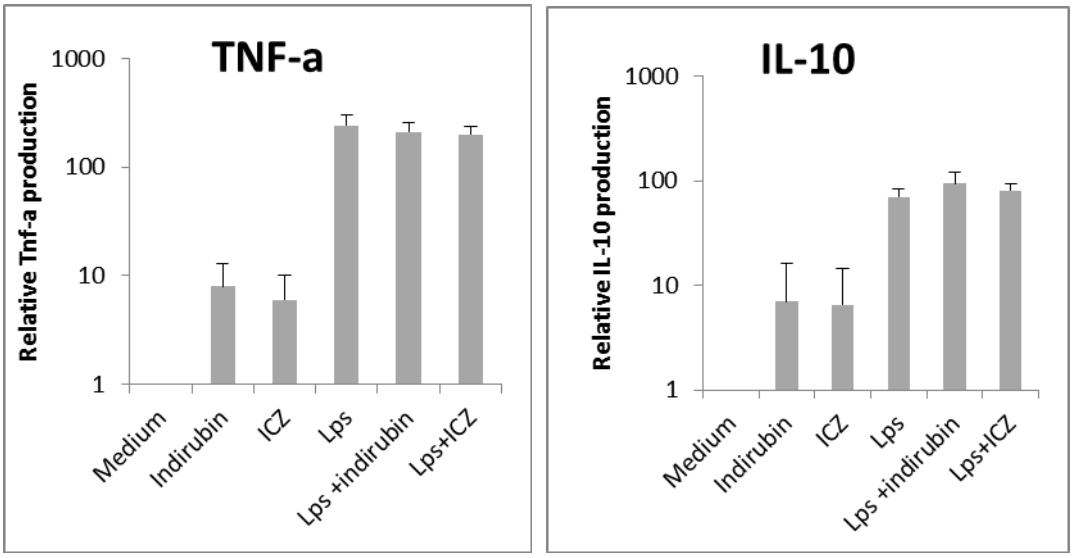


Figure 46: Panel (A): Monocyte derived dendritic cells were exposed for 24 hours to 100nM of indirubin or ICZ, TLR ligands (LPS 100ng/ml or R848 4μg/ml), combinations or were left unstimulated. IL6 and IL12p70 levels were determined by ELISA as described. Indoles down regulated the cytokines expression in inflammatory monocyte derived dendritic cells. Panel (B): The inhibition caused by indoles in IL-6 and IL12 production is dose-depended. Higher concentrations provided a higher inhibition. Panel (C): After indolic stimulation of Lps exposed dendritic cells a Tnf-a production was prohibited while IL-10 production was amplified. However the aforementioned trends remained insignificant (*Statistical significance at $p<0.05$; Bars display standard deviation, results are averages of at least 3 separate experiments)

Effects of indolic compounds in maturation of monocyte derived DCs are mediated through AhR.

As indicated in the aforementioned results the simultaneous exposure of moDCs to TLR and AhR ligands limits TLR induced phenotypic and secretive maturation of these cells. The putative role of AhR stimulation in the above process was further strengthened with the use of the specific AhR inhibitor CH223191. MoDCs were exposed either to R848 or LPS (4 μg/ml or 100ng/ml) and 100nM of indirubin or ICZ in the presence or absence of CH223191

(10μM). IL-6 and CD80 were tested as secretive and phenotypic index markers of maturation respectively. The addition of CH223191 partially ''restored'' the maturation of the monocyte derived dendritic cells when they were pre-incubated with the inhibitor (Figure 47).

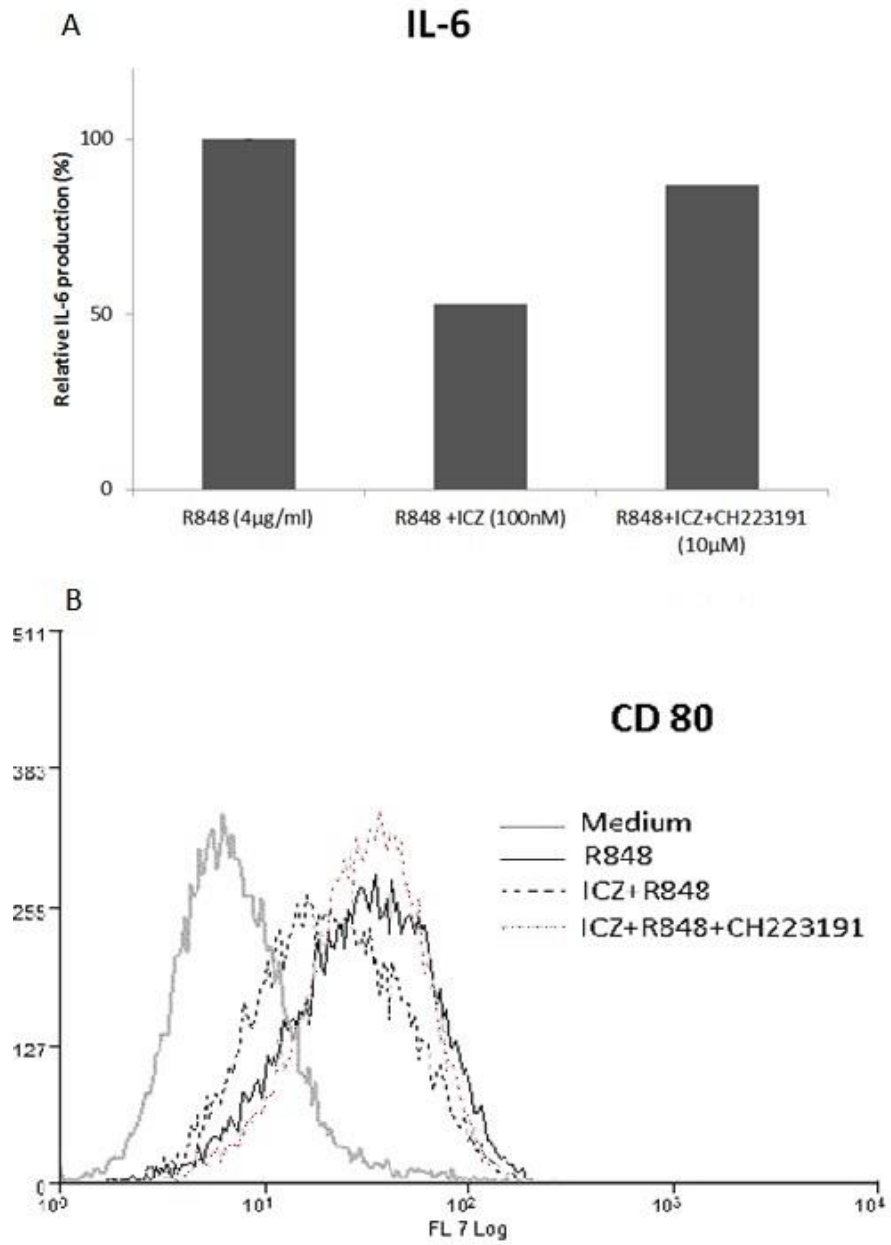


Figure 47: R848 upregulates the expression of the surface marker CD 80 in monocyte derived dendritic cells (moDCs). The simultaneous exposure to the AhR inhibitor CH223191 partially inhibits this effect of R848. moDCs were incubated with R848 (4μg/ml), ICZ (100 nM) and their combination with or without CH223191 (10μM) for 24h. The inhibitor was added 1 hour before the rest of the ligands. The reduced expression of IL-6 (Panel A) and CD 80 (Panel B) caused by co-exposure to ICZ was partially rescued when the AhR inhibitor CH223191 was also present. (Above diagrams are representative experiments)

Indoles limit the ability of dendritic cells to induce lymphocyte proliferation

Mixed lymphocyte reaction assay was employed in order to determine the ability of moDCs to stimulate T cell proliferation after maturing in the presence or absence of AhR-ligands for 24h. Exposure of DCs to the TLR ligands LPS and R848 increased their ability to induce lymphocyte proliferation. This proliferation was not observed when moDCs were exposed only to either indirubin or ICZ. Concurrent exposure of DCs to indirubin or ICZ and the TLR ligands significantly inhibited the proliferation of lymphocytes by the DCs. ($p<0.05$; Figure 48).

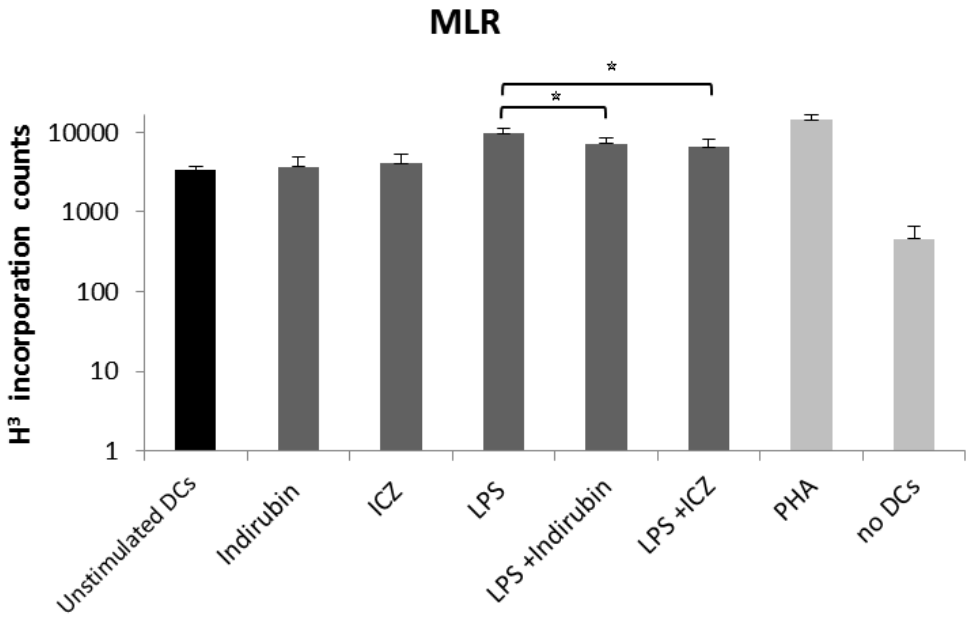
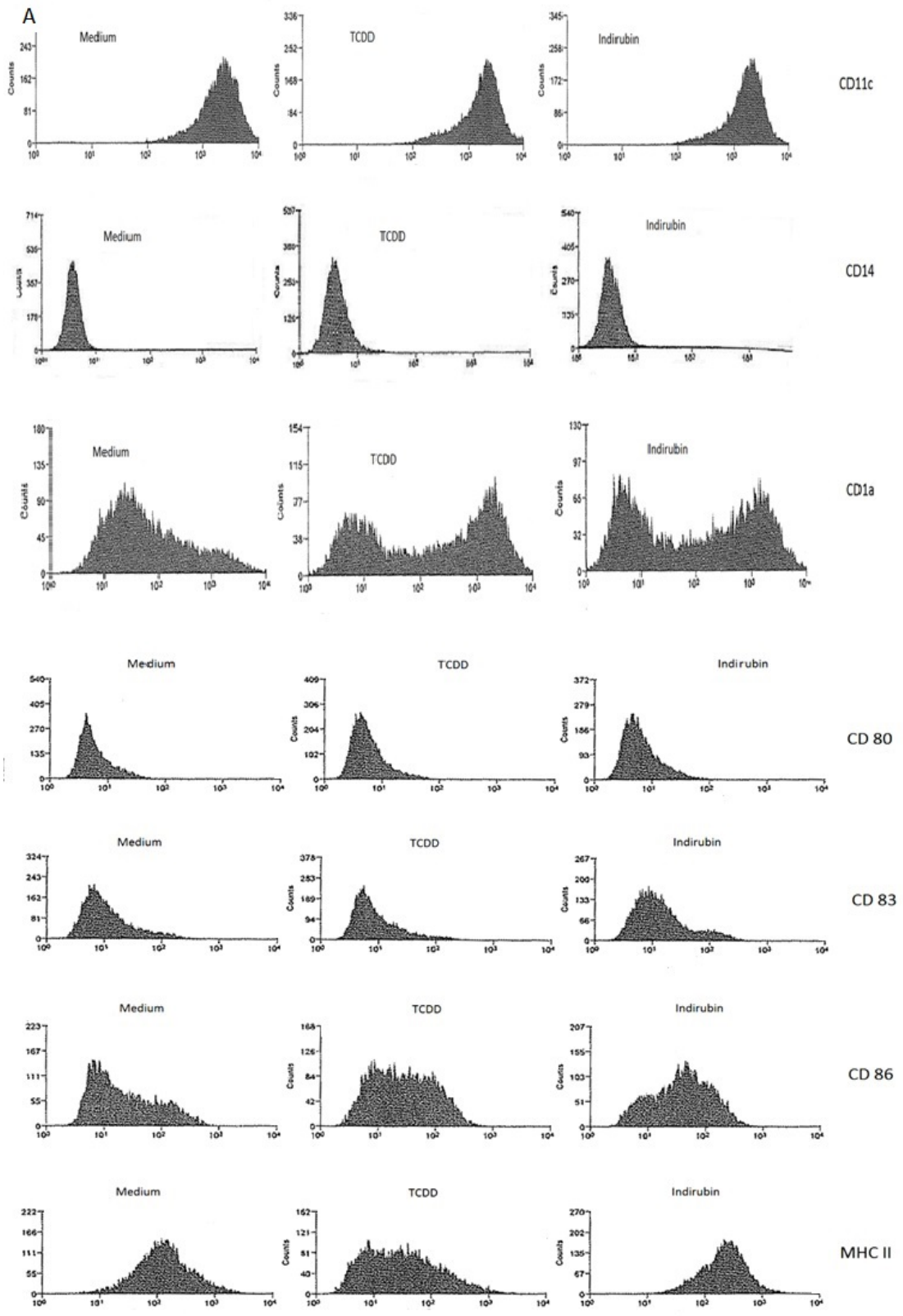


Figure 48: Indirubin or ICZ impair the allostimulatory ability of LPS exposed monocyte derived dendritic cells (moDCs) in mixed lymphocyte reaction assay. moDCs were exposed for 24 hours to 100nM of either indirubin or ICZ and the TLR ligand LPS (100ng/ml) or their combination. Subsequently, cells were harvested and their allostimulatory capacity was assessed by Mixed Lymphocyte Reaction. PHA (Phytohaemagglutinin) was used as a positive control. (*Statistical significance at $p<0.05$; Bars demonstrate standard deviation, results are averages of at least three independent experiments).

The *in vitro* differentiation process of peripheral blood mononuclear cells to DCs displays a variable alteration after exposure to AhR ligands.

The previously described impact of AhR activation on the differentiation of DCs and other cell lines (Platzer et al, 2009) led us to examine the effect of indirubin, ICZ or TCDD on the differentiation of peripheral blood monocytes (PBMCs) to DCs. PBMCs are converted *in vitro* to dendritic cells by incubation with GM-CSF (400u/ml) and IL-4 (300u/ml) for a period of six days. Differentiation of PBMCs to DCs was determined with the use of cell surface markers whose expression is altered during this process (CD1a, CD14, and CD11c). Addition of 100nM of indirubin, or TCDD on days 0 and 3 during the *in vitro* differentiation process displayed a variable effect regarding the final expression of CD1a, CD11c, and CD14 (Figure 49). In some donors the expected alteration (increase for CD1a-CD11c, decrease for CD14) in the surface markers expression was strengthened while in others it was prohibited or no change was observed. Additionally, the basic expression of the previously used maturation markers (CD80, CD83, CD86, and MHC II) also exhibited variance after exposure to the different AhR agonists (Figure 49) and also between donors. During all the experiments no toxic influence of the tested indoles on DCs viability was observed.


A
Medium
TCDD
Indirubin
CD11c
Medium
TCDD
Indirubin
CD14
Medium
TCDD
Indirubin
CD1a
Medium
TCDD
Indirubin
CD 80
Medium
TCDD
Indirubin
CD 83
Medium
TCDD
Indirubin
CD 86
Medium
TCDD
Indirubin
MHC II

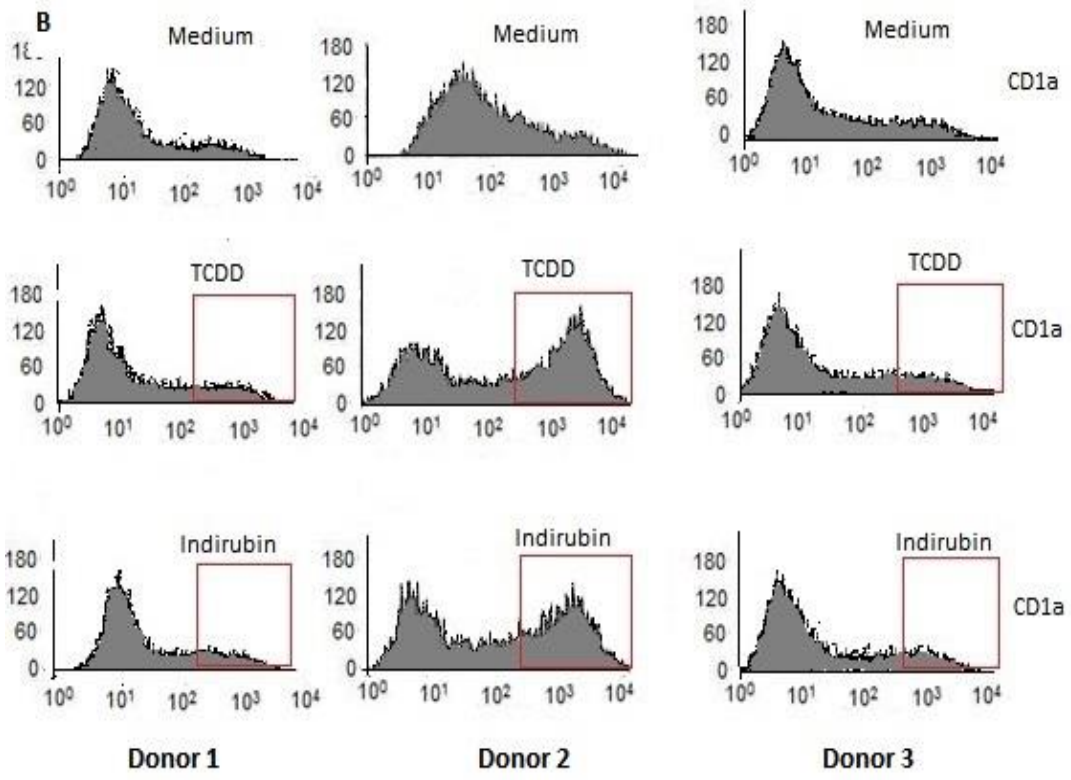


Figure 49: Expression of surface markers in *in-vitro* differentiated dendritic cells displayed variation with the addition of AhR agonists. Monocytes were exposed to indirubin (100Nm) or TCDD (100Nm), in addition to IL-4 (300u/ml) and GM-CSF (400u/ml) during their *in vitro* differentiation to dendritic cells. Panel A: For the displayed donor the mean expression of CD11c CD14 was marginally increased, in contrast to the evident CD1a upregulation. In the same donor the markers CD80, CD83, CD86 and MHCII were moderately increased by indirubin but not by TCDD. Panel B: Comparison of the alteration in CD1a expression in monocyte derived dendritic cells after exposure to TCDD or indirubin during their in vitro differentiation. In donors 1 and 3 no significant difference to control (medium) is observed, while in the second donor both TCDD and indirubin augmented the CD1a expression (representative experiments).

Discussion of the effect of the in vitro *Malassezia* produced indolic compounds in dendritic cells

The data presented herein indicate that indolic substances produced *in vitro* and potentially *in vivo* (Magiatis et al, 2013) by *Malassezia* yeasts have the ability, in appropriate concentrations, to activate AhR in moDCs and modify the response of these cells to simultaneous TLR stimulation. Furthermore, our data shows that indirubin and ICZ are more potent inducers of *Cyp1a1* and *Cyp1b1* transcription in human moDCs than TCDD, at least at the tested time points. Human AhR differs from homologous rodent receptors (Flaveny et al, 2009) by displaying a 17-fold lower affinity to TCDD (Kawanishi et al, 2003). On the other hand, ligands like indirubin demonstrate higher affinity to the human AhR as compared to the rodent (Flavenyl et al, 2009). This difference in ligand affinity between human and rodent AhR might be responsible for the herein observed higher AhR activation by indirubin or ICZ in comparison to TCDD, a result that is the opposite to the one observed in rodents. Furthermore, the *Cyp1a1* mRNA induction caused by TCDD displayed a smaller decrease between 6 and 24 hours, in contrast to the effect of indirubin and ICZ that displayed a more evident decrease between the two time points This could be attributed to the induced Cyp1a1 enzyme, which is able to metabolize the latter two ligands, yet has practically no effect on the TCDD levels within the accounted time (Adatchi et al, 2004; Chen et al, 1995) (resulting in a more sustained activation of the AhR downstream pathway (Kerkvliet, 2009)). Thus regarding AhR activation, direct comparison of the naturally occurring ligands, like indirubin and ICZ, with the anthropogenic TCDD is complex and the outcome varies between humans and experimental animals (Procházková et al, 2011). Differences in the expression or the characteristics of the aryl hydrocarbon receptor that give a species specific result in the various agonists should also be accounted (Karchner et al, 2000). Therefore, studying naturally occurring AhR ligands that can be degraded by P450 enzymes may produce physiologically more relevant information that aids in understanding better the AhR function in/on the skin.

A novel finding herein, was the observed increase in the transcription of *Cyp1a1* and *Cyp1b1* by the combination of the TLR ligands LPS, PIC or R848 with indirubin or ICZ. In previous experiments with murine splenocytes LPS-associated induction of *Cyp1a1* and *Cyp1b1* was considered as secondary to increased AhR levels upon TLR ligand exposure (Markus et al, 1998) This was not that evident in our study (Figure 29). The underlying mechanism that

causes the enhanced induction of *Cyp1a1* in human moDC upon TLR plus AhR stimulation remains to be elucidated. However, these findings demonstrate a link between maturation of the dendritic cells and the activity of xenobiotic metabolizing enzymes.

Maturation impairment of moDCs has also been observed with other AhR ligands e.g. VAF347 and benzopyrene (Lawrence et al, 2008; Laupeze et al, 2002). In our results this was evident by the impairment of the increased expression of MHC II and co-stimulatory molecules on the cell surface in addition to the decreased production of inflammatory cytokines by the AhR ligand exposed cells (Figure 44, 46A). Likewise, VAF347 and benzopyrene (Lawrence et al, 2008; Laupeze et al,2002) were reported to down-regulate the production of IL6 and IL12 in inflammatory moDCs, although their inhibitory effect on phenotypic maturation markers was restricted to CD86 for VAF347 (Lawrence P, 2008) and CD83 for benzopyrene (Laupeze et al, 2002). This inhibitory effect of AhR ligands on DC maturation and on their capacity to subsequently stimulate T cells is mediated through a yet unrevealed mechanism. Literature proposed theories include AhR interaction with the key mediator of inflammation, NF-κB, at the protein level (Verhasselt et al, 1997), or additionally, through antagonism for common transcription co-activators, such as p300, or through modification of the IKBa activity (Tian et al, 2009). In any case, the observed anti-inflammatory action of these AhR ligands is at least to some degree AhR mediated since the addition of an AhR inhibitor partially restores the maturation ability of moDCs. The reduced induction of IL-6 upon TLR stimulation in the presence of indirubin or ICZ might be a more direct effect, since the IL-6 promoter possess up-stream non-consencus xenobiotic responsive element sequences and modifications of IL-6 transcription via AhR activation have been already described (DiNatale et al, 2010). Alternatively, IL6 transcription may be secondarily modified through AhR-mediated alterations primarily in the NF-κB pathway, as mentioned above. It is worth noting that xenobiotic responsive elements have also been reported for the mice IL-12 gene-complex and the respective receptors (Kerkvliet et al, 2009), but this has not been shown for human IL-12 gene-complex yet.

Going back to the situation on the human skin, we could speculate that the production of these immunomodulatory AhR ligands by skin-colonizing *Malassezia in vivo* could represent at least one aspect out of the multiple strategies employed by this yeast in order to ensure survival and growth on the surface of the skin. It has been previously shown that *Malassezia* possess a lipid wall that assists evasion of the immune system and that it can also produce melanin, which has immunomodulatory and ‘defense’ (free radical scavenger) properties (Mayser and Gaitanis, 2010, Gaitanis et al, 2010). Within this hypothetical framework the

production of AhR ligands *in loco* may ameliorate occasional activation of DCs by incidentally exposed *Malassezia* antigens (Wollenberg et al, 2002) or down-regulate the skin immune response in the heavily infected lesions of PV (Crespo-Erchiga and Hay, 2010).

In conclusion, the *Malassezia* derived AhR ligands demonstrate a significant activation potential of the AhR pathway in moDCs. Furthermore, they can inhibit the ability of TLR ligands to induce moDC maturation and T cell proliferation. These results combined with the potential of *Malassezia* yeasts to synthesize these ligands on the skin surface, underscore the biological significance of the modification of the AhR pathway on the skin and warrants further investigation.

Synopsis and conclusions

Malassezia is a constitutional part of the human skin microflora. As is the predominant eukaryotic organism of the skin microflora, a complex interaction with the skin is anticipated. This complexity is highlighted by the yet mostly unraveled mechanisms that underlie the transformation of *Malassezia* yeasts from a harmless commensal to a persistent pathogen.

The pathogenetic potential of *Malassezia* species in the development of pityriasis versicolor is well supported by the association of this disease with the hyphal form of the yeast, a distinct, disease associated transformation. Regarding seborrheic dermatitis, direct solid evidence is lacking, yet the previous observation on the effectiveness of antifungal formulations on this condition is supported by a distinct metabolic profile of the *M. furfur* seborrheic dermatitis isolates (Gaitanis et al, 2008) which is confirmed by *in vivo* findings (Magiatis et al, 2013). In the present Thesis, seborrheic dermatitis patients and their family members, as well as healthy volunteers were studied in respect to the distribution of *Malassezia* species and the corresponding molecular and metabolic profile of selected strains. Although *Malassezia* yeasts were isolated from all the patients and the majority of healthy volunteers, no **particular pattern or correlation was identified between the isolated species and the epidemiological characteristics of the host population. More specifically no specific *Malassezia* species could be correlated with host's gender, sampling site, disease state or disease severity. The strains of *M. furfur,* the most commonly isolated species, were** further subtyped by PCR fingerprinting, yet no particular pattern could be recorded in strains isolated from members of the same family irrespective of the underlying skin condition or healthy skin. Conclusively it is intriguing that in the present study, seborrheic dermatitis could not be connected to a specific *Malassezia* species and furthermore, despite that part of the seborrheic pathogenesis can be attributed to the action of skin microbes, neither *Malassezia* yeasts, no seborrheic dermatitis can be transmitted within family members.

However the metabolic profile of the isolated strains, as assessed by phospholipase production, seems to contribute to the pathogenetic potential of *Malassezia*. For species like *Candida* or *Saccharomyces cerevisiae* increased virulence and adherence has been related to

increased phospholipase activity. Also for the zoophilic *Malassezia* species *M. pachydermatis,* lipolytic activity has been directly connected the yeasts ability to cause dermatitis in animals. Additionally, modification in the production of phospholipase enzymes by *M. pachydermatis* through a possible opioid receptor on the yeast cell membrane has been described. In the present study**, exposure of seborrheic dermatitis isolates to b-endorphin increased b-phospholipase activity in strains of *M. furfur* and *M. restricta,* in contrast to *M. globosa* and *M. sympodialis*. Furthermore, higher phospholipase enzymatic activity was recorded *in vitro* for strains isolated from seborrheic dermatitis lesional skin in comparison to strains from healthy volunteers after b endorphin exposure. These pathogenic stains responded more to b-endorphin stimulation with increased phospholipase production (phospholipase inducibility). Overall, these observations are in favor of the existence of pathogenic *Malassezia* species within the skin flora of susceptible individuals.**

Although production of various indolic substances has been shown for different non-skin associated microorganisms, the quantitative production of *Malassezia* indolic compounds in vitro and its association with a skin disease is a novel observation. The herein presented data on the effect of *Malassezia* indoles on the maturation and antigen presenting ability of dendritic cells complement the aforementioned findings as they propose a functional role of these ligands on skin physiology. Indoles are produced by all known *Malassezia* species and interestingly in higher quantities by strains originating from diseased skin. These biologically active compounds have variable effects on cellular subsets of the immune system including the differentiation of dendritic cells and T-cells as well as the phagocytosis ability of macrophages. In this Thesis, the **tested indoles, which can be produced *in vitro* and *in vivo* by *Malassezia*, were shown to activate the aryl hydrocarbon receptor (AhR) pathway in monocyte derived dendritic cells as well as in myeloid dendritic cells**. **Moreover, when monocyte derived dendritic cells are conditioned towards a pro-inflammatory phenotype this induction is significantly higher and it is coupled by down-regulation of the functional ability of dendritic cells to mature, present antigens and induce inflammation. This effect is at least partly mediated through AhR activation as inhibition of this pathway restores the ability of dendritic cells to respond to TLR ligand stimulation.**

The metabolic profile of *Malassezia* yeasts and the response of the skin barrier and immune function to it represent an interesting area of future research. There is an increasing array of autoimmune and autoinflammatory conditions that have their trigger on epithelia that are in

direct contact with microbial populations, as is the integument, the gut and the lungs. Differences and similarities on the epithelia and the relevant microbial populations could constitute a unique area of comparative studies that would assist in the comprehension of aspects of human physiology as well as the development of disease. Likewise, in the skin, the in loco production of the *Malassezia* associated indoles has been proposed as the explanation of the absence of inflammation in pityriasis versicolor and the hyperproliferative response in seborrheic dermatitis. The present Thesis has assessed yeast descriptive characteristics (epidemiology) and functional traits (phospholipase production) as well as the effect of *Malassezia* metabolites on human immune cells (dendritic cells). The findings further support the role of *Malassezia* yeasts in a common skin disease as is seborrheic dermatitis and highlight the need of a comprehensive approach for understanding skin physiology in health and disease.

Ελληνική Περίληψη Διδακτορικής Διατριβής

Εισαγωγή

Οι μικροοργανισμοί που βρίσκονται στο δέρμα και αποτελούν τη μικροβιακή του χλωρίδα εμφανίζουν μία εντυπωσιακή ποικιλία ανάλογη της ποικιλίας της φυσιολογίας και τοπογραφίας του δέρματος. Η πλειονότητα των οργανισμών αυτών είναι βακτήρια που ταξινομούνται σε περισσότερα από χίλια είδη. Η μυκητιακή χλωρίδα αντίθετα είναι ιδιαίτερα περιορισμένη και τον κύριο εκπρόσωπό της αποτελεί το γένος *Malassezia.*

Οι μύκητες του γένους *Malassezia* είναι ευκαρυωτικοί οργανισμοί. Η αλληλεπίδρασή τους με το δέρμα δεν περιορίζεται απλά στη συλλογή βιομορίων, τα οποία χρησιμοποιούνται για ενέργεια και ως δομικά στοιχεία, αλλά επεκτείνεται και στην παραγωγή βιολογικά δραστικών ουσιών που έχουν τόσο αυτόλογη όσο και ετερόλογη δράση.

Μέχρι σήμερα έχουν αναγνωριστεί 14 είδη *Malassezia*. Τα στελέχη του γένους αυτού θεωρούνται αιτιολογικοί παράγοντες σε δερματικά νοσήματα όπως η σμηγματορροϊκή δερματίτιδα και η ποικιλόχροη πιτυρίαση, ενώ εμπλέκονται σε άλλα νοσήματα όπως η ψωρίαση και η ατοπική δερματίτιδα. Οι ακριβείς παθογενετικοί μηχανισμοί ωστόσο, δεν έχουν ακόμα αποσαφηνιστεί.

Σκοπός της παρούσας μελέτης είναι η διεύρυνση και κατανόηση παθοφυσιολογικών μηχανισμών σε σχέση με τη συμμετοχή των ζυμών *Malassezia* στη σμηγματορροϊκή δερματίτιδας. Πιο συγκεκριμένα διερευνήθηκαν τα ακόλουθα: 1) η κατανομή των ειδών του γένους *Malassezia* σε οικογένειες που περιείχαν πάσχοντες από σμηγματορροϊκή δερματίτιδα β) η παραγωγή λιπολυτικών ενζύμων από τα απομονωθέντα στελέχη *Malassezia* και η επίδραση της β ενδορφίνης σε αυτή και γ) η *in vitro* δράση ινδολικών παραγώγων της *Malassezia* σε ανθρώπινα δενδριτικά κύτταρα .

Ιστορία, βιολογία και οικολογία του γένους *Malassezia*

Το γένος *Malassezia* πήρε το όνομά του από τον παθολογοανατόμο του 19ου αιώνα Malassez, που περιέγραψε ένα μύκητα με τη χαρακτηριστική οβάλ μορφολογία στο δέρμα ασθενών με ποικιλόχροη πιτυρίαση. Για ένα μεγάλο χρονικό διάστημα το γένος αποκαλούνταν επίσης με τον όρο πιτυρόσπορο, ο οποίος εισήχθη από τον Sabouraud το 1902. Η σύγχρονη χρήση των 2 όρων προκαλούσε σύγχυση και αμφιβολία μεταξύ δερματολόγων και βιολόγων για την ταυτόσημη φύση των ειδών. Το 1970 αναγνωρίστηκαν 3 είδη του μύκητα *Malassezia* (*P. ovale*, *P. orbiculare*, and *P. pachydermatis)* και το 1977 επιβεβαιώθηκε η ικανότητα του μύκητα να μετατρέπεται από μυκήλιο σε υφή. Τελικά, το 1986 η επιστημονική κοινότητα αποδέχθηκε την ενοποίηση του γένους υπό το όνομα *Malassezia*. Η χρήση μοριακών μεθόδων οδήγησε στην αύξηση των ταξινομημένων στο γένος ειδών σε 14.

Η μορφολογία των στελεχών ποικίλει μεταξύ των ειδών, τόσο σε κυτταρικό επίπεδο όσο και σε επίπεδο αποικίας. Το κυτταρικό σχήμα μπορεί να είναι κυκλικό όπως στην *M. restricta* ή *M. globosa,* επίμηκες όπως στην *M. furfur*, κυλινδρικό όπως στην *M. pachydermatis* ή οβάλ όπως στην *M. yamatoensis.* Επιπλέον διαφορές υπάρχουν και στο μοτίβο πολλαπλασιασμού, το οποίο έδωσε στην *M.sympodialis* το όνομά της. Μακροσκοπικά οι αποικίες εμφανίζονται λείες και κρεμώδεις, επιδεικνύοντας όμως διαφορές μεταξύ των ειδών κυρίως στην υφή.

Στο ηλεκτρονικό μικροσκόπιο η *Malassezia* εμφανίζει ένα κυτταρικό τοίχωμα με πολλαπλές φολίδες σε σπειροειδή διάταξη. Τα θυγατρικά κύτταρα εκφύονται από την έσω επιφάνεια του κυτταρικού τοιχώματος του μητρικού κυττάρου αφήνοντας ένα χαρακτηριστικό σημάδι. Η έκφυση του θυγατρικού κυττάρου είναι συνήθως μονοπολική. Ένα επιπλέον ιδιαίτερο χαρακτηριστικό του γένους είναι η παρουσία ενδοσπορίων.

Δέκα από τα 14 είδη του μύκητα ανευρίσκονται κυρίως σε ανθρώπους (*M. furfur, M. globosa, M. symbodialis, M. restricta, M. slooffiae, M. nana, M. yamatoensis, M. obtusa, M. japonica, M. dermatis*). Τα είδη αυτά έχουν απομονωθεί από ποικίλες δερματικές περιοχές όπως το τριχωτό της κεφαλής, το πρόσωπο ή ο κορμός αλλά υπό συγκεκριμένες συνθήκες και από τα ούρα ή το αίμα. Τα υπόλοιπα 4 είδη (*M. pachydermatis, M. carpae, M. equina, M. nana*) απομονώνονται κυρίως από οικιακά ζώα, χωρίς ωστόσο να αποκλείεται και ο παροδικός αποικισμός του ανθρώπινου δέρματος.

Ο μεταβολισμός της *Malassezia*

Η εξάρτηση του γένους *Malassezia* από τα λιπίδια οφείλεται στην ανικανότητα σύνθεσης του μυριστικού οξέος, ενός πρόδρομου των λιπαρών οξέων μακράς αλύσου. Η ανάλυση του γονιδιώματος της *M. furfur* επιβεβαίωσε την έλλειψη των γονιδίων της συνθάσης των λιπαρών οξέων αλλά ταυτόχρονα ανέδειξε και την ύπαρξη πολυάριθμων γονιδίων που κωδικοποιούν λιπάσες και φωσφολιπάσες. Συνεπώς, οι μύκητες του γένους *Malassezia* είναι σε θέση να μεταβολίζουν λιπαρά οξέα χρησιμοποιώντας τα είτε σαν δομικά συστατικά είτε σαν πηγή ενέργειας.

Η οξεϊκή ζύμωση εκχυλίσματος λεκίθου αβγού (egg yolk assimilation) αποτελεί μια αξιόπιστη και επιστημονικά αποδεκτή μέθοδο για τον έλεγχο παραγόμενων λιπασών. Η χρήση της μεθόδου εδραιώθηκε με την ανίχνευση της δράσης φωφολιπασών από την *Candida albicans* και τον *Cryptococcus neoformans* και επεκτάθηκε αργότερα σε είδη του γένους *Malassezia* όπως η *M. furfur* και η *M. pachydermatis*. Βασική αρχή της μεθόδου αποτελεί η παραδοχή ότι η ζώνη καθίζησης γύρω από την αποικία του μύκητα αποτελεί προϊόν της δράσης λιπασών και είναι ευθέως ανάλογη της ποσότητας αυτών. Κατά συνέπεια ο λόγος της διαμέτρου της αποικίας προς την διάμετρο της ζώνη καθίζησης αποτελεί ένα δείκτη της παραγωγικότητας λιπασών.

Υποστρώματα ανάπτυξης για τη *Malassezia* αποτελούν και υδατάνθρακες όπως η σορβιτόλη και η μανιτόλη. Αυτοί αποτελούν εναλλακτικό των λιπιδίων τόσο σαν δομικό συστατικό όσο και σαν πηγή ενέργειας Αντίθετα, οι μύκητες του γένους *Malassezia* δε μπορούν να μεταβολίσουν σάκχαρα.

Με τη χρήση κατάλληλου υποστρώματος (minimal essential medium) και αυξανογράμματος νατρίου αποδείχθηκε ότι ο μύκητας μπορεί να χρησιμοποιεί όλα τα απαραίτητα αμινοξέα, με την εξαίρεση της κυστείνης, σαν πηγές νατρίου για την ανάπτυξή του. Ο μεταβολισμός της τρυπτοφάνης από τον μύκητα αποδίδει ινδολικά παράγωγα, ουσίες με υψηλή βιολογική σημασία. Παραδείγματα ουσιών που προκύπτουν από το μεταβολισμό της τρυπτοφάνης, in vitro, από τη *Malassezia* αποτελούν η pitiriacitrin, η indirubin, η malassezin και η 6-Formylindolo(3,2-b)carbazole. Τα φυσικά και βιολογικά χαρακτηριστικά των ουσιών αυτών έχουν συσχετιστεί με την κλινική σημασία του γένους *Malassezia.*

Συμβατικές και μοριακές μέθοδοι απομόνωσης και ταυτοποίησης της *Malassezia*

Οι μακροσκοπικές διαφορές των αποικιών και οι μικροσκοπικές διαφορές των κυττάρων, δεν είναι επαρκείς για το διαχωρισμό μεταξύ των ειδών. Επιπλέον βιοχημικά χαρακτηριστικά αλλά και γονιδιακή ανάλυση είναι απαραίτητα για να ταυτοποιηθούν τα 14 είδη της *Malassezia*.

Η λιποφιλία του γένους αποτέλεσε τη βάση για την δημιουργία πολυάριθμων εκλεκτικών υποστρωμάτων ανάπτυξης, με πλέον διαδεδομένα το Leeming Notman και το Dixons modified agar. Επιπλέον της ανάπτυξης σε εκλεκτικά μέσα, οι μεταβολικές ιδιότητες ορισμένων ειδών ,όπως ο καταβολισμός Tween 40/60/80, εσκουλίνης και υπεροξειδίου του υδρογόνου αποτέλεσαν τη βάση για την αναγνώριση και ταυτοποίηση 7 ειδών.

Τη δεκαετία του 90 οι συμβατικές μέθοδοι άρχισαν να συμπληρώνονται με μοριακές τεχνικές. Οι μέθοδοι ανάλυσης της γονιδιακής ακολουθίας (sequencing analysis) οδήγησαν στην αναγνώριση επιπλέον ειδών όπως η *M. japonica, M. carpae* και *M. equina.* Η ανάλυση συνήθως πραγματοποιείται στις περιοχές ITS-1/2, IGS-1 του ριβοσωμικού RNA, που παρέχουν την απαραίτητη ποικιλότητα στις νουκλεοτιδικές τους ακολουθίες, για διάκριση μεταξύ ειδών εντός του γένους.

Η PCR πραγματικού χρόνου έχει χρησιμοποιηθεί επιτυχώς στην ανίχνευση και επιβεβαίωση της *Malassezia* απευθείας από λέπια δέρματος. Η μέθοδος αυτή χρησιμοποιήθηκε για τη συσχέτιση ορισμένων ειδών *Malassezia* με δερματικά νοσήματα όπως η ψωρίαση. Επιπλέον μέθοδοι ενίσχυσης πολυμορφικών δεικτών του DNA έχουν χρησιμοποιηθεί κυρίως στην αναγνώριση υποτύπων εντός των ειδών και συσχέτισής τους με άλλα επιδημιολογικά χαρακτηριστικά των φορέων όπως την κατάσταση νόσου του ξενιστή ή ακόμα και τη γεωγραφική προέλευση.

Προσφάτως τα γονιδιώματα των ειδών *M. globosa* και *M. restricta* αναλύθηκαν πλήρως αποκαλύπτοντας πολυάριθμα μεταβολικά μονοπάτια, σχετικά με τη γλυκόλυση, τη σύνθεση αμινοξέων και νουκλεϊκών οξέων και την παραγωγή λιπασών.

Νοσήματα που σχετίζονται με τη *Malassezia*

Η **ποικιλόχροη πιτυρίαση** αποτελεί μια συχνή δερματοπάθεια που εμφανίζεται στο 2-8% του γενικού πληθυσμού. Η νόσος έχει συσχετιστεί με την αυξημένη δραστηριότητα των σμηγματογόνων αδένων και την παρουσία της *Malassezia*. Πιο συγκεκριμένα ο παθοφυσιολογικός μηχανισμός της ποικιλοχρόου πιτυριάσεως περιλαμβάνει τη μετατροπή του μύκητα από μυκήλιο σε υφή, γεγονός που θεωρητικά τουλάχιστον διευκολύνει τη διείσδυση του μύκητα στη κερατίνη στιβάδα. Μάλιστα, η χαρακτηριστική μικροσκοπική εικόνα της συνύπαρξης μυκηλίων και υφών αποτελεί διαγνωστικό κριτήριο της νόσου. Ένα επιπλέον χαρακτηριστικό της νόσου, ο φθορισμός των βλαβών κάτω από τη λυχνία Wood,αποδίδεται επίσης σε μεταβολικά παράγωγα του μύκητα.

Το είδος *Malassezia* που απομονώνεται πιο συχνά από βλάβες ποικιλόχροης πιτυρίασης είναι η *M. globosa*. Ο ρόλος του μυκητιακού φορτίου ως προκλητικού παράγοντα αποτελεί ακόμη αιτία διχογνωμίας μεταξύ των ερευνητών. Παρά όμως την παρουσία της *Malassezia* τόσο υπό μορφή μυκηλίου όσο και υπό μορφή υφής στις βλάβες της ποικιλοχρόου πιτυρίασεως, παρατηρείται ελάχιστη φλεγμονώδης αντίδραση από το ανοσοποιητικό σύστημα του δέρματος.

Η **σμηγματορροϊκή δερματίτιδα** αποτελεί μια έτερη συχνή δεματοπάθεια που αφορά το 1-5% του πληθυσμού. Χαρακτηρίζεται από την παρουσία ερυθηματωδών πλακών με συνοδό απολέπιση. Η ηπιότερη μορφή της νόσου, η οποία περιορίζεται στο τριχωτό της κεφαλής ονομάζεται πιτυρίδα και αποτελεί αρκετά συχνότερη εκδήλωση. Ένα κύριο επιχείρημα συσχέτισης της νόσου με την *Malassezia* αποτελεί η κοινή αντιμυκητιακή δραστικότητα όλων των θεραπευτικών μεθόδων που έχουν χρησιμοποιηθεί. Μεταξύ άλλων, ο παθογενετικός μηχανισμός της νόσου συνδέεται με την παραγωγή λιπασών από τον μύκητα που μεταβολίζοντας το σμήγμα παράγει ελεύθερα λιπαρά οξέα. Αυτά όπως έχει αποδειχθεί πειραματικά ευθύνονται κατά ένα μέρος για τον τοπικό ερεθισμό. Η παρουσία αυξημένων επιπέδων RNA λιπασών του μύκητα σε βλάβες σμηγματορροϊκής έχει επιβεβαιωθεί πειραματικά. Ενδιαφέρον έχει η παρατήρηση ότι τα ελεύθερα λιπαρά οξέα προκαλούν απολέπιση στο δέρμα των πασχόντων αλλά όχι των υγιών. Επίσης στη σμηγματορροϊκή δερματίτιδα το είδος που απομονώνεται συχνότερα είναι η *M. globosa*.

Η **θυλακίτιδα από *Malassezia*** χαρακτηρίζεται από την ύπαρξη πολυάριθμων μονόμορφων βλατίδων ή φλυκταινιδίων τα οποία συνοδεύονται από τοπικό ερεθισμό. Σε ιστολογικό επίπεδο παρουσιάζονται διατεταμένα τριχοθυλάκια που περιέχουν άφθονα κύτταρα του μύκητα *Malassezia*, ουδετερόφιλα, λεμφοκύτταρα και ιστιοκύτταρα. Η θυλακίτιδα από *Malassezia* μπορεί να επιτευχθεί πειραματικά, αν σε ασθενείς με ιστορικό θυλακίτιδας τοποθετηθούν στο δέρμα μύκητες του γένους *Malassezia* με κλειστή περίδεση. Επιπλέον οι πάσχοντες εμφανίζουν υψηλότερο τίτλο αντισωμάτων έναντι του μύκητα σε σχέση με τους υγιείς ή τους πάσχοντες από ποικιλόχροη πιτυρίαση.

Η **ατοπική δερματίτιδα** αποτελεί μια συχνή δερματοπάθεια με πολυάριθμες κλινικές εκδηλώσεις και σαφές γονιδιακό υπόβαθρο. Η *Malassezia* απομονώνεται από τα δέρμα των πασχόντων από ατοπική δερματίτιδα σχεδόν στο 100% των περιπτώσεων. Το συχνότερα απομονωθέν είδος είναι η *M. restricta* στα παιδιά και η *M. furfur* στους ενήλικες. Η κύρια υπόθεση εμπλοκής της *Malassezia* στην ατοπική δερματίτιδα αφορά την δράση της ως αλλεργιογόνο με εντονότερη αντίδραση σε άτομα που έχουν ευαισθητοποιηθεί. Πειραματικά έχουν επιβεβαιωθεί η θετική αντίδραση σε έλεγχο με patch test και τα υψηλά επίπεδα της IgE έναντι της *Malassezia* σα συχνό εύρημα (20-100%) σε ασθενείς με ατοπική δερματίτιδα.

Τέλος η **ψωρίαση** αποτελεί χρόνιο δερματικό νόσημα με συχνότητα 3% στο γενικό πληθυσμό. Σε ιστολογικό επίπεδο χαρακτηρίζεται από υπερκεράτωση, παρακεράτωση, υπερπλασία των αγγείων της επιδερμίδας και φλεγμονή. Το 1982 ο Lober έθεσε τις βάσεις συσχέτισης της νόσου με τη *Malassezia* αναπαράγοντας ψωριασιόμορφες βλάβες με την επίθεση εναιωρήματος *Malassezia* στην επιφάνεια του δέρματος. Σήμερα γνωρίζουμε ότι η *Malassezia* μπορεί να δράσει σαν χημειοτακτικός παράγοντας για τα πολυμορφοπύρηνα ασθενών που πάσχουν από ψωρίαση, σε αντίθεση με αυτά των υγιών. Επιπλέον έχει αποδειχθεί ότι το προφίλ κυτταροκινών που παρουσιάζουν τα κερατινοκύτταρα ασθενών με ψωρίαση εμφανίζει πολλές ομοιότητες με αυτό κερατινοκυττάρων υγειών που έχουν εκτεθεί στη *Malassezia*. Το στέλεχος που κυρίως απομονώνεται από βλάβες ψωρίασης είναι η *M. restricta* ακολουθούμενη από την *M. globosa*.

Υποδοχέας ξενοβιοτικού μεταβολισμού (AhR)

Ο AhR είναι ένας κυτταροπλασματικός υποδοχέας, μέλος μιας οικογένειας μεταγραφικών παραγόντων γνωστή ως PAS (Per-Arnt-Sim). Υπό συνθήκες ηρεμίας ο AhR βρίσκεται στο κυτταρόπλασμα σε ένα ανενεργό σύμπλοκο. Κατόπιν πρόσδεσης του αγωνιστή μεταβαίνει στον πυρήνα, διμερίζεται με τον AhRR και επάγει την μεταγραφή μιας σειράς γονιδίων μεταξύ των οποίων και εκείνα του ξενοβιοτικού μεταβολισμού όπως τα *Cyp1A2*, *Cyp1B1* και *Gstp1*. Ο ρόλος του AhR επεκτείνεται πέραν της επαγωγής γονιδίων του μεταβολισμού και σε άλλες βασικές κυτταρικές διεργασίες όπως τη ρύθμιση του κυτταρικού κύκλου, την απόπτωση, τη λειτουργία των υποδοχέων οιστρογόνων, τον κιρκάδιο ρυθμό και την οργανογένεση.

Οι αγωνιστές του υποδοχέα του ξενοβιοτικού μεταβολισμού έχουν συνήθως μια χαρακτηριστική αρχιτεκτονική που περιλαμβάνει επίπεδα, υδρόφοβα και αρωματικά μόρια. Προσφάτως ένας μεγάλος αριθμός μορίων με διαφορετική δομή από την προαναφερθείσα περιγράφηκαν να ενεργοποιούν τον AhR. Το γεγονός αυτό αποδόθηκε στην ύπαρξη πολυάριθμων θέσεων πρόσδεσης επί του AhR ή στην ενεργοποίηση χωρίς πρόσδεση.

Οι αγωνιστές του AhR διακρίνονται σε εξωγενείς και ενδογενείς. Η πλειονότητα των εξωγενών ανήκει στην οικογένεια των αρωματικών υδρογοναθράκων όπως οι διβενζοδιοξίνες ή τα διφενίλια. Πρόκειται για υδρόφοβα, συμμετρικά μόρια που αποτελούν ισχυρούς αγωνιστές και είναι κυρίως βιομηχανικά προϊόντα. Στην ομάδα των εξωγενών ανήκουν και ουσίες με διαφορετική δομή όπως η ομερπραζόλη ή η ιαβενδαζόλη που συνήθως αποτελούν ασθενείς αγωνιστές.

Κύρια πηγή ενδογενών αγωνιστών του AhR αποτελεί η δίαιτα. Ήδη από το 1992 είχε παρατηρηθεί ότι κάποια φρούτα και λαχανικά μπορούσαν να οδηγήσουν σε επαγωγή του *Cyp1a1* σε πειραματόζωα. Συνήθως οι ενδογενείς αγωνιστές είναι ασθενείς αγωνιστές με κυριότερες ομάδες τις ινδόλες, τις τετραπυρόλες, τα φλαβινοειδή, τους μεταβολίτες του αραχιδονικού οξέος, τα καροτενοειδή και τις οξυστερόλες.

Το 2001 ο Wille χρησιμοποιώντας την τρυπτοφάνη ως πηγή αζώτου σε καλλιέργειες της *M. furfur* απομόνωσε την ινδόλη malassezin. Ο μεταβολίτης αυτός λόγω της δομικής του ομοιότητας με άλλους γνωστούς αγωνιστές του AhR όπως το ICZ και το FICZ ελέγχθηκε ως προς την ικανότητα επαγωγής του P450 και εμφάνισε δραστικότητα αντίστοιχη με αυτή άλλων ενδογενών αγωνιστών. Προοδευτικά επιπλέον αγωνιστές του Ahr προστέθηκαν στους παραγόμενους από την *Malassezia* όπως οι indirubin, tryptanthrin, ICZ, FICZ. Μάλιστα μερικοί από αυτούς συνδέθηκαν με την ύπαρξη ή όχι νόσου (σχετιζόμενης με τη *Malassezia*) στους φορείς καθώς και με κλινικά χαρακτηριστικά των νόσων.

Το ανοσοποιητικό σύστημα του δέρματος και η αλληλεπίδραση του με τη *Malassezia*.

Το δέρμα αποτελεί την πρώτη γραμμή άμυνας του οργανισμού έναντι των επιθέσεων των παθογόνων και των φυσικών αιτίων. Εξαιτίας αυτής της εκθέσεώς του αλλά και του μεγάλου μεγέθους του, η ανοσοεπιτήρηση αποτελεί μια πρόκληση. Δομικά διακρίνεται σε 3 μέρη: το υπόδερμα, το χόριο (δερμίδα) και την επιδερμίδα. Τα κύρια κύτταρα της επιδερμίδας είναι τα κερατινοκύτταρα, τα μελανινοκύτταρα, τα Langerhans και τα λεμφοκύτταρα ενώ η δερμίδα περιέχει ινοβλάστες, λιποκύτταρα και μακροφάγα.

Τα κερατινοκύτταρα είναι εξοπλισμένα με υποδοχείς ανίχνευσης παθογόνων τόσο στην κυτταρική μεμβράνη όσο και στο κυτταρόπλασμά τους και μπορούν να παράγουν κατόπιν διεγέρσεως φλεγμονώδεις κυτταροκίνες όπως IL-1/6 και Tnf-a και χημειοκίνες όπως CXCL1 και CXCL8. Επιπλέον είναι σε θέση να παράγουν αντιμικροβιακά πεπτίδια όπως οι β-defencin, αλλά και να εκφράσουν στην επιφάνειά τους αντιγόνα ιστοσυμβατότητας (MHC) δρώντας εν μέρει ως αντιγονοπαρουσιαστικά κύτταρα.

Τα κύτταρα Langerhans είναι τα κύρια αντιγονοπαρουσιαστικά κύτταρα της επιδερμίδας. Ρόλος τους είναι η φαγοκυττάρωση αντιγόνων και η παρουσίασή τους στα Τ λεμφοκύτταρα επάγοντας τη διαφοροποίηση των τελευταίων προς τον Th2 φαινότυπο. Πέρα από αυτή τη δράση η παρουσία τους έχει πρόσφατα συνδεθεί και με την προώθηση της ανοχής (tolerance) σε αντιδράσεις υπερευαισθησίας. Άλλο πληθυσμό δενδριτικών κυττάρων του δέρματος αποτελούν τα φλεγμονώδη δενδριτικά επιδερμιδικά κύτταρα (idecs) τα οποία είναι μονοκυτταρικής προελεύσεως και δεσμεύουν IgE σύμπλοκα. Στο χόριο υπάρχουν επιπλέον τα δενδριτικά κύτταρα του χορίου με αντιγονο-παρουσιαστικό ρόλο και τα πλασματοειδή δενδριτικά κύτταρα με δυνατότητα παραγωγής ιντερφερόνης 1.

Τα Τ λεμφοκύτταρα της επιδερμίδας βρίσκονται κυρίως στα επιφανειακά στρώματα, πλησίον των Langerhans κυττάρων. Επιδερμιδικά επικρατεί ο CD 8 τύπος ενώ στο χόριο ισοδυναμούν οι CD4 και CD8 τύποι. Τα λεμφοκύτταρα κατά την διάρκεια φλεγμονωδών αντιδράσεων μπορούν να επαχθούν προς τους φαινοτύπους Th 1,2,17,22.

Ο διττός ρόλος της *Malassezia* ως φυσιολογική χλωρίδα του δέρματος αλλά και ως ευκαιριακό παθογόνο αναδεικνύει τη σύνθετη αλληλεπίδραση του μύκητα με το ανοσοποιητικό σύστημα.

Τα στελέχη της *Malassezia* μπορούν να ενεργοποιήσουν τόσο το κλασσικό όσο και το εναλλακτικό μονοπάτι του συμπληρώματος, γεγονός που θα μπορούσε να αποτελέσει συνδετικό κρίκο του μύκητα με διάφορες δερματοπάθειες όπως η ψωρίαση. Επιπλέον ανοσολογική αντίδραση εκδηλώνεται και με αντιμικροβιακά πεπτίδια όπως το LL 37, που έχει βρεθεί αυξημένο σε βλάβες ποικιλοχρόου πιτυρίασεως ή το HBD-22 που παράγεται από τα κερατινοκύτταρα *in vitro* κατά την παρουσία του μύκητα .

Σε κυτταρικό επίπεδο έχει επιβεβαιωθεί η ταχεία φαγοκυττάρωση των στελεχών της *Malassezia* από ουδετερόφιλα η οποία όμως συνοδεύεται από πολύ χαμηλά επίπεδα ενδοκυτταρίου θανάτου. Η πρόσδεση της *Malassezia* από τα μονοκύτταρα γίνεται μέσω των υποδοχέων μανόζης και β-γλυκανών. Τα κύρια φλεγμονώδη κύτταρα που ανευρίσκονται στις βλάβες νοσημάτων που σχετίζονται με την *Malassezia* όπως στην ποικιλόχροη πιτυρίαση είναι τα Τ λεμφοκύτταρα του βοηθητικού φαινότυπου συνοδευόμενα από μακροφάγα, μονοκύτταρα και κύτταρα Langerhans. Η συγκαλλιέργεια κυττάρων *Malassezia* με κερατινοκύτταρα επιδρά στο προφίλ των παραγομένων, από τα δεύτερα, κυτταροκινών ανάλογα του λόγου *Malassezia*/KCs και του είδους του μύκητα. Η αξιολόγηση της ανοσολογικής απάντησης έναντι της *M. furfur* με τις τεχνικές lymphocyte transformation assay (LT) και leucocyte migration inhibition assay (LMI) ανέδειξαν μειωμένη ανοσογονικότητα των στελεχών εκείνων που προέρχονταν από πάσχοντες με ποικιλόχροη πυτιρίαση έναντι των στελεχών που προέρχονταν από υγιείς ή έπασχαν από σμηγματορροική δερματίτιδα. Τέλος θα πρέπει να αξιολογηθεί το γεγονός ότι η σμηγματορροική δερματίτιδα είναι πολύ συχνή σε ανοσοκατεσταλμένους ασθενείς όπως σε πάσχοντες απο AIDS ή μεταμοσχευμένους.

Στόχοι της διδακτορικής διατριβής

Σκοπός της παρούσης διδακτορικής διατριβής είναι η διεύρυνση και κατανόηση παθοφυσιολογικών μηχανισμών σε σχέση με τη συμμετοχή των ζυμών *Malassezia* στη σμηγματορροϊκή δερματίτιδα. Πιο συγκεκριμένα μελετήθηκαν τα ακόλουθα α) απομόνωση και ταυτοποίηση των ειδών *Malassezia* από οικογένειες πασχόντων με σμηγματορροική δερματίτιδα και σύγκριση αυτών με στελέχη απομονωθέντα από ομάδα ελέγχου β) παραγωγή λιπολυτικών ενζύμων από στελέχη απομονωθέντα από πάσχοντες σμηγματοροικής δερματίτιδας και υγιείς και η μεταβολή της παραγωγής αυτής κατόπιν εκθέσεως των στελεχών σε β ενδορφίνη γ) *in vitro* επίδραση ινδολικών παραγώγων που παράγονται από στελέχη *Malassezia* σε δενδριτικά κύτταρα.

Υλικά και μέθοδοι

Συνολικά 84 εθελοντές περιελήφθησαν στην μελέτη εκ των οποίων 43 πάσχοντες από σμηγματορροϊκή δερματίτιδα και 41 υγιείς. Ο πληθυσμός συλλέχθηκε από τα εξωτερικά ιατρεία της δερματολογικής κλινικής του Πανεπιστημιακού γενικού νοσοκομείου Ιωαννίνων. Στον πληθυσμό περιλαμβάνονται 27 οικογένειες πασχόντων καθώς και μία ομάδα ελέγχου. Επιπλέον, ως ομάδα ελέγχου χρησιμοποιήθηκαν και 11 στελέχη αναφοράς από το διεθνές κέντρο αναφοράς μυκητιακής βιοποικιλότητας((Centraalbureau voor Schimmelcultures (CBS). Η δειγματοληψία πραγματοποιήθηκε με τη μέθοδο του βαμβακοφόρου στυλεού σε προκαθορισμένες περιοχές στους υγιείς και σε θέσεις βλάβης στους πάσχοντες. Επί υπάρξεως λεπίων αυτά αφαιρούνταν, μικροσκοπούνταν και καλλιεργούνταν. Τα συλεχθέντα δείγματα καλλιεργούνταν σε modified Dixons agar.

Για την ταυτοποίηση των ειδών *Malassezia* χρησιμοποιήθηκαν συμβατικές και μοριακές μέθοδοι. Οι συμβατικές μέθοδοι περιελάμβαναν α) την επιβεβαίωση εξάρτησης των στελεχών από την παρουσία λιπιδίων στο θρεπτικό υλικό β) την ύπαρξη καταλάσης με την διάσπαση του υπεροξειδίου του υδρογόνου γ) την ικανότητα μεταβολισμού υποστρωμάτων όπως τα Tween 20/40/80 και Cremophor el και τη δημιουργία ανάλογου φαινοτυπικού αποτελέσματος δ) την μετατροπή της εσκουλίνης σε εσκουλετίνη από την παρουσία του ενζύμου β-γλυκουρονιδάση.

Με βάση τα παραπάνω χαρακτηριστικά μπορούμε να διακρίνουμε μεταξύ 9 διαφορετικών ειδών *Malassezia*. Ωστόσο οι παραπάνω τεχνικές συμπληρώθηκαν με μοριακές μεθόδους προς επιβεβαίωση των αποτελεσμάτων. Εφαρμόστηκε PCR στις περιοχές ITS 3 και ITS 4 του ριβοσωμικού DNA. Στα προϊόντα πολυμερισμού έγινε επεξεργασία με περιοριστικά ένζυμα. Τα προϊόντα ακολούθως ηλεκτροφορήθηκαν και αναλόγως του αποτελέσματος ταυτοποιήθηκαν τα στελέχη σε είδη.

Τα στελέχη που ανήκουν στο είδος *M furfur* υποβλήθηκαν σε επιπλέον γενετική ανάλυση με ανάλυση αποτυπωμάτων του DNA (STR–PCR:short tandem repeat PCR) επιτρέποντας την ανάλυση εντός του είδους σε υποτύπους. Τα δεδομένα της PCR επεξεργάστηκαν με την χρήση κατάλληλου λογισμικού (bionumerics version 4) προκειμένου να συσχετιστούν με τα επιδημιολογικά χαρακτηριστικά του πληθυσμού.

Η αξιολόγηση της παραγωγής φωσφολιπάσης πραγματοποιήθηκε με τη μέθοδο egg yolk plate. Τα στελέχη καλλιεργήθηκαν σε Dixons agar με ή χωρίς την προσθήκη 100 nM β-ενδορφίνης. Ακολούθως οι αποικίες μεταφέρονταν σε egg yolk agar όπου και ελέγχονταν η παραγωγή λιπασών ανάλογα με μία ζώνη καθίζησης που δημιουργούνταν γύρω από την αποικία. Η ζώνη αυτή αποτελεί το φαινοτυπικό αποτέλεσμα δράσης των λιπασών και το εύρος της είναι ανάλογο της παραγώμενης ποσότητάς τους.

Τα ινδολικά παράγωγα της *Malassezia* (indirubin,ICZ, malassezin, tryptnthrin) συντέθηκαν *in vitro* και χρησιμοποιήθηκαν κατόπιν τιτλοποίησης.

Τα χρησιμοποιηθέντα δενδριτικά κύτταρα προέκυψαν από την *in vitro* εξεργασία με IL-4 και G-CSF μονοκυττάρων, τα οποία απομονώθηκαν από μονάδες περιφερικού αίματος υγιών δοτών. Τα δενδριτικά κύτταρα εκτέθηκαν σε TLR ή AhR αγωνιστές, συνήθως κατά την 6^{η} μέρα ωρίμανσης τους για προκαθορισμένα χρονικά διαστήματα. Η επίδραση των ινδολικών παραγώγων εξετάσθηκε επιπλέον σε μυελοειδή δενδριτικά κύτταρα που απομονώθηκαν απευθείας από το περιφερικό αίμα υγιών δοτών, μέσω επιλογής με μαγνητικά αντιγόνα.

Η αξιολόγηση της δράσης των ινδολών στα δενδριτικά κύτταρα μετρήθηκε σε επίπεδο RNA με PCR και σε επίπεδο πρωτεϊνών με western blotting. Η μικροσκοπική παρατήρηση της μετακίνησης του AhR μετά τη δράση των ινδολικών παραγόντων πραγματοποιήθηκε με την τεχνική της συνεστιακής μικροσκοπίας. Τέλος η επίδραση των ινδολικών παραγόντων στη φαινοτυπική και εκκριτική ωρίμανση των δενδριτικών αξιολογήθηκε με κυτταρομετρία ροής στην έκφραση των επιφανειακών δεικτών και με Eliza για τις παραγόμενες κυτταροκίνες. Η μεικτή λεμφοκυτταρική αντίδραση εφαρμόστηκε προκειμένου να ελεγχθεί η επίδραση των ινδολικών παραγώγων στην ικανότητα των δενδριτικών να επάγουν φλεγμονή.

Αποτελέσματα

Ενδοοικογενειακή κατανομή των ειδών και φυλογενετική ανάλυση των απομονωθέντων ειδών *Malassezia*

Από τους 85 ασθενείς που περιελήφθησαν στη μελέτη απομονώθηκαν 65 στελέχη που κατανέμονται σε 5 είδη (*M. furfur* (N=30)*, M. sympodialis* (N=20)*, M. globosa* (N=8)*, M. restricta* (N=6) και *M. sloofiae* (N=1)). Σε 2 ασθενείς απομονώθηκαν περισσότερα του ενός είδη. Σε κανένα από τα απομονωθέντα είδη δεν παρατηρήθηκε ενδοοικογενειακή κατανομή. Σε 10 από τις 18 οικογένειες ένα μόνο είδος *Malassezia* απομονώθηκε από όλα τα μέλη. Σε 7 από τις 10 οικογένειες το είδος αυτό ήταν η *M. furfur*. Η συχνότητα απομόνωσης των επιμέρους ειδών δεν συσχετίστηκε με το φύλο, τη θέση δειγματοληψίας και την ύπαρξη ή όχι νόσου. Τα απομονωθέντα στελέχη *M. furfur* αναλύθηκαν με την μέθοδο αποτυπωμάτων του DNA. Τα στελέχη με ενδοοικογενειακή προέλευση ή εκείνα που απομονώθηκαν από πάσχοντες δεν ταξινομήθηκαν σε συγκεκριμένους υπότυπους εντός των ειδών.

Παραγωγή φωσφολιπάσης από στελέχη *Malassezia* σε συνάρτηση με το είδος, την ύπαρξη σμηγματορροϊκής δερματίτιδας και την έκθεση των στελεχών σε β-ενδορφίνη.

Από τα στελέχη αναφοράς που ελέγχθηκαν, φωσφολιπάση παρήγαγαν τα: *M. furfur* CBS 6001*, M. caprae* CBS10434*, M. pachydermatis* CBS1879*, M. dermatis* CBS 9170*, M. slooffiae* CBS7956*, M. globosa* CBS7966. Από τα κλινικά στελέχη το 3% δεν παρήγαγε φωσφολιπάση ανεξάρτητα από το υλικό στο οποίο επωάστηκε (Dixons/ Dixons + b-endorphin). Τα μη παραγωγά στελέχη κατανέμονται εξίσου μεταξύ πασχόντων και υγιών. Δεν υπήρξε συσχέτιση μεταξύ της παραγωγής φωσφολιπάσης του φύλου, της οικογενούς κατανομής και της θέσης δειγματοληψίας. Τα διαφορετικά είδη δεν εμφάνισαν στατιστικά σημαντική διαφορά μεταξύ της βασικής παραγωγής φωσφολιπάσης.

Η έκθεση των στελεχών σε β-ενδορφινη (100 nM) αύξησε την παραγωγή φωσφολιπάσης από τα είδη *M. furfur* και *M. restricta* σε αντίθεση με την *M. globosa* και την *M sympodialis*. Σε σχέση με την προέλευση των στελεχών από υγιείς ή πάσχοντες, η έκθεση σε β-ενδορφίνη αύξησε την έκκριση φωσφολιπάσης στα στελέχη των πασχόντων ενώ δεν άλλαξε την έκκριση από τα στελέχη των υγιών. Τέλος τα στελέχη από πάσχοντες εμφανίζουν στατιστικά αυξημένη επαγωγιμότητα της παραγώγης της β φωσφολιπάσης (παραγωγή φωσφολιπάσης μετά από έκθεση σε β-ενδορφίνη/ βασική παραγωγή φωσφολιπάσης).

Η μεταγραφή των γονιδίων *Cyp1a1* kai *Cyp1b1* σε ανθρώπινα δενδριτικά κύτταρα εξαρτάται από τη δομή και συγκέντρωση των ινδολικών παραγώγων της *Malassezia*.

Δενδριτικά κύτταρα εκτέθηκαν για 6 h σε συγκεντρώσεις 100pM, 1nM, 10nM, 100nM και 1μM indirubin, malassezin, tryptanthrin, ICZ και TCDD. Η μέγιστη επαγωγή των υπό έλεγχο γονιδίων προέκυψε από τη δράση της indiroubin στη συγκέντρωση των 100nM. Δεύτερος πιο ισχυρός αγωνιστής αποδείχθηκε το ICZ. Αξίζει να σημειωθεί ότι η επαγωγή που προκλήθηκε από την indirubin ήταν τετραπλάσια εκείνης από το TCDD που αποτελούσε ως τώρα τον πιο ισχυρό γνωστό αγωνιστή. Στο χρονικό σημείο των 24 ωρών η επαγωγή από τις ινδόλες παρέμεινε στατιστικά σημαντική αν και μειωμένη σε σχέση με τις 6 ώρες. Η επαγωγή από τις ινδόλες επιβεβαιώθηκε και σε μυελοειδή δενδριτικά κύτταρα για το γονίδιο *Cyp1a1*. Η χρήση του εκλεκτικού αναστολέα του AhR CH223191 περιορίζει την προκαλούμενη επαγωγή δείχνοντας ότι πρόκειται για ένα AhR εξαρτώμενο αποτέλεσμα. Η έκφραση των γονιδίων του AhR ή του ARNT(aryl hydrocarbon nuclear translocator) δεν επηρεάστηκε από τη δράση των ινδολών.

Η συνδυασμένη έκθεση των δενδριτικών κυττάρων σε AhR και TLR αγωνιστές ενισχύει περαιτέρω τη μεταγραφή των γονιδίων *Cyp1a1* και *Cyp1b1.*

Η ινδιρουμπίνη και το ICZ περιορίζουν τη φαινοτυπική ωρίμανση και την έκκριση φλεγμονωδών κυτταροκινών από τα δενδριτικά κύτταρα

Η έκθεση ανώριμων (6^{ης} μέρας) δενδριτικών κυττάρων σε ινδολικά παράγωγα δε μεταβάλλει την έκφραση των επιφανειακών δεικτών ωρίμανσης (co-stimulatory molecules (CD80, CD83, CD86) ή του μείζονος συμπλέγματος ιστοσυμβατότητας. Αντίθετα η επίδραση αγωνιστών TLR προκαλεί στατιστικά σημαντική αύξηση αυτών των δεικτών. Ο συνδυασμός της indirubin ή του ICZ με TLR αγωνιστές (Lps ή R848) προκαλεί ένα στατιστικά σημαντικό περιορισμό της αναμενόμενης από τους TLR αγωνιστές επαγωγής.

Όπως είναι αναμενόμενο η έκθεση δενδριτικών κυττάρων σε TLR αγωνιστές διεγείρει την έκκριση φλεγμονωδών κυτταροκινών όπως οι IL-6, IL-12, TNFα και IL-10. Αντίθετα η έκθεση δενδριτικών κυττάρων σε ινδιρουμπίνη ή ICZ δεν επηρεάζει την έκφραση των προαναφερθέντων κυτοκινών. Ο συνδυασμός TLR αγωνιστών (LPS ή R848) με AhR αγωνιστές περιορίζει στατιστικά σημαντικά την έκφραση της IL6 και IL12. Ο περιορισμός στην παραγωγή Tnf-a και η αύξηση στην παραγωγή IL-10 εμφανίστηκαν ως τάση αλλά δεν επιβεβαιώθηκαν στατιστικά.

Η δράση των ινδολικών παραγόντων στην ωρίμανση των δενδριτικών κυττάρων διαμεσολαβείται από τον υποδοχέα AhR

Ο πιθανολογούμενος ρόλος του υποδοχέα AhR στην φαινοτυπική και εκκριτική αναστολή ωρίμανσης των δενδριτικών κυττάρων επιβεβαιώθηκε με τη χρήση του CH213191, εκλεκτικού αναστολέα του AhR. Δενδριτικά κύτταρα εκτέθηκαν σε συνδυασμούς R848/Lps, ινδιρουμπίνης/ICZ και CH223191. Η προσθήκη του CH312191 περιόρισε την ανασταλτική δράση των ινδολών στην ωρίμανση.

Τα ινδολικά παράγωγα περιορίζουν την ικανότητα των δενδριτικών κυττάρων να διεγείρουν των πολλαπλασιασμό των λεμφοκυττάρων

Η ικανότητα των δενδριτικών να επάγουν τον πολλαπλασιασμό των λεμφοκυττάρων αξιολογήθηκε με τη χρήση της μικτής λεμφοκυτταρικής αντίδρασης. Η έκθεση των δενδριτικών κυττάρων σε TLR αγωνιστές και η συνεπαγόμενη ωρίμανσή τους αυξάνει την ικανότητα των δενδριτικών να προκαλούν τον πολλαπλασιασμό των λεμφοκυττάρων σε αντίθεση με τους AhR αγωνιστές οι οποίοι δεν έχουν καμία επίδραση. Ο συνδυασμός AhR και TLR αγωνιστών περιορίζει την ικανότητα επαγωγής του πολλαπλασιασμού σε σχέση με την επίδραση μόνο TLR αγωνστών.

Η *in vitro* ωρίμανση των μονοκυττάρων του περιφερικού αίματος προς δενδριτικά κύτταρα επηρεάζεται ποικιλοτρόπως από την έκθεση σε ινδολικά παράγωγα

Τα μονοκύτταρα του περιφερικού αίματος ωριμάζουν προς δενδριτικά κύτταρα μετά από έκθεση σε GM-CSF (400u/ml) και IL-4 (300u/ml) για μια περίοδο 6 ημερών. Από την βιβλιογραφία είναι γνωστό ότι η ενεργοποίηση του AhR κατά τη διάρκεια ωρίμανσης των μονοκυττάρων αναστέλλει την ωρίμανση τους προς δενδριτικά κύτταρα. Η δράση των ινδολικών παραγώγων σε αυτή την διαδικασία επηρέασε τη διαδικασία διαφοροποίησης με τρόπο που ποικίλει ανάλογα με το δότη και την εξεταζόμενη ινδόλη.

Συζήτηση αποτελεσμάτων

Όπως αναφέρθηκε η κατανομή των ειδών της *Malassezia* σε σχέση με την ύπαρξη ή μη νόσου, τη βαρύτητα αυτής και την οικογενειακή ή μη σχέση των φορέων δεν εμφάνισε κάποια στατιστική σημαντικότητα. Η παρουσία περισσοτέρων του ενός είδους στους εθελοντές που συμμετείχαν δείχνει την πολυπλοκότητα της σχέσης ανάμεσα στα επιδημιολογικά χαρακτηριστικά του πληθυσμού και την κατανομή των ειδών. Η *Malassezia furfur* αποτέλεσε το συχνότερα απομονωθέν είδος και έδειξε τάσεις ενδοοικογενειακής κατανομής χωρίς όμως στατιστική σημαντικότητα.

Από την υπάρχουσα βιβλιογραφία είναι γνωστό ότι η β-endorfin όσο και οι πρόδρομες ουσίες είναι παρούσες στην ανθρώπινη επιδερμίδα. Έχει ήδη αποδειχθεί ότι η δράση της β-endorphin επηρεάζει την παραγωγή φωσφολιπασών σε ζωόφιλα είδη του γένους *Malassezia* (*M. pachydermatis*). Επιπλέον το γεγονός αυτό έχει συνδεθεί με την ύπαρξη δερματίτιδος στους συγκεκριμένους ξενιστές. Τα δεδομένα που παρουσιάζονται στην παρούσα μελέτη συσχετίζουν τη δράση της β-endorphin με την παραγωγή φωσφολιπάσης και την κατάσταση υγείας του ξενιστή. Πιο συγκεκριμένα, τα στελέχη που προέρχονται από ασθενείς με σμηγματορροϊκή δερματίτιδα έχουν χαμηλότερη βασική παραγωγή πριν την έκθεση σε β-endorphin και υψηλότερη μετά, εμφανίζοντας στατιστικά σημαντική επαγωγιμότητα. Τα ανωτέρω αναδεικνύουν τη σημαντικότητα της φωσφολιπάσης στην παθογένεια της νόσου.

Τα παρουσιαζόμενα αποτελέσματα αποδεικνύουν ότι τα ινδολικά παράγωγα αποτελούν *in vitro* και εν δυνάμει *in vivo* ισχυρούς αγωνιστές του υποδοχέα AhR. Η indirubin αποτελεί τον ισχυρότερο αγωνιστή και εμφανίζεται σε συγκεκριμένη συγκέντρωση 4 φορές ισχυρότερη του TCDD. Προηγούμενα δεδομένα που παρουσιάζουν ισχυρότερο το TCDD έναντι της ινδιρουμπίνης σε τρωκτικά μπορούν να αποδοθούν σε διαφορές στην δομή των υποδοχέων μεταξύ των ειδών. Ένα νέο εύρημα της μελέτης ήταν η αυξημένη επαγωγή που προκαλείται από το συνδύασμο ινδολικών παραγόντων και TLR αγωνιστών στην επαγωγή γονιδίων ξενοβιοτικού μεταβολισμου όπως το *Cyp1a1*. Το εύρημα αυτό δεν μπορεί να αποδοθεί στην αύξηση των υποδοχέων AhR καθώς κάτι τέτοιο δεν επιβεβαιώθηκε πειραματικά.

Η αναστολή ωρίμανσης στα δενδριτικά κύτταρα που παρατηρήθηκε από τη δράση των ινδολικών παραγόντων έχει προηγούμενα αναφερθεί και για άλλους αγωνιστές όπως το βενζοπυρένιο. Οι προτεινόμενες θεωρίες από τη βιβλιογραφία περιλαμβάνουν την αλληλεπίδραση του AhR και του βασικού μεσολαβητή της φλεγμονής NF-kB σε πρωτεϊνικό επίπεδο ή τον ανταγωνισμό τους για μεταγραφικούς παράγοντες όπως ο p 300. Η επίδραση του AhR στην παραγωγή κυτοκινών μπορεί να είναι πιο άμεση, καθώς θέσεις πρόσδεσης του AhR έχουν αναφερθεί πλησίων των θέσεων μεταγραφής της IL6 ή IL12 σε διάφορα είδη.

Σε επίπεδο δέρματος θα μπορούσε να υποτεθεί οτι η παραγωγή ανοσοτροποποιητικών ουσιών όπως οι ινδόλες αποτελεί μία από τις πολλές στρατηγικές που εφαρμόζει ο μύκητας προκειμένου να εξασφαλίσει την επιβίωση και ανάπτυξη του. Έτσι η παραγωγή ινδολών θα μπορούσε να μειώσει την ενεργοποίηση δενδριτικών κυττάρων μετά από έκθεση σε αντιγόνα της *Malassezia* ή να μειώσει τη φλεγμονή σε θέσεις βλάβης όπως στην ποικιλόχροη πιτυρίαση και τη σμηγματορροϊκή δερματίτιδα.

References

Adachi J, Mori Y, Matsui S. (2004) Comparison of gene expression patterns between 2,3,7,8-tetrachlorodibenzo-p-dioxin and a natural arylhydrocarbon receptor ligand, indirubin *Toxicol Sci*. 80(1):161-9.

Adachi J, Mori Y, Matsui S. (2001) Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. J Biol Chem. 24;276(34):31475-8.

Aggarwal BB, Sethi G, Ahn KS. (2006) Targeting signal-transducer-and-activator-of-transcription-3 forprevention and therapy of cancer: modern target but ancient solution. Ann N Y Acad Sci.; 1091:151-69.

Aizawa T, Kano R, Nakamura Y. (2001) The geneticdiversity of clinical isolates of Malassezia pachydermatis from dogs and cats. Med Mycol;39:329e33

AOAC International. 2000. *Official methods of analysis of AOAC International*. 17th edition. Gaithersburg, MD, USA, Association of Analytical Communities.

Archer-Dubon C, Icaza-Chivez ME, Orozco-Topete. (1999) An epidemic outbreak of Malassezia folliculitis in three adult patients in an intensive care unit: a previously unrecognized nosocomial infection. Int J Dermatol. ;38(6):453-6.

Ashbee HR. (2007) Update on the genus of Mallassezia Medical Mycology 45, 287_303

Ashbee HR, Evans EG. (2002) Immunology of diseases associated with Malassezia species. Clin Microbiol Rev. ;15(1):21-57.

Ashbee HR, Ingham E, Holland KT, (1993) The carriage of Malassezia furfur serovars A, B and C in patients with pityriasis versicolor, seborrhoeic dermatitis and controls. Br J Dermatol. ;129(5):533-40.

Astorg P, Gradelet S, Leclerc J. (1997) Effects of provitamin A or non-provitamin A carotenoids on liver xenobiotic-metabolizing enzymes in mice. Nutr Cancer.;27(3):245-9.

Astorg P, Gradelet S, Leclerc J. (1994) Effects of beta-carotene and canthaxanthin on liver xenobiotic-metabolizing enzymes in the rat. Food Chem Toxicol ; 32 : 735 -42

Back O, Scheynius A, Johansson SG.(1995) Ketoconazole in atopic dermatitis: therapeutic response is correlated with decrease in serum IgE. Arch Dermatol Res;287:448-51.

Backlund M, Ingelman-Sundberg M. (2004) Different structural requirements of the ligand binding domain of the aryl hydrocarbon receptor for high- and low-affinity ligand binding and receptor activation. Mol Pharmacol. ;65(2):416-25.

Bankoti J, Rase B, Simones T. (2010) Functional and phenotypic effects of AhR activation in inflammatory dendritic cells. Toxicol Appl Pharmacol. ;246(1-2):18-28.

Baroni A, Orlando M, Donnarumma G. (1999)Toll-like receptor 2 (TLR2) mediates intracellular signalling in human keratinocytes in response to Malassezia furfur. Arch Dermatol Res. ;297(7):280-8.

Baroni A, Paoletti I, Ruocco E. (1996) Possible role of Malassezia furfur in psoriasis: modulation of TGF-beta1, integrin, and HSP70 expression in human keratinocytes and in the skin of psoriasis-affected patients. J Cutan Pathol. ;31(1):35-42.

Baroni A, Perfetto B, Paoletti I. (2001) *Malassezia* furfur invasiveness in a keratinocyte cell line (HaCat): effects on cytoskeleton and on adhesion molecule and cytokine expression. Arch Dermatol Res 293:414–419

Baroni A, Paoletti I, Ruocco E. (2004) Possible role of Malassezia furfur in psoriasis: modulation of TGF-beta1, integrin, and HSP70 expression in human keratinocytes and in the skin of psoriasis-affected patients. J Cutan Pathol. ;31(1):35-42.

Barrett-Bee K, Hayes Y, Wilson RG. (1985) A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. J Gen Microbiol. ;131(5):1217-21.

Benham RW.(1939) The cultural characteristics of Pityrosporum ovale a lipophilic fungus. J Invest Dermatol; 2: 187_203.

Begerow D, Stoll M, Bauer R. (2006) A phylogenetic hypothesis of Ustilaginomycotina based on multiple gene analyses and morphological data1 Mycologia, 98(6): 906–916.

Bergbrant IM, Andersson B, Faergemann J. (1999) Cell-mediated immunity to *Malassezia* furfur in patients with seborrhoeic dermatitis and pityriasis versicolor. Clin Exp Dermatol 24:402–406

Bieber T. (2007) The pro- and anti-inflammatory properties of human antigen-presenting cells expressing the high affinity receptor for IgE (Fc epsilon RI). Immunobiology.;212(6):499-503.

Bigliardi PL, Bigliardi-Qi M, Buechner S. (1998) Expression of mu-opiate receptor in human epidermis and keratinocytes. J Invest Dermatol, 111: 297-301.

Bigliardi-Qi M, Bigliardi PL, Surnanovski LT. (2004) Expression of μ-opiate receptor and endorphin in peripheral nerve endings in dermis and epidermis. Dermatology , 209: 183-189

Binder RL, Jonelis FJ. (1984) Seborrheic dermatitis: a newly reported side effect of neuroleptics. J Clin Psychiatry. ;45(3):125-6.

Bock KW, Köhle C. (2009) The mammalian aryl hydrocarbon (Ah) receptor: from mediator of dioxin toxicity toward physiological functions in skin and liver. Biol Chem. ;390(12):1225-35.

Bock KW, Köhle C. (2010) Contributions of the Ah receptor to bilirubin homeostasis and its antioxidative and atheroprotective functions. Biol Chem. ;391(6):645-53.

Bock KW (1994) ;.Aryl hyd rocarbon or dioxin receptor: biologic and toxic responses.Rev Physiol Biochem Pharmacol. 125:1-42.

Boekhout T, Kamp M, Guého E. (1998) Molecular typing of Malassezia species with PFGE and RAPD. Med Mycol ;36:365e72.

Bond R, Kim Y, Lloyd DH. (2005) Bovine and canine transferrin inhibit the growth of *Malassezia* pachydermatis in vitro. Med Mycol 43:447–451

Bond R, Stevens K, Perrins N. (2008) Carriage of *Malassezia* spp. yeasts in Cornish Rex, Vet Dermatol. ;19(5):299-304.

Bos JD, De Rie MA. (1999) The pathogenesis of psoriasis: immunological facts and speculations. Immunol Today. ;20(1):40-6.

Bos JD, Kapsenberg ML. (1993) The skin immune system: progress in cutaneous biology. Immunol Today. ;14(2):75-8.

Bos JD, Zonneveld I, Das PK. (1987) The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. J Invest Dermatol. ;88(5):569-73.

Bos WE, Thio HB, Neumann HA. (2006) The pathogenesis of inflammatory dermatoses, especially psoriasis Ned Tijdschr Geneeskd. 28;150(4):179-83.

Boyman O, Conrad C, Tonel G. (2007) The pathogenic role of tissue-resident immune cells in psoriasis. Trends Immunol. ;28(2):51-7.

Bowcock AM, Krueger JG. (2005) Gettingunder the skin: the immunogenetics ofpsoriasis. Nat Rev Immunol;5:699-711.

Bongiovanni L, Müller EJ, Della Salda L. (2011) Survivin in skin pathologies. Exp Dermatol.; 20(6): 457-63.

Brasch J, Martens H, Sterry W (1993) Langerhans cell accumulation in chronic tinea pedis and pityriasis versicolor. Clin Exp Dermatol 18:329–332

Breathnach AS, Gross M, Martin B (1976) Freeze-fracture replication of cultured *Pityrosporum orbiculare*. Sabouraudia 14:105–113 AS

Brenninkmeijer EE, Schram ME, Leeflang MM. (2008) Diagnostic criteria for atopic dermatitis: a systematic review. Br J Dermatol. ;158(4):754-65.

Bunse T, Mahre G. (1996) Soluble Pityrosporum-derived chemoattractant for polymorphonuclear leukocytes of psoriatic patients. Acta Derm Venereol. ;76(1):10-2.

Burton J, Pye RJ. (1983) Seborrhoea is not a feature of seborrhoeic dermatitis. Br Med J (Clin Res Ed). 9;286 (6372):1169-70.

Cabañes FJ, Theelen B, Castellá G (2007) Two new lipid-dependent *Malassezia* speciesfrom domestic animals. FEMS Yeast Res 7:1064–1076

Cabañes FJ, Vega S, Castellá G. (2011) Malassezia cuniculi sp. nov., a novel yeast species isolated from rabbit skin. Med Mycol. ;49(1):40-8.

Cafarchia C, Dell'aquila ME, Capelli G. (2007). Role of beta endorphin on phospholipase production in *Malassezia* pachydermatis in dogs: new insights into the pathogenesis of this yeast. Medical Mycology, 45: 11-15

Cafarchia C, Dell'aquila ME, Traversa D. (2009) Expression of the micro-opioid receptor on *Malassezia* pachydermatis and its effects in modulating phospholipase production, Medical mycology, 18: 1-6

Cafarchia C, Dell'Aquila ME, Traversa D, (2010) Expression of the micro-opioid receptor on Malassezia pachydermatis and its effect in modulating phospholipase production. Med Mycol. ;48(1):73-8.

Cafarchia C, Latrofa MS, Figueredo LA, (2011) Physiological and molecular characterization of atypical lipid-dependent Malassezia yeasts from a dog with skin lesions: adaptation to a new host? Med Mycol. ;49 (4):365-74

Cafarchia C, Gasser RB, Latrofa MS, (2008) Geneticvariants of Malassezia pachydermatis from canine skin: body distribution andphospholipase activity. FEMS Yeast Res;8:451e9.

Cafarchia C, Gasser RB, Latrofa MS. (2008) Genetic variants of *Malassezia pachydermatis* from canine skin: body distribution and phospholipase activity. FEMS Yeast Res. ;8(3):451-9.

Cafarchia C, Otranto D. (2004) Association between phospholipase production by *Malassezia* pachydermatis and skin lesions. J Clin Microbiol. ;42(10):4868-9.

Cannon P. F. (1986) International Commission on the taxonomy of fungi (ICTF): name changes in fungi of microbiological, industrial and medicalimportance. Microbiol. Sci. 3:285–287.

C-H. Lee, H-Y. Chuang, C-C. Shih. (2006) Transepidermal water loss, serum IgE and b-endorphin asimportant and independent biological markers fordevelopment of itch intensity in atopic dermatitis, British Journal of Dermatology, 154:1100–1107

Chang HJ, Miller HL, Watkins N. (1998) An epidemic of *Malassezia pachydermatis* in an intensive care nursery associated with colonization of health care workers' pet dogs. NewEngl J Med 338:706–711

Chatproedprai S, Wananukul S. (2008) Survey of common cutaneous lesions in healthy infants at the well baby clinic. J Med Assoc Thai. Sep;91(9):1356-9.

Chatzikokkinou P, Sotiropoulos K, Katoulis A. (2008) Seborrheic dermatitis - an early and common skin manifestation in HIV patients. Acta Dermatovenerol Croat.;16(4):226-30

Chelikani P, Fita I, Loewen PC. (2004) "Diversity of structures and properties among catalases". *Cell. Mol. Life Sci.* 61 (2): 192–208.

Chen SC, Muller M, Zhou JZ. (1997) Phospholipase activity in Cryptococcus neoformans: a new virulence factor? J Infect Dis. ;175(2):414-20.

Chen YH, Riby J, Srivastava P. (1995) Regulation of CYP1A1 by indolo[3,2-b]carbazole in murine hepatoma cells *J Biol Chem* 270(38):22548-55

Ciolino HP, Daschner PJ, Wang TT. (1998) Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. Biochem Pharmacol;56: 197–206.

Ciolino HP, Daschner PJ, Yeh GC. (1999) Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. Biochem J. 15;340 :715-22.

Ciolino HP, Wang TT, Yeh GC. (1998) Diosmin and diosmetin are agonists of the aryl hydrocarbon receptor that differentially affect cytochrome P450 1A1 activity. Cancer Res. 1;58 (13):2754-60.

Ciolino HP, Yeh GC. (1999) The flavonoid galangin is an inhibitor of CYP1A1 activity and an agonist/antagonist of the aryl hydrocarbon receptor. Br J Cancer. ;79(9-10):1340-6.

Clark RA, Chong BF. (2006) A novel method for the isolation of skin resident T cells from normal and diseased human skin. J Invest Dermatol. ;126(5):1059-70.Crespo MJ, Abarca ML, Cabañes FJ. (2000) Atypical lipid-dependent *Malassezia* species isolated from dogs with otitis externa. J Clin Microbiol 38:2383–2385

Crespo MJ, Abarca ML, Cabañes FJ. (2002) Occurrence of *Malassezia* spp. in horses and domestic ruminants. Mycoses 45:33–337

Crespo-Erchiga V, Hay RJ. (2010) Pityriasis versicolor and other *Malassezia* skin diseases. In *Malassezia* and the skin. Science and clinical practice (Boekhout T, Gueho E, Mayser P, J Velegraki A eds) Springer, Berlin, 175-199

Crews ST, Thomas JB, Goodman CS. (1988) The Drosophila single-minded gene encodes a nuclear protein with sequence similarity to the per gene product. Cell. 15;52(1):143-51.

Cunningham A. C., Leeming J. P., Ingham E. (1990) Differentiation of three serovars of *Malassezia furfur*. J. Appl. Bacteriol. 68:439–446.

Dagdeviren M, Cerikcioglu N, Karavus M. (2005) Acid proteinase, phospholipase and adherence properties of Candida parapsilosis strains isolated from clinical specimens of hospitalised patients. Mycoses. ;48(5):321-6.

Dai X, Sayama K, Yamasaki K. (2006) SOCS1-negative feedback of STAT1 activation is a key pathway in the dsRNA-induced innate immune response of human keratinocytes. J Invest Dermatol. ;126(7):1574-81.

Damiens E, Meijer L. (2000) [Chemical inhibitors of cyclic-dependent kinases: preclinical and clinicalstudy]. Pathol Biol (Paris).;48 (3):340-51.

Darabi K, Hostetler SG, Bechtel MA, (2009).The role of Malassezia in atopic dermatitis affecting the head and neck of adults. J Am Acad Dermatol. ;60 (1):125-36

Davis PD, Elliott LH, Harris W. (1992) Inhibitors of protein kinase C. Substituted bisindolylmaleimides with improved potency and selectivity. J Med Chem. Mar 20; 35(6):994-1001

DaerM, ObermaierB, HertenJ.(2003)Mature dendritic cells derived from human monocytes within 48 hours:a novel strategy for dendritic cell differentiation from blood precursors. J Immunol. 15;170 (8): 4069-76.

Dawson TL Jr. (2007) Malassezia globosa and restricta: breakthrough understanding of the etiology and treatment of dandruff and seborrheic dermatitis through whole-genome analysis. J Investig Dermatol Symp Proc. ;12(2):15-9

DeAngelis YM, Gemmer CM, Kaczvinsky JR. (2005) Three etiologic facets of dandruff and seborrheic dermatitis: Malassezia fungi, sebaceous lipids, and individual sensitivity.J Investig Dermatol Symp Proc. 10(3):295-7.

De Angelis YM, Saunders CW, Johnstone KR. (2007) Isolation and expression of a Malassezia globosa lipase gene, LIP1. 127(9):2138-46

De D, Kanwar AJ, Handa S.(2006) Comparative efficacy of Hanifin and Rajka's criteria and the UK working party's diagnostic criteria in diagnosis of atopic dermatitis in a hospital setting in North India. J Eur Acad Dermatol Venereol. ;20(7):853-9.

Denison MS, Nagy SR. (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol. ;43:309-34.

Diani-Moore S, Labitzke E, Brown R. (2006) Sunlight generates multiple tryptophan photoproducts eliciting high efficacy CYP1A induction in chick hepatocytes and in vivo. Toxicol Sci. ;90(1):96-110.

Difonzo EM, Faggi E. (2008) Skin diseases associated with Malassezia species in humans. Clinical features and diagnostic criteria. Parassitologia. ;50(1-2):69-71

DiNatale BC, Schroeder JC, Francey LJ. (2010) Mechanistic insights into the events that lead to synergistic induction of interleukin 6 transcription upon activation of the aryl hydrocarbon receptor and inflammatory signaling Biol Chem. ;285(32):24388-97.

Dominguez-Soto L, Hojyo-Tomoka. (1996) Pigmentary problems in the tropics. Dermatol Clin. Oct;12(4):777-84.

Donnarumma G, Paoletti I, Buommino E. (2004) Malassezia furfur induces the expression of beta-defensin-2 in human keratinocytes in a protein kinase C-dependent manner. Arch Dermatol Res 295:474–481

Doostdar H, Burke MD, Mayer RT. (2000) Bioflavonoids: selective substrates and inhibitors for cytochrome P450 CYP1A and CYP1B1. Toxicology. 3;144(1-3):31-8.

Dorn M, Roehnert K. (1977) Dimorphism of Pityrosporum orbicularein a defined culture medium. J Invest Dermatol; 69: 244_ 248.

Dolwick KM, Schmidt JV, Carver LA. (1993) Cloning and expression of a human Ah receptor cDNA. Mol Pharmacol. ;44(5):911-7.

Emery A. (1986) Methodology in Medical Genetics

Eisenbrand G, Hippe F, Jakobs S. (2004) Molecular mechanisms of indirubin and its derivatives: novel anticancer molecules with their origin in traditional Chinese phytomedicine. J Cancer Res Clin Oncol. ;130(11):627-35.

Elewski B. (1990) Does pityrosporum ovale have a role in psoriasis? Arch Dermatol. ;126 (8):1111-2.

Ercis M, Balci S, Atakan N. (1996) Dermatological manifestations of 71 Down syndrome children admitted to a clinical genetics unit. Clin Genet. ;50 (5):317-20.

Esser C, Rannug A, Stockinger B. (2009) The aryl hydrocarbon receptor in immunity *Trends Immunol* 30(9):447-54

Faergemann J, Bergbrant IM, Dohse M. (2001) Seborrhoeic dermatitis and Pityrosporum (*Malassezia*) folliculitis: characterization of inflammatory cells and mediators in the skin by immunohistochemistry. Brit J Dermatol 144:549–556

Faergemann J, Bernander S. (1979) Tinea versicolor and Pityrosporum orbiculare: a mycological investigation. Sabouraudia;17:171-9.

Faergemann J, Johansson S, Back O. (1986) An immunologic and cultural study of Pityrosporum folliculitis.\ J Am Acad Dermatol;14:429-33.

Farr PM, Krause LB, Marks JM. (1985). Response of scalp psoriasis to oral ketoconazole. Lancet 26;2(8461):921-2.

Flaveny CA, Murray IA, Chiaro CR. (2009) Ligand selectivity and gene regulation by the human aryl hydrocarbon receptor in transgenic mice *Mol Pharmacol* 75(6):1412-20

Fontaine F, Delescluse C, de Sousa G. (1999) Cytochrome 1A1 induction by primaquine in human hepatocytes and HepG2 cells: absence of binding to the aryl hydrocarbon receptor. Biochem Pharmacol. 1;57 (3):255-62.

Foster C, Yokozeki H, Rapperberger K. (1990) Human epidermal T cells predominantly belong to the lineage expressing alpha/beta T cell receptor. J Exp Med. 1990 Apr 1;171(4):997-1013.

Fritsche E, Schäfer C, Calles C. (2007). Lightening up the UV response by identification of the arylhydrocarbon receptor as a cytoplasmatic target for ultraviolet B radiation *Proc Natl Acad Sci U S A*. 22; 104(21):8851-6

Fry L, Baker BS. (2007) Triggering psoriasis: the role of infections and medications. Clin Dermatol. ;25 (6):606-15.

Gaitanis G, Magiatis P, Hantschke M. (2012) The Malassezia genus in skin and systemic diseases. Clin Microbiol Rev. ;25 (1):106-41.

Gaitanis G, Magiatis P, Stathopoulou K. (2008) AhR ligands, malassezin, and indolo[3,2-b]carbazole are selectively produced by *Malassezia furfur* strains isolated from seborrheic dermatitis *J Invest Dermatol* 128(7):1620-5

Gaitanis G, Mayser P, Scheynius A. (2010) *Malassezia* yeasts in Seborrheic and Atopic Eczema. *Malassezia* and the skin. Science and clinical practice (Boekhout T, Guého E, Mayser P, Velegraki A eds) Springer, Berlin, 201-228

Gaitanis G, Robert V, Velegraki A. (2006) Verifiable single nucleotide polymorphisms of the internal transcribed spacer 2 region for the identification of 11 Malassezia species. J Dermatol Sci.; 43(3):214-7.

Gaitanis G, Velegraki A, Alexopoulos EC. (2009) Malassezia furfur fingerprints as possible markers for human phylogeography. ISME J. ;3(4):498-502.

Gaitanis G, Velegraki A, Alexopoulos EC. (2006) Distribution of Malassezia species in pityriasis versicolor and seborrhoeicdermatitis in Greece. Typing of the major pityriasis versicolor isolate M. globosa.Br J Dermatol ;154:854-9.

Gaitanis G, Velegraki A, Frangoulis E. (2002) Identification of Malassezia species from patient skin scales by PCR-RFLP. Clin Microbiol Infect. ;8(3):162-73.

Gaitanis G, Velegraki A, Magiatis P. (2011) Could Malassezia yeasts be implicated in skin carcinogenesis through the production of aryl-hydrocarbon receptor ligands? Med Hypotheses. ;77(1):47-51.

Gaitanis G, Mayser P. Malassezia yeasts in seborrheic and atopic eczemas, Malassezia and the skin. Springerlink, 2010

Gambichler T, Krämer HJ, Boms S. (2007) Quantification of ultraviolet protective effects of pityriacitrin in humans. Arch Dermatol Res. ;299(10):517-20.

Gandra RF, Simão RC, Matsumoto FE. (2006) Genotyping by RAPD-PCR analyses of Malassezia furfur strains from pityriasisversicolor and seborrhoeic dermatitis patients. Mycopathologia;162: 273e80.

Garside H, Stewart A, Brown N. (2008) Quantitative analysis of aryl hydrocarbon receptor activation using fluorescence-based cell imaging--a high-throughput mechanism-based assay for drug discovery. Xenobiotica. ;38(1):1-20.

Gemmer CM, DeAngelis YM, Theelen B. (2002) Fast, noninvasive method for molecular detection and differentiation of Malassezia yeast species on human skin and application of the method to dandruff microbiology.J Clin Microbiol. ;40 (9):3350-7.

Ghannoum MA. (1998) Extracellular phospholipases as universal virulence factor in pathogenic fungi. Nihon Ishinkin Gakkai Zasshi.;39 (2):55-9.

Ghannoum MA. (2000) Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev. ;13(1):122-43

Giakoumaki D, Stathopoulou K, Melliou E. (2008) Identification of indirubin as a metabolite of *Malassezia furfur* strains isolated from diseased skin *Planta Medica* 74 (9): 1081

Gillner M, Bergman J, Cambillau C. (1993) Interactions of indolo[3,2-b]carbazoles and related polycyclic aromatic hydrocarbons with specific binding sites for 2,3,7,8-tetrachlorodibenzo-p-dioxin in rat liver. Mol Pharmacol. ;44(2):336-45.

Glinski W, Brodecka H, Glinska-Ferenz M. (1994) Increased concentration of beta-endorphin in sera of patients with psoriasis and other inflammatory dermatoses, Br J Dermatology, 131 :260-264

Glinski W, Brodecka H, Glinska-Ferenz M. (1995) Increased concentration of beta-endorphin in the sera of patients with severe atopic dermatitis. Acta Derm Venereol. ;75(1):9-11.

Goodfield MJ, Saihan EM, Crowley J. (1987) Experimental folliculitis with Pityrosporum orbiculare: the influence of host response. Acta Derm Venereol.;67(5):445-7.

Gordon, M. A. (1951) Lipophilic yeast organism associated with tinea versicolor. J. Investig. Dermatol. 17:267–272.

Gradelet S, Astorg P, Leclerc J. (1996). Effectsof canthaxanthin, astaxanthin, lycopeneand lutein on liver xenobiotic-metabolizingenzymes in the rat. Xenobiotica; 26 : 49 -63

Gradelet S, Astorg P, Pineau T. (1997) Ah receptor-dependent CYP1A induction by two carotenoids, canthaxanthin and beta-apo-8'-carotenal, with no affinity for the TCDD binding site. Biochem Pharmacol. 15;54(2):307-15.

Grice EA, Kong HH, Conlan SD. (2009) Topographical and temporal diversity of the human skin microbiome. Science, 29;324(5931):1190-2.

Guého E, Midgley G, Guillot J. (1996) The genus *Malassezia* with description of four new species. Antonie van Leeuwenhoek 69:337–35

Guillot J, Guého E, Mialot M. (1998) Importance des levures du genre *Malassezia* endermatologie vétérinaire. Point Vét 29:21–31

Guillot J,Hadina S, Guého E. (2008) The genus *Malassezia*: old facts and new concepts. Parassitologia 50(1-2):77-9

Guillot J. and Gueho E. (1995) The diversity of *Malassezia* yeasts confirmed by rRNA sequence and nuclear DNAcomparisons. Antonie Leeuwenhoek 67:297–314.

Guillot J, Gueho E, Lesourd M. (1996) Identification of *Malassezia* species. A practical approach. J. Mycol.Med. 6:103–110.

Gupta AK, Batra R, Bluhm R. (2004) Skin diseases associated with Malassezia species. J Am Acad Dermatol. ;51(5):785-98

Gupta AK, Batra R, Bluhm R. (2003) Pityriasis versicolor. Dermatol Clin. ;21(3):413-29

Gupta AK, Boekhout T, Theelen B. (2004) Identification andtyping of Malassezia species by amplified fragment length polymorphism andsequence analyses of the internal transcribed spacer and large-subunitregions of ribosomal DNA. J Clin Microbiol;42:4253e60.

Gupta AK, Kohli Y, Summerbell RC. (2001) Quantitative culture of Malassezia species from different body sites of individuals with or without dermatoses. Med Mycol. ;39(3):243-51.

Guttman-Yassky E, Lowes MA. (2007) Major differences in inflammatory dendritic cells and their products distinguish atopic dermatitis from psoriasis. J Allergy Clin Immunol. ;119(5):1210-7.

Haarmann-Stemmann T, Abel J. (2010) An introduction to the molecular basics of aryl hydrocarbon receptor biology. Biol Chem. ;391(11):1235-48.

Harrison CA, Layton CM, Hau Z. (2007) Transglutaminase inhibitors induce hyperproliferation and parakeratosis in tissue-engineered skin. Br J Dermatol;156:247-57

Habermann E, Hardt K.L (1972) A sensitive and specific plate test for the quantitation of phospholipases Anaalytical biochemistry 50, 163-173

Hajdúch M, Weigl E, Jezdínská V. (1993) Biochemical aspects of immunomodulation by Propionibacterium acnes. Acta Univ Palacki Olomuc Fac Med.;136:9-12.

Hale FE, Melia TW. (1911) The value of the esculin test. J Am Public Health Assoc. ;1(11):833-6.

Hanifin JM, Rajka G. (1980) Diagnostic features of atopic dermatitis. *ActaDermatol Venereol*; 92 (suppl): 44–47.

He SM, Du WD, Yang S. (2008) The genetic epidemiology of tinea versicolor in China.Mycoses. ;51(1):55-62.

Heng MC, Henderson CL, Barker DC, (1990) Correlation of Pityosporum ovale density with clinical severity of seborrheic dermatitis as assessed by a simplified technique J Am Acad Dermatol. ;23(1):82-6.

Hedayati MT, Hajheydari Z, Hajjar F. (2010) Identification of Malassezia species isolated from Iranian seborrhoeic dermatitis patients. Eur Rev Med Pharmacol Sci. ;14(1):63-8.

Henseler RA, Romer EJ, Sulentic CE. (2009) Diverse chemicals including aryl hydrocarbon receptor ligands modulate transcriptional activity of the 3'immunoglobulin heavy chain regulatory region. Toxicology. 30;261(1-2):9-18.

Hoessel R, Leclerc S, Endicott J. (1999) Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nat Cell Biol. ;1(1):60-7.

Hong KK, Cho HR, Ju WC. (2007) A study on altered expression of serine palmitoyltransferase and ceramidase in psoriatic skin lesion.J Korean Med Sci;22:862-7.

Herzog MG, Hollman PCH, Katan MB. (1992) Content of potentially anticarcinogenic flavonoids of 28 vegetable and 9 fruits commonly consumed in the Netherlands. J. Agr. Food Chem. 41:2379–83

Hier S, Cornbleet T, Bergeim O. (1946) The aminoacids of human sweat. *J Biol Chem* 166:327–33

Hirai A, Kano R, Makimura K. (2004) *Malassezia nana* sp. a novel lipid-dependentyeast species isolated from animals. Int J Syst Evol Microbiol 54:623–627

Hollman PC, Katan MB. (1997) Absorption, metabolism and health effects of dietary flavonoids in man. Biomed Pharmacother.;51(8):305-10.

Hossain HM, Landgraf V, Weiß R. (2007) The genetic and biochemical characterization of the species *Malassezia* pachydermatis with particular attention on pigment-producing subgroups. Med Mycol 45:41–49

Huang ZJ, Edery I, Rosbash M. (1993) PAS is a dimerization domain common to Drosophila period and several transcription factors. Nature. 15;364(6434):259-62.

Hunger RE, Sieling PA, Ochoa MT. (2004) Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. J Clin Invest. ;113(5):701-8.

Ibrahim AS, Mirbod F, Filler SG. (1995) Evidence implicating phospholipase as a virulence factor of Candida albicans. Infect Immun. May;63(5):1993-8.

Ikuta T, Namiki T, Fujii-Kuriyama Y. (2009) AhR protein trafficking and function in the skin *Biochemical Pharmacology* 7 7 : 588 – 596

Ishibashi Y, Kato H, Asahi Y. (2009) Identification of the major allergen of *Malassezia* globosa relevant for atopic dermatitis. J Dermatol Sci. ;55(3):185-92.

Jena DK, Sengupta S, Dwari BC. (2005) Pityriasis versicolor in the pediatric age group. Indian J Dermatol Venereol Leprol. ;71(4):259-61.

Juntachai W, Oura T, Murayama SY. (2009) The lipolytic enzymes activities of *Malassezia* species. Med Mycol.; 47(5):477-84.

Kaga M, Sugita T, Nishikawa A. (2011) Molecular analysis of the cutaneous *Malassezia* microbiota from the skin of patients with atopic dermatitis of different severities. Mycoses ;54(4):e24-8

Kaneko T, Makimura K, Abe M. (2007) Revised culture-based system for identification of Malassezia species. J Clin Microbiol; 45:3737e42.

Kaneko T, Makimura K, Onozaki M. (2005) Vital growth factors of *Malassezia* species on modified CHROMagar Candida. Med Mycol;43:699e704.

Kaneko T, Makimura K, Sugita T. (2006) Tween 40-based precipitate production observed on modified chromogenic agar and development of biological identification kit for *Malassezia* species. Med Mycol; 44: 227e31.

Kantardjiev TB, Zisova LG, Kuzmanov AH. (2001) Study of the titer of common antibodies against superficial antigens of Pityrosporum ovale in dermatitis seborrhoides. Folia Med (Plovdiv).;43(3):37-40.

Karchner SI, Kennedy SW, Trudeau S. (2000) Towards molecular understanding of species differences in dioxin sensitivity: initial characterization of Ah receptor cDNAs in birds and an amphibian. Mar Environ Res. ;50(1-5):51-6.

Kaplan DH, Kissenpfennig A, Clausen BE. (2008) Insights into Langerhans cell function from Langerhans cell ablation models. Eur J Immunol. ;38(9):2369-76.

Karaoui R, Bou-Resli M, Al-Zaid NS. (1981) Tinea versicolor: ultrastructural studies on hypopigmented and hyperpigmented skin. Dermatologica. 162(2):69-85

Kato H, Sugita T, Ishibashi Y. (2006) Detection and quantification of specific IgE antibodies against eight Malassezia species in sera of patients with atopic dermatitis by using an enzyme-linked immunosorbent assay. Microbiol Immunol. ;50(11):851-6.

Kaur I, Handa S, Kumar B. (2009) Tinea versicolor: involvement of unusual sites. Int J Dermatol. 1996 ;35(8):604-5.

Kauser S, Thody AJ, Schallreuter KU. (2004) Beta-endorphin as a regulator of human hair follicle melanocyte biology, J Invest Dermatol, 123: 184–95.

Kawai K, Shimura H, Minagawa M. (2002) Expression of functional Toll-like receptor 2 on human epidermal keratinocytes. J Dermatol Sci. ;30(3):185-94.

Kawanishi M, Sakamoto M, Ito A. (2003) Construction of reporter yeasts for mouse aryl hydrocarbon receptor ligand activity Mutation Research 540 99–105

Keddie FM. (1966) Electron microscopy of *Malassezia furfur* in tinea versicolor. Sabouraudia5:134–13.

Kerkvliet N. (2009) AHR-mediated immunomodulation: the role of altered gene transcription *Biochem Pharmacol* 77(4):746-60.

Kerr LM, Levvy GA. (1947) B-Glucuronidase and tissue damage. Nature. 4;160(4066):463

Klechevsky E, Morita R, Liu M. (2008) Functional specializations of human epidermal Langerhans cells and CD14+ dermal dendritic cells. Immunity. ;29(3):497-510.

Kobayashi A, Numayama-Tsuruta K, Sogawa K. (1997) CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). J Biochem. ;122(4):703-10.

Krämer HJ, Kessler D, Hipler UC. (2005) Pityriarubins, novel highly selective inhibitors of respiratory burst from cultures of the yeast *Malassezia* furfur: comparison with the bisindolylmaleimide arcyriarubin A. Chembiochem. ;6(12):2290-7.

Krämer HJ, Podobinska M, Bartsch A. (2005) Malassezin, a novel agonist of the aryl hydrocarbon receptor from the yeast *Malassezia* furfur, induces apoptosis in primary human melanocytes. Chem biochem. ;6(5):860-5.

Kramer M, Schulte B, Toonen L. (2007) Echovirus infection caused rapid loss-of-function and cell death in human dendritic cells. *Cell Microbiol* 9(6): 1507-1518.

Krisanty RI, Bramono K, Made Wisnu I.(2009) Identification of *Malassezia* species from pityriasis versicolor in Indonesia and its relationship with clinical characteristics. Parassitologia. ;50(1-2):69-71.

Kusunoki T, Morimoto T, Nishikomori R. (2009) Changing prevalence and severity of childhood allergic diseases in kyoto, Japan, from 1996 to 2006. Allergol Int. ;58(4):543-8.

Lambert D, Beylot C, Mazet J. (1984) [Drug eruptions caused by lithium salts]. Ann Med Interne.;135(8):637-8.

Larocco M, Dorenbaum A, Robinson A. (1988) Recovery of *Malassezia* pachydermatis from eight infants in a neonatal intensive care nursery: clinical and laboratory features. Pediatr Infect Dis J. 7(6):398-401.

Larsen FS, Holm NV, Henningsen K. (1986) Atopic dermatitis. A genetic-epidemiologic study in a population-based twin sample. J Am Acad Dermatol.;15(3):487-94.

Laupeze B, Amiot L, Sparfel L, Le Ferrec E, (2002). Polycyclic aromatic hydrocarbons affect functional differ- entiation and maturation of human monocyte-derived dendritic cells. J Immunol, 168: 2652-58.

Lawrence BP, Denison MS, Novak H. (2008) Activation of the aryl hydrocarbon receptor is essential for mediating the anti-inflammatory effects of a novel low-molecular-weight compound *Blood* 112(4):1158-65

Lee J-H and Lee J. (2010) Indole as an intercellular singal in microbial communities *FEMS Microbiol Rev* 34; 426-444

Lee YW, Byun HJ, Kim BJ. (2011) Distribution of *Malassezia* species on the scalp in korean seborrheic dermatitis patients. Ann Dermatol. ;23(2):156-61.

Leeming JP, Notman FH. (1987) Improved methods for isolation and enumeration of *Malassezia* furfur from human skin. J Clin Microbiol;25:2017e9.

Leidich SD, Ibrahim AS, Fu Y. (1998) Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of Candida albicans. J Biol Chem. 2;273(40):26078-86.

León B, Ardavín C. (2008) Monocyte-derived dendritic cells in innate and adaptive immunity. Immunol Cell Biol. May-Jun;86(4):320-4.

Lim SL, Lim CS. (2008) New contrast stain for the rapid diagnosis of pityriasis versicolor. Arch Dermatol.;144(8):1058-9.

Lober CW, Belew PW, Rosenberg EW, (1982) Patch tests with killed sonicated microflora in patients with psoriasis. Arch Dermatol. ;118(5):322-5.

López-Bravo M, Ardavín C. (2008) *In vivo* induction of immune responses to pathogens by conventional dendritic cells *Immunity* 19;29(3):343-51.

Lopez-Garcia B, Lee PH, Gallo RL. (2006) Expression and potential function of cathelicidin antimicrobial peptides in dermatophytosis and tinea versicolor. J Antimicrob Chemother 57:877–882

Luciana C. Paulino, Chi-Hong Tseng. (2006) Molecular Analysis of Fungal Microbiota in Samples from Healthy Human Skin and Psoriatic Lesions J Clin Microbiol.;44(8):2933-41.

Luecke S, Backlund M, Jux B, (2010) The aryl hydrocarbon receptor (AHR), a novel regulator of human melanogenesis. Pigment Cell Melanoma Res. ;23(6):828-33.

MacDonald CJ, Ciolino HP, Yeh GC. (2001). Dibenzoylmethane modulates aryl hydrocarbon receptor function and expression of cytochromes P50 1A1, 1A2, and 1B1. Cancer Res. 613919–24

Machowinski A, Krämer HJ, Hort W. (2006) Pityriacitrin--a potent UV filter produced by Malassezia furfur and its effect on human skin microflora. Mycoses. ;49(5):388-92.

Magiatis P, Gaitanis G, Mexia N. (2010) Chemistry and biology of *Malassezia* metabolites related to skin diseases *Planta Medica* 76 (12): 1293-1293

Maines MD. (2003) Bile pigments: newcomers to the cell signaling arena. Toxicol Sci. ;71(1):9-10.

Maietta G, Rongioletti F, Rebora A. (1991) Seborrheic dermatitis and daylight. Acta Derm Venereol. ;71(6):538-9.

Makimura K, Tamura Y, Kudo M. (2000) Species identification and strain typingof Malassezia species stock strains and clinical isolates based on the DNAsequences of nuclear ribosomal internal transcribed spacer 1 regions. J MedMicrobiol; 49:29–35.

Marco-Llorente J, Rojo-Martínez E. (2010) [Other non-motor disorders in Parkinson's disease]. Rev Neurol. Feb 8; 50 Suppl 2:S75-83.

Marcus RS, Holsapple MP, Kaminski NE. (1998) Lipopolysaccharide activation of murine splenocytes and splenic B cells increased the expression of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator *J Pharmacol Exp Ther* 287(3):1113-8.

Martinon F, Mayor A, Tschopp J. (2009) The inflammasomes: guardians of the body. Annu Rev Immunol. ;27:229-65.

Marples, M. (1965) The Ecology of the Human Skin. Bannerstone; Springfield, IL:

Mathov I, Plotkin L, Abatangelo C, Antibodies from patients with psoriasis recognize N-acetylglucosamine terminals in glycoproteins from Pityrosporum ovale. Clin Exp Immunol. ;105(1):79-83.

Mayser P., Gaitanis G. (2010) Physiology and Biochemistry. In *Malassezia* and the skin. Science and clinical practice (Boekhout T, Guého E, Mayser P, Velegraki A eds) Springer, Berlin, 121-138.

Mayser P, Imkampe A, Winkeler M. (1998) Growth requirements and nitrogen metabolism of Malassezia furfur. Arch Dermatol Res 290:277–282

Mayser P, Laabs S, Heuer KU. (1996) Detection of extracellular phospholipase activity in Candida albicans and Rhodotorula rubra. Mycopathologia.;135(3):149-55

Mayser P, Schäfer U, Krämer HJ. (2002) Pityriacitrin --an ultraviolet-absorbing indole alkaloid from the yeast Malassezia furfur. Arch Dermatol Res. ;294(3):131-4.

Mayser P, Stapelkamp H, Krämer HJ. (2003); Pityrialactone- a new fluorochrome from the tryptophan metabolism of Malassezia furfur. Antonie Van Leeuwenhoek. 84(3):185-9

Mayser P, Wille G, Imkampe A, (1998) Synthesis of fluorochromes and pigments in Malassezia furfur by use of tryptophan as the single nitrogen source. Mycoses;4:265e71.

McKinney JD, Singh P. (1981) Structure-activity relationships in halogenated biphenyls: unifying hypothesis for structure specificity Chem Biol Interact. ;33(2-3):271-83.

McNally NJ, Williams HC, Phillips DR. (2000) Is there a geographical variation in eczema prevalence in the UK? Evidence from the 1958 British Birth Cohort Study. Br J Dermatol. ;142(4):712-20

Mendez-Tovar LJ. (2010) Pathogenesis of dermatophytosis and tinea versicolor. Clin Dermatol. Mar 4;28(2):185-9

Mempel M, Kalali BN, Ollert M, Ring J. (2007) Toll-like receptors in dermatology. Dermatol Clin. ;25(4):531-40,

Meyer BK, Pray-Grant MG, Vanden Heuvel JP. (1998) Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. Mol Cell Biol. ;18 (2):978-88

Miller LS, Modlin RL. (2007) Toll-like receptors in the skin. Semin Immunopathol. ;29(1):15-26.

Miller LS, Sorensen OE, Liu PT. (2005) TGF-alpha regulates TLR expression and function on epidermal keratinocytes. J Immunol. 15;174(10):6137-43.

Mittag H. (1994) An approach of the fine structure of *Malassezia furfur*. XII Congress of the International Society for Human and Animals Mycology,D139

Mittag H. (1995) Fine structural investigation of Malassezia furfur II. The envelope of the yeast cells. Mycoses. ;38(1-2):13-21.

Millington G. (2006) Proopiomelanocortin (POMC): the cutaneous roles of its melanocortin products and receptors Clin Exp Dermatol. ;31(3):407-12.

Moore M. (1940) Malassezia furfur the cause of tinea versicolor:cultivation of the organism and experimental production of the disease. Arch Dermatol; 41: 253_261.

Moon YJ, Wang X, Morris ME. (2006) Dietary flavonoids: effects on xenobiotic and carcinogen metabolism. Toxicol In Vitro. ;20(2):187-210.

Morar N, Willis-Owen SA, Moffatt MF. (2006) The genetics of atopic dermatitis J Allergy Clin Immunol. l;118(1):24-34

Morrien JJ. (1962) The morphology of Malassezia furfur (Robin) (Baillon, 1889) Ned Tijdschr Geneeskd. 106:1049-50.

Mukherjee PK, Seshan KR, Leidich SD. (2001) Reintroduction of the PLB1 gene into Candida albicans restores virulence in vivo. Microbiology. ;147(Pt 9):2585-97.

Murphy KA, Quadro L, White LA. (2007) The intersection between the aryl hydrocarbon receptor (AhR)- and retinoic acid-signaling pathways. Vitam Horm.;75:33-67.

Nagao T, Adachi K, Nishida F. (1999) The new ultraviolet ray absorbing material and its manufacturing method. Japanese Kokai Tokkyo Koho CODEN: JKXXAF JP 11269175 A2 1005 Heisei.

Nakabayashi A, Sei Y. (2001) [Relationship between Malassezia Yeast and Infantile Seborrhoeic Dermatitis]. Nihon Ishinkin Gakkai Zasshi. ;42(4):218-20.

Nakabayashi A, Sei Y, Guillot J. (2000) Identification of Malassezia species isolated from patients with seborrhoeic dermatitis, atopic dermatitis, pityriasis versicolor and normal subjects. Med Mycol. ;38(5):337-41.

Nair S, Kekatpure VD, Judson BL. (2009) UVR exposure sensitizes keratinocytes to DNA adduct formation. Cancer Prev Res (Phila). ;2(10):895-902.

Narang T, Dogra S, Kaur I. (2004) Malassezia and psoriasis: Koebner's phenomenon or direct causation? J Eur Acad Dermatol Venereol. ;21(8):1111-2.

Nazzaro-Porro M, Passi S, Caprilli F. (1977) Induction of hyphae in cultures in *Pityrosporum* by cholesterol and cholesterol esters. J. Investig. Dermatol. 69:531–534.

Nestle F, Di Meglio P, Qin J-P. (2009) Skin immune sentinels in health and disease *Nat Rev Immunol* 9(10): 679–691

Nickoloff BJ, Qin JZ, Nestle FO. 2007 Immunopathogenesis of psoriasis. Clin Rev Allergy Immunol.;33(1-2):45-56.

Nestle FO, Kaplan DH, Barker J. (2009) Psoriasis N Engl J Med. 30;361(5):496-509.

Nestle, F.O., Zheng, X.G., Thompson, C.B. (1993) Characterizationof dermal dendritic cells obtained from normal human skin reveals phenotypic and functionallydistinctive subsets. J. Immunol. 151:6535–6545

Netea MG, Van der Graaf C, (2004) Recognition of fungal pathogens by Toll-like receptors Eur J Clin Microbiol Infect Dis Sep;23(9):672-6

Nguyen TA, Hoivik D, Lee JE. (1999) Interactions of nuclear receptor coactivator/corepressor proteins with the aryl hydrocarbon receptor complex. Arch Biochem Biophys. 15;367(2):250-7.

Nickoloff BJ, Mitra RS, Green J. (1993) Activated keratinocytes present bacterial-derived superantigens to T lymphocytes: relevance to psoriasis. J Dermatol Sci. ;6(2):127-33.

Nickoloff, B.J. (1993) Dermal immune system. CRC Press. Boca Raton.

Nickoloff BJ, Wrone-Smith T, Bonish B. (1999) Response of murine and normal human skin toinjection of allogeneic blood-derived psoriatic immunocytes: detection of T cells expressing receptors typically present on natural killer cells, including CD94, CD158, and CD161. Arch Dermatol 1999;135:546-52.

Nickoloff BJ, Schröder JM. (2000) Is psoriasis a T-cell disease? Exp Dermatol;9:359-75.

Nograles KE, Zaba LC, Shemer A. (2009) IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. J Allergy Clin Immunol. ;123(6):1244-52.e2.

Nozaki S, Feliciani C, Sauder DN. (1992) Keratinocyte cytokines. Adv Dermatol.;7:83-100; 101.

Nyirjesy P, Nixon JM, Jordan CA. (1994) Malassezia furfur folliculitis of the vulva: olive oil solves the mystery Obstet Gynecol. ;84(4 Pt 2):710-1.

Odom RB. (1994) Common superficial fungal infections in immunosuppressed patients. J Am Acad Dermatol. ;31(3 Pt 2):S56-9.

Oh BH, Lee YW, Choe YB. (2010) Epidemiologic Study of Malassezia Yeasts in Seborrheic Dermatitis Patients by the Analysis of 26S rDNAPCR-RFLP.Ann Dermatol.;22(2):149-55.

Oh BH, Song YC, Lee YW. (2009) Comparison of Nested PCR and RFLPfor identification and classification of Malassezia yeasts from healthy humanskin. Ann Dermatol ;21:352e7.

Olesen AB, Ellingsen AR, Olesen H. (1997) Atopic dermatitis and birth factors: historical follow up by record linkage. BMJ. 5;314(7086):1003-8.

Oliveri S, Trovato L, Betta P. (2011) Malassezia furfur fungaemia in a neonatal patient detected by lysis-centrifugation blood culture method: first case reported in Italy. Mycoses. ;54(5):e638-40

Ong PY, Leung DY. (2010) The infectious aspects of atopic dermatitis. Immunol Allergy Clin North Am.;30(3):309-21.

Panja G. (1927) The Malassezia of the skin, their cultivation, morphology and species. Trans 7th Cong Far East Assoc Trop Med ; 2: 442_456.

Parry ME, Sharpe GR. (1998) Seborrhoeic dermatitis is not caused by an altered immune response to Malassezia yeast. Br J Dermatol. ;139(2):254-63.

Patten CL, Glick BR. (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol. ;42(3):207-20.

Paulino LC, Tseng CH, Blaser MJ. (2008) Analysis of Malassezia microbiota in healthy superficial human skin and in psoriatic lesions by multiplex real-time PCR. FEMS Yeast Res.;8(3):460-71.

Paulino LC, Tseng CH, Strober BE. (2006) Molecular analysis of fungal microbiota in samples from healthy human skin and psoriatic lesions. J Clin Microbiol.;44(8):2933-41.

Pennisi E. (2008) Microbiology:Bacteria are picky about their homes on human skin. Science: May23;320(5879):1001.

Pechère M, Krischer J, Remondat C. (1999) Malassezia spp carriage in patients with seborrheic dermatitis. J Dermatol. ;26(9):558-61.

Phelan D, Winter GM, Rogers WJ. (1998) Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin. Arch Biochem Biophys. 1;357(1):155-63.

Pierard-Franchimont C, Hermanns JF, Degreef H, (2000) From axioms to new insights into dandruff. Dermatology; 200:93-8.

Pivarcsi A, Bodai L, Réthi B. (2003) Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. Int Immunol. ;15(6):721-30.

Pini G, Faggi E. (2011) Extracellular phospholipase activity of Malassezia strains isolated from individuals with and without dermatological disease. Rev Iberoam Micol. ;28(4):179-82.

Platzer B, Richter S, Kneidinger D. (2009) Aryl hydrocarbon receptor activation inhibits in vitro differentiation of human monocytes and Langerhans dendritic cells J Immunol 183(1):66-74

Pollenz RS, Sattler CA, Poland A. (1994) The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. Mol Pharmacol. ;45(3):428-38.

Porro MN, Passi S, Caprilli f. (1977) Induction of hyphae in cultures of Pityrosporum by cholesterol and cholesterol esters. J Invest Dermatol. ;69(6):531-4

Porro MN, Passi S, Caprill F. (1976) Growth requirements and lipid metabolism of Pityrosporum orbiculare. J Invest Dermatol. ;66(3):178-82.

Potter BS, Burgoon CF, Johnson WC. (1973) Pityrosporum folliculitis. Report of seven cases and review of the Pityrosporum organism relative to cutaneous disease. Arch Dermatol 107:388–391

Prakobphol A, Leffler H, Hoover CI. (1997) Palmitoyl carnitine, a lysophospholipase-transacylase inhibitor, prevents Candida adherence in vitro. FEMS Microbiol Lett. 1;151(1):89-94.

Price MF, Wilkinson ID, Gentry LO. (1982) Plate method for detection of phospholipase activity in Candida albicans. Sabouraudia. ;20(1):7-14.

Procházková J, Kozubík A, Machala M, (2011) Differential effects of indirubin and 2,3,7,8-tetrachlorodibenzo-p-dioxin on the aryl hydrocarbon receptor (AhR) signalling in liver progenitor cells. Toxicology. 11;279(1-3):146-54.

Prohić A. (2003) Identification of Malassezia species isolated from scalp skin of patients with psoriasis and healthy subjects. Acta Dermatovenerol Croat.;11(1):10-6.
Prohic A. (2010) Distribution of Malassezia species in seborrhoeic dermatitis: correlation with patients' cellular immune status. Mycoses. ;53(4):344-9.

Prohic A, Kalilovic E. (2009) Identification of Malassezia pachydermatis species from healthy and diseased human skin Med Arh. ;63(6):317-9.

Prohic A, Kasumagic-Halilovic E. (2010) Identification of Malassezia species from immunocompetent and immunocompromised patients with seborrheic dermatitis. Eur Rev Med Pharmacol Sci. ;14(12):1019-23.

Puga A. (2011) Perspectives on the potential involvement of the AH receptor-dioxin axis in cardiovascular disease. Toxicol Sci. ;120(2):256-61.

Raabe P, Mayser P, Weiss R. (1998) Demonstration of Malassezia furfur and M. sympodialis together with M. pachydermatis in veterinary specimens. Mycoses. 1998 Dec;41(11-12):493-500.

Ramesh, A.V. Prabhudesai, Viswanathan C.V. (1978) Simultaneous Extraction and Preparative Fractionation of Egg Yolk Lipids Using the Principle of Adsorption. Journal of the American Chemist's Society 55:501-5028.

Ran Y, He X, Zhang H. (2008) Seborrheic dermatitis flare in a Dutch male due to commensal Malassezia furfur overgrowth. Med Mycol. ;46(6):611-4.

Rasool O, Zargari A, Almqvist J, (2000) Cloning, characterization and expression of complete coding sequences of three IgE binding Malassezia furfur allergens, Mal f 7, Mal f 8 and Mal f 9. Eur J Biochem. ;267(14):4355-61.

Ray S, Swanson H. (2009) Activation of the Aryl Hydrocarbon Receptor by TCDD Inhibits Senescence: A Tumor Promoting Event? *Biochem Pharmacol* 15; 77(4): 681–688

Rendic E, Díaz C, Fich F. (2003) [Characterization of species of the gender Malassezia in patients with seborrheic dermatitis and subjects without skin lesions]. Rev Med Chil. ;131(11):1295-300.

Reyes H, Reisz-Porszasz S, Hankinson O. (1992) Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNAbinding form of the Ah receptor. Science. 22;256(5060):1193-5

Richardson MD, Shankland GS. (1991) Enhanced phagocytosis and intracellular killing of Pityrosporum ovale by human neutrophils after exposure to ketoconazole is correlated to changes of the yeast cell surface. Mycoses 34:29–33

Riciputo RM, Oliveri S, Micali G. (1996) Phospholipase activity in Malassezia furfur pathogenic strains. Mycoses.;39(5-6):233-5

Rincón S, Celis A, Sopó L. (2005) Malassezia yeast species isolated from patients with dermatologic lesions. Biomedica. ;25(2):189-95.

Roberts BJ, Whitelaw ML. (1999) Degradation of the basic helix-loop-helix/Per-ARNT-Sim homology domain dioxin receptor via the ubiquitin/proteasome pathway. J Biol Chem. 17;274(51):36351-6.

Rocha N, Velho G, Horta M. (2005) Cutaneous manifestations of familial amyloidotic polyneuropathy.J Eur Acad Dermatol Venereol. Sep;19(5):605-7.

Rosenberg EW, Noah PW, Skinner RB Jr. (1989) Microbial associations of 167 patients with psoriasis. Acta Derm Venereol Suppl .;146:72-4; 75.

Rosenthal M, Goldberg D. (2011) Skin microbiota: microbial community structure and its potential association with health and disease. Infect Genet Evol ; 11(5) :839-48

Saadatzadeh MR, Ashbee HR, Holland KT. (2001) Production of the mycelial phase of Malassezia in vitro. Med Mycol; 39: 487_493.

Sabat R, Philipp S, Höflich C, (2007) Immunopathogenesis of psoriasis. Exp Dermatol. ;16(10):779-98.

Sabouraud R. (1904). Maladies du cuir chevulu. Masson et cie, Paris, France.

Salkin, F., Gordon M. (1977) Polymorphism of *Malassezia furfur*. Can J Microbiol. ;23(4):471-5.

Samaranayake LP, Raeside JM, MacFarlane TW. (1984) Factors affecting the phospholipase activity of Candida species in vitro. Sabouraudia.;22(3):201-7.

Sandhu K, Jain R, Kaur I. (2003) Role of Pityrosporum ovale in guttate psoriasis. J Dermatol.;30(3):252-4.

Sandström Falk MH, Tengvall Linder M, Johansson C. (2005) The prevalence of Malassezia yeasts in patients with atopic dermatitis, seborrhoeic dermatitis and healthy controls. Acta Derm Venereol. ;85(1):17-23.

Savin R. (1996) Diagnosis and treatment of tinea versicolor. J Fam Pract. ;43(2):127-32.

Savolainen J, Lintu P, Kosonen J. (2001) Pityrosporum and Candida specific and non-specific humoral, cellular and cytokine responses in atopic dermatitis patients.Clin Exp Allergy. ;31(1):125-34.

Savouret JF, Antenos M, Quesne M. (2001) 7-ketocholesterol is an endogenous modulator for the arylhydrocarbon receptor. J Biol Chem. 2;276(5):3054-9.

Scalbert A, Williamson G.(2000) Dietary intake and bioavailability of polyphenols. J Nutr. ;130(8S Suppl):2073S-85S.

Scheynius A, Faergemann J, Forsum U. (1984) Phenotypic characterization in situ of inflammatory cells in pityriasis (tinea) versicolor. Acta Derm Venereol 64:473–479

Schechtman RC, Midgley G, Hay RJ. (1995) HIV disease and Malassezia yeasts: a quantitative study of patients presenting with seborrhoeic dermatitis. Br J Dermatol. ;133(5):694-8

Schmidt JV, Bradfield CA. (1996) Ah receptor signaling pahtways *Annu Rev Cell Dev Biol* 12:55-89.

Schrenk D, Riebniger D, Till M. (1999) Tryptanthrins and other tryptophan-derived agonists of the dioxin receptor *Adv Exp Med Biol* 467:403-8

Schröder JM. (2010) The role of keratinocytes in defense against infection. Curr Opin Infect Dis.;23(2):106-10.

Seidel SD,Winters GM, Rogers WJ. (2001) Activation of the Ah receptor signaling pathway by prostaglandins. J. Biochem. Molec. Toxicol. 15:187–96

Serbina NV, Salazar-Mather TP, Biron CA. (2003) TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity. ;19(1):59-70.

Shanle EK, Xu W. (2011) Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action. Chem Res Toxicol. 14;24(1):6-19.

Shimba S, Watabe Y. (2009) Crosstalk between the AHR signaling pathway and circadian rhythm. Biochem Pharmacol. 15;77(4):560-5.

Shuster S. (1984) The aetiology of dandruff and the mode of action of therapeutic agents. Br J Dermatol. ;111(2):235-42

Simmons RB. (1989) Comparison of chitin localization in *Saccharomyces cerevisiae, Crytococcus neoformans*, and *Malassezia* spp. Mycol Res 94:551–553

Simmons RB, Buffington JR, Ward M. (1986) Morphology and ultrastructure of fungi in extended-wear soft contact lenses. J Clin Microbiol. 24(1):21-5

Simmons RB, Guého E. (1990) A new species of *Malassezia*. Mycol Res 94:1146–1149

Simones T, Shepherd DM. (2011) Consequences of AhR Activation in Steady-State Dendritic *Cells Toxicol Sci* 119(2):293-307.

Sina B, Kauffman CL, Samorodin CS. (1995) Intrafollicular mucin deposits in Pityrosporum folliculitis. J Am Acad Dermatol;32:807-9.

Sinal CJ, Bend JR. (1997) Aryl hydrocarbon receptor-dependent induction of cyp1a1 by bilirubin in mouse hepatoma hepa 1c1c7 cells. Mol Pharmacol. ;52(4):590-9.

Slooff, W. C. **(**1970) *Pityrosporum* sabouraud, the yeasts—a taxonomic study, 2nd ed. *In* J. Lodder (ed.), North Holland Publishing Co. p.1167–1186

Sohnle PG, Collins-Lech C. (1983) Activation of complement by Pityrosporum orbiculare. J Invest Dermatol 80:93–97

Sonesson A, Bartosik J, Christiansen J. (2012) Sensitization to skin-associated microorganisms in adult patients with atopic dermatitis is of importance for disease severity. Acta Derm Venereol. ;93(3):340-5

Stein DH. (1983) Superficial fungal infections. Pediatr Clin North Am ;30:545-61.

Sugita T, Kodama M, Saito M. (2003) Sequence diversity of the intergenicspacer region of the rRNA gene of Malassezia globosa colonizing the skin ofpatients with atopic dermatitis and healthy individuals. J Clin Microbiol;41:3022–3027.

Sugita T, Tajima M, Takashima M. (2004) A new yeast, *Malassezia yamatoensis*, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. Microbiol Immunol 48:579–583

Sugita T, Tajima M, Takashima M. (2004) A new yeast, *Malassezia yamatoensis*, isolated from a patient with seborrheic dermatitis, and its distribution in patients and healthy subjects. Microbiol Immunol 48:579–583

Sugita T, Tajima M, Tsubuku H. (2006) Quantitative analysis ofcutaneous *Malassezia* in atopic dermatitis patients using real-time PCR.Microbiol Immunol 2006;50:549e52.

Sugita T, Takashima M, Kodama M. (2003) A Description of a new yeast species, *Malassezia* japonica , and its detection in patients with atopic dermatitis and healthy subjects.J Clin Microbiol; 41: 4695_4699.

Sugita T, Takashima M, Shinoda T. (2002) New yeast species, *Malassezia* dermatis, isolated from patients with atopic dermatitis. J Clin Microbiol. 40(4):1363-7.

Sun LD, Cheng H, Wang ZX.(2010) Association analyses identify six new psoriasis susceptibility loci in the Chinese population. Nat Genet;42:1005-9.

Suzuki T, Ohno N, Oshima Y. (1998) Activation of the complement system, alternative and classical pathways, by *Malassezia* furfur. Pharm Pharmacol Lett 8:133–136

Tagami H. (1992) The role of complement-derived mediators in inflammatory skin diseases. Arch Dermatol Res.;284 Suppl 1:S2-9.

Tajima M, Sugita T, Nishikawa A. (2008) Molecular analysis of *Malassezia* microflora in seborrheic dermatitis patients: comparison with other diseasesand healthy subjects. J Invest Dermatol ;128:345e51.

Takahata Y, Sugita T, Hiruma M. (2007) Quantitative analysis of *Malassezia* in the scale of patients with psoriasis using a real-time polymerase chain reaction assay. Br J Dermatol.;157(4):670-3.

Takahata Y, Sugita T, Kato H. (2007) Cutaneous *Malassezia* flora in atopic dermatitis differs between adults and children. Br J Dermatol. ;157(6):1178-82.

Takahashi M, Ushijima T, Ozaki Y. (1984) Biological activity of Pityrosporum. I. Enhancement of resistance in mice stimulated by Pityrosporum against Salmonella typhimurium. Immunology. ;51(4):697-702

Takahashi M, Ushijima T, Ozaki Y. (1986) Biological activity of Pityrosporum. II. Antitumor and immune stimulating effect of Pityrosporum in mice. J Natl Cancer Inst. ;77(5):1093-7.

Tengvall Linder M, Johansson C, Scheynius A. (2000) Positive atopy patch test reactions to Pityrosporum orbiculare in atopic dermatitis patients. Clin Exp Allergy. ;30(1):122-31

Theelen B, Silvestri M, Gueho E. (2001) Identification andtyping of *Malassezia* yeasts using amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA(RAPD) and denaturing gradientgel electrophoresis (DGGE). FEMS Yeast Res ;1:79e86.

Thiboutot D, Sivarajah A, Gilliland K. (2000) The Melanocortin 5 Receptor is Expressed in Human Sebaceous Glands and Rat Preputial Cells *Journal of Investigative Dermatology* (2000) **115**, 614–619

Thomas DS, Ingham E, Bojar RA. (2008) In vitro modulation of human keratinocyte pro- and anti-inflammatory cytokine production by the capsule of *Malassezia* species. FEMS Immunol Med Microbiol.;54(2):203-14

Thompson E. and Colvin J. R.. (1970) Composition of the cell wall of *Pityrosporum ovale* (Bizzozero) Castellani and Chalmers. Can. J. Microbiol. 16:263–265

Tian Y. (2009) Ah receptor and NF-kappaB interplay on the stage of epigenome *Biochem Pharmacol* 77(4):670-80

Tragiannidis A, Bisping G, Koehler G. (2010) Minireview: *Malassezia* infections in immunocompromised patients. Mycoses. ;53(3):187-95

Travers JB, Hamid QA, Norris DA. (2005). Epidermal HLA-DR and the enhancement of cutaneous reactivity to superantigenic toxins in

Toebak MJ, Gibbs S, Bruynzeel DP. (2009) Dendritic cells: biology of the skin. Contact Dermatitis 60(1):2-20.

Tollesson A, Frithz A, Stenlund K. (1997) *Malassezia* furfur in infantile seborrheic dermatitis. Pediatr Dermatol. ;14(6):423-5.

Uenishi T, Sugiura H, Uehara M. (2001) Changes in the seasonal dependence of atopic dermatitis in Japan. J Dermatol.;28 (5):244-7.

Ushijima T, Takahashi M, Ozaki Y. (1981) Selective and differential media for isolation and tentative identification of each species of Pityrosporum residing on normal or diseased human skin. Microbiol Immunol;25:1109e18.

Van de Kerkhof PC, Franssen ME. (2001) Psoriasis of the scalp. Diagnosis and management. Am J Clin Dermatol. ;2(3):159-65.

Vecchini F, Lenoir-Viale MC, Cathelineau C, (1994) Presence of a retinoid responsive element in the promoter region of the human cytochrome P4501A1 gene. Biochem Biophys Res Commun. 30;201(3):1205-12.

Verhasselt V, Buelens C, Willems F. (1997) Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway *J Immunol* 158(6):2919-25

Vidotto V, Leone R, Sinicco A. (1998) Comparison of phospholipase production in Cryptococcus neoformans isolates from AIDS patients and bird droppings. Mycopathologia.;142(2):71-6.

Vilhelmsson M, Zargari A, Crameri R. (2007) Crystal structure of the major *Malassezia* sympodialis allergen Mala s 1 reveals a beta-propeller fold: a novel fold among allergens.J Mol Biol. 15;369(4):1079-86.

Vogel CF, Sciullo E, Matsumura F. (2004) Activation of inflammatory mediators and potential role of ah-receptor ligands in foam cell formation. Cardiovasc Toxicol.;4(4):363-73.

Waersted A, Hjorth N. (1985) Pityrosporum orbiculare--a pathogenic factor in atopic dermatitis of the face, scalp and neck? Acta Derm Venereol Suppl .;114:146-8.

Waller CL, McKinney JD. (1995) Three-dimensional quantitative structure-activity relationships of dioxins and dioxin-like compounds: model validation and Ah receptor characterization. Chem Res Toxicol;8:847–858.

Wang, X. and Seed B. (2003). A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res.31:e154.

Watanabe S, Kano R, Sato H. (2001) The effects of *Malassezia* yeasts on cytokine production by human keratinocytes. J Invest Dermatol 116:769–773

Weary PE. (1970) Comedogenic potential of the lipid extract of Pityrosporum ovale. Arch Dermatol 102:84–91

Wei YD, Helleberg H, Rannug U. (1998) Rapid and transient induction of CYP1A1 gene expression in human cells by the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole. Chem Biol Interact. 12;110(1-2):39-55.

Wei YD, Rannug U, Rannug A. (1999) UV-induced CYP1A1 gene expression in human cells is mediated by tryptophan. Chem Biol Interact. 1;118(2):127-40.

Wells PG, Lee CJ, McCallum GP. (2010) Receptor- and reactive intermediate-mediated mechanisms of teratogenesis. Handb Exp Pharmacol.;(196):131-62.

White TJ, Bruns T, Lee S. (1990) Amplification anddirect sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH,Sninsky JJ, White TJ, eds. PCR protocols. A guide to methods and applications. San Diego: Academic Press, Inc. ; 315–22.

Wille G, Mayser P. (2001) Malassezin--A novel agonist of the arylhydrocarbon receptor from the yeast Malassezia furfur. Bioorg Med Chem. ;9(4):955-60.

Wikler JR, Nieboer C, Willemze R. (1992) Quantitative skin cultures of Pityrosporum yeasts in patients seropositive for the human immunodeficiency virus with and without seborrheic dermatitis. J Am Acad Dermatol. ;27(1):37-9.

Williams HC. (1992) Is the prevalence of atopic dermatitis increasing? Clin Exp Dermatol. ;17(6):385-91.

Williams HC. (2000) Epidemiology of atopic dermatitis. Clin Exp Dermatol. ;25(7):522-9.

Williamson MI, Samaranayake LP, MacFarlane TW. (1986) Phospholipase activity as a criterion for biotyping Candida albicans. J Med Vet Mycol. ;24(5):415-7.

Wincent E, Amini N, Luecke S. (2009) The suggested physiologic aryl hydrocarbon receptor activator and cytochrome P4501 substrate 6-formylindolo[3,2-b]carbazole is present in humans. J Biol Chem. 30; 284(5):2690-6.

Winiarczyk S. (1992) The ultrastructure of *Pityrosporum pachydermatis*. Arch Vet Polonicum System Evol Microbiol ; 54: 623_627

Wollenberg A, Mommaas M, Oppel T. (2002) Expression and function of the mannose receptor CD206 on epidermal dendritic cells in inflammatory skin diseases *J Invest Dermatol* 118(2):327-34**.**

Wollenberg A, Wagner M, Günther S. (2002) Plasmacytoid dendritic cells: a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. J Invest Dermatol. 119(5):1096-102

Wroblewski N, Bär S, Mayser P. (2005) [Missing granulocytic infiltrate in pityriasis versicolor--indication of specific anti-inflammatory activity of the pathogen?]. Mycoses.;48 Suppl 1:66-71.

Xu J, Saunders CW, Hu P. (2007) Dandruff-associated Malassezia genomes reveal convergent and divergent virulence traits shared with plant and human fungal pathogens.Proc Natl Acad Sci U S A. Nov 20;104(47):18730-5.

Yim SM, Kim JY, Ko JH. (2010) Molecular analysis of malassezia microflora on the skin of the patients with atopic dermatitis. Ann Dermatol. ;22(1):41-7.

Youn SW, Na JI, Choi SY. (2005). Regional and seasonal variations in facial sebum secretions: a proposal for the definition of combination skin type. Skin Res. Technol. 11:189–195.

Yurayart C, Chindamporn A, Suradhat S. (2011) Comparative analysis of the frequency, distribution and population sizes of yeasts associated with canine seborrheic dermatitis and healthy skin. Vet Microbiol. 24;148(2-4):356-62.

Zaba L.C, Fuentes-Duculan J, Steinman R.M. (2007) Normalhuman dermis contains distinct populations ofCD11c+BDCA-1+ dendritic cells and CD163+FXIIIA+macrophages. J. Clin. Invest. 117:2517–2525.

Zaidi Z, Wahid Z, Cochinwala R. (2002) Correlation of the density of yeast Malassezia with the clinical severity of seborrhoeic dermatitis. J Pak Med Assoc. ;52(11):504-6.

Zargari A, Eshaghi H, Bäck O. (2001) Serum IgE reactivity to Malassezia furfur extract and recombinant M. furfur allergens in patients with atopic dermatitis. Acta Derm Venereol. ;81(6):418-22

Zelzer E, Wappner P, Shilo BZ. (1997) The PAS domain confers target gene specificity of Drosophila bHLH/PAS proteins. Genes Dev. 15;11(16):2079-89.

Zenz R, Eferl R, Kenner L. (2005) Psoriasis-like skin disease and arthritis caused by inducible epidermal deletion of Jun proteins. Nature; 437:369-75.

Zhai S, Dai R, Friedman FK, Vestal RE. (1998) Comparative inhibition of human cytochromes P450 1A1 and 1A2 by flavonoids. Drug Metab Dispos. ;26(10):989-92.

Zhang E Tanaka T Tajima M. (2011) Characterization of the skin fungal microbiota in patients with atopic dermatitis and in healthy subjects. Microbiol Immunol. ;55(9):625-32.

Zhao Y, Rodriguez-Barbosa JI, Shimizu A. (2003) Despite efficient intrathymic negative selection of host-reactive T cells, autoimmune disease may develop in porcine thymus-grafted athymic mice: evidence for failure of regulatory mechanisms suppressing autoimmunity. Transplantation. 15;75(11):1832-40.

Zouboulis CC. (2004) Acne and sebaceous gland function. Clin Dermatol. ;22(5):360-6.