

Article ID: 566
DOI: 10.5586/am.566

Publication History
Received: 2020-08-01
Accepted: 2020-12-07
Published: 2021-05-17

Handling Editor
Dorota Hilszczańska; Forest
Research Institute, Poland;
<https://orcid.org/0000-0002-4363-704X>

Authors' Contributions
FFK: research design,
conduction of experiments, data
analysis, data presentation,
statistical analysis, writing the
manuscript; KG: supervision of
the project, research design,
verification of methods,
conduction of experiments, data
analysis, writing the manuscript;
EBL: supervision of the project,
verification of methods, data
analysis, writing the manuscript




Funding
The research was funded from
statutory funds by the
Department of Biomedicine and
Genetics, Medical University
of Lodz.

Competing Interests
No competing interests have
been declared.

Copyright Notice
© The Author(s) 2021. This is an
open access article distributed
under the terms of the [Creative
Commons Attribution License](#),
which permits redistribution,
commercial and
noncommercial, provided that
the article is properly cited.

ORIGINAL RESEARCH PAPER in MICROSCOPIC FUNGI

Detection of Cross-Resistance Between Methotrexate and Azoles in *Candida albicans* and *Meyerozyma guilliermondii*: An In Vitro Study

Filip Franciszek Karuga ^{1*}, Katarzyna Góralaska ²,
Ewa Brzezińska-Lasota ¹

¹Department of Biomedicine and Genetics, Chair of Biology and Medical Microbiology, Medical University of Lodz, Poland

²Department of Biology and Parasitology, Chair of Biology and Medical Microbiology, Medical University of Lodz, Poland

* To whom correspondence should be addressed. Email: filipfranciszek439@gmail.com

Abstract

In recent years, there has been a rapid increase in the incidence of *Candida* infections. The different species of the genus *Candida* vary in their virulence abilities and susceptibility to antifungal agents, depending on several external factors. The result of such modifications may be cross-resistance, which is understood as an acquired resistance to a certain antimicrobial agent after exposure to another drug. The aim of this study was to determine the possibility of cross-resistance between fluconazole, voriconazole, itraconazole, and methotrexate in *Candida albicans* and *Meyerozyma guilliermondii* (syn. *Candida guilliermondii*). Fifteen strains of *M. guilliermondii* and eight strains of *C. albicans*, including the standard strains, were tested. For all strains, the minimum inhibitory concentrations (MICs) for fluconazole, voriconazole, and itraconazole were determined before and after stimulation with methotrexate. The median MICs in *M. guilliermondii* before and after stimulation were 9.333 and 64 mg/L ($p = 0.005$) for fluconazole; 0.917 and 1.667 mg/L ($p = 0.001$) for itraconazole, respectively. No significant change in MIC was observed for voriconazole. For *C. albicans* strains, the median MICs before and after stimulation were 0.917 and 64 mg/L ($p = 0.012$) for fluconazole; 0.344 and 1.135 mg/L ($p = 0.018$) for voriconazole, respectively. There was no significant change in MIC values for itraconazole. Thus, this study demonstrates the presence of cross-resistance between voriconazole, itraconazole, fluconazole, and methotrexate for the selected strains. Methotrexate exposure induces different responses when certain drugs are used for various species. Therefore, if a patient was previously exposed to methotrexate, there may be a higher risk of treatment failure with fluconazole than with other azoles such as voriconazole for fungemia caused by *M. guilliermondii* or itraconazole for *C. albicans* infection.

Keywords

antifungal treatment; drug resistance; fluconazole; inhibitor

1. Introduction

Fungi of *Candida* spp. can be a part of the natural microbiota of immunocompetent individuals and may cause superficial and systemic fungal infections and fungemia. In recent years, there has been a rapid increase in the incidence of *Candida* infections. An estimated 750,000 people worldwide are affected by candidiasis annually, with a mortality rate of up to 40% (Ben-Ami, 2018; Bongomin et al., 2017). Symptomatic, invasive candidiasis concerns patients with severely weakened

immune system, including those with cancer, organ transplantation, major abdominal surgeries, and implanted catheters as well as premature neonates and patients treated with aggressive chemotherapy or broad-spectrum antibiotics and corticosteroids (Ben-Ami, 2018; Yapar, 2014).

Candida albicans is the most commonly isolated species from patients. However, its frequency has been decreasing in recent years. The worldwide incidence of *C. albicans* has decreased from 73% in 1997 to 65% in 2007. In certain countries, this phenomenon is still being observed; for instance, the prevalence of *C. albicans* has decreased from 52% to 38% between 1999 and 2012 in the United States and from 51% to 45% between 2005 and 2013 in Spain (Guinea, 2014). However, there has been a gradual increase in the frequency of infections caused by species other than *C. albicans*, mainly *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (Ben-Ami, 2018; Guinea, 2014). There are differences in the prevalence of individual species depending on the type of predisposing factors; for example, *C. parapsilosis* is more common in newborns and young children than in adults, whereas *C. glabrata* is more common in stem cell recipients (Guinea, 2014).

It is becoming increasingly challenging to successfully treat patients with candidiasis, especially those with invasive candidemia (Sharafutdinov et al., 2020). The limited number of antifungal agents and their widespread use are thought to be the main factors causing drug resistance, which further restricts treatment options (Antonovics et al., 2007; Scorzoni et al., 2017).

Azoles (fluconazole – FCZ, itraconazole – ICZ, and voriconazole – VCZ) are the most commonly used antifungals. They are known to inhibit ergosterol biosynthesis via the cytochrome P450 enzyme 14- α demethylase, which catalyzes the conversion of lanosterol to ergosterol, thereby affecting the integrity of fungal cell membranes (Choi et al., 2016; Jensen, 2016; Scorzoni et al., 2017). There has been a very rapid increase in the prevalence of azole resistance, especially FCZ, in recent years, especially among species other than *C. albicans*. In particular, three species, namely, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*, are considered as FCZ-resistant (Berkow & Lockhart, 2017; Castanheira et al., 2017; Wiederhold, 2017). Azole resistance in *Candida* spp. has been associated with overexpression or mutations of *ERG11*, *CDR1*, and *MDR1* genes, the products of which are active cell membrane transporters, “efflux pumps” (de Oliveira Santos et al., 2018). In fungi, two different types of drug efflux systems modulate azole resistance, the ATP-binding cassette (ABC) superfamily and major facilitator superfamily (MFS). Overactivity of efflux pumps can lead to multidrug resistance (MDR) or cross-resistance (Cowen et al., 2015; Jensen, 2016).

Cross-resistance can be defined as the acquired resistance to a certain antimicrobial agent after exposure to another drug (Figure 1). Cross-resistance can result from the induced expression of one of the ABC or MFS transporters by the first drug. After induction, ABC or MFS transporters can pump out another type of drug from the cell (Brody, 2016). An example of already detected cross-resistance between antifolates and azoles is the correlation between FCZ and methotrexate (MTX) in *C. albicans* (Ben-Yaacov et al., 1994; Kohli et al., 2001). MTX is frequently used in patients who are at an increased risk of mycosis. Indications for the use of MTX include acute lymphocytic leukemia in children, acute lymphoblastic and myeloid leukemia in adults, patients with breast, ovarian, cervical, testicular, lung, or bladder cancer, those with bone sarcoma, epithelium and chorionic adenoma, advanced mycosis fungoides, meningeal forms of leukemia and lymphoma, severe psoriasis, psoriatic arthritis, severe active rheumatoid arthritis, osteosarcoma, and non-Hodgkin’s lymphoma (Abolmaali et al., 2013; Bello et al., 2017; Huennekens, 1994; Polańska et al., 2016; Roszkiewicz et al., 2020, 2021).

The cross-resistance between FCZ and MTX is mediated through the overexpression of *CaMDR1* gene, which encodes an MFS protein (Ben-Yaacov et al., 1994; Jensen, 2016; Kohli et al., 2001). Whether the same mechanism is responsible for the cross-resistance between MTX and other azoles is, however, unclear. The available literature does not indicate whether analogous membrane transporters found in other species of the genus *Candida* can also transport MTX. Stimulation with MTX leads to the overexpression of MFS transporters, which may mediate resistance to FCZ and other azoles in strains that have never been in contact with azoles.

Therefore, we decided to evaluate the possibility of cross-resistance between selected azoles and MTX for *C. albicans* and *Meyerozyma guilliermondii* (syn. *Candida guilliermondii*).

Meyerozyma guilliermondii is a relatively less studied species that can be a part of the natural microbiota; however, its prevalence is increasing among patients at the risk of mycosis. Some authors have indicated that *M. guilliermondii* as a re-emerging pathogen, especially in patients with cancer. The documented cases of infection indicate a mortality rate of up to 66% (Ahangarkani et al., 2019; Castillo-Bejarano et al., 2020; Tseng et al., 2018). In *M. guilliermondii*, cross-resistance between MTX and FCZ has never been observed before. As the species is considered to have a reduced sensitivity to azoles (Marcos-Zambrano et al., 2017), it is possible that it may acquire resistance more quickly following exposure to MTX.

The aim of this study was to determine the possibility of cross-resistance between azoles and MTX in selected strains of *M. guilliermondii* and *C. albicans*. We investigated this phenomenon using the azoles FCZ, VCZ, and ICZ.

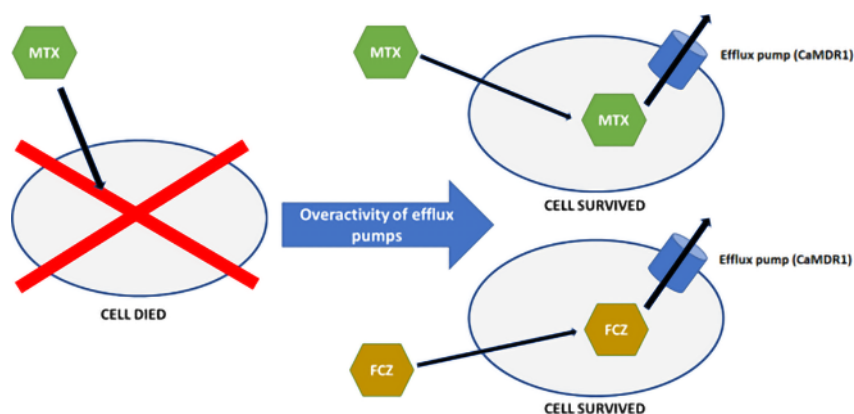


Figure 1 Cross-resistance mechanism with the examples of fluconazole and methotrexate in *Candida albicans*. Fungal cell that has never been exposed to fluconazole acquires resistance to fluconazole after being exposed to methotrexate. This phenomenon occurs because both methotrexate and fluconazole leave the cell through the cell membrane transporters, “efflux pumps.” MTX – methotrexate; FCZ – fluconazole; CaMDR1 – membrane transporter.

2. Material and Methods

In this study, 15 strains of *M. guilliermondii* (Wick.) Kurtzman & M. Suzuki were used, including one standard ATCC 6260 strain and 14 isolates from the collection of the Department of Biology and Parasitology. In addition, eight strains of *C. albicans* (C. P. Robin) Berkhout, including standard strains CBS 2312, L 45, and ATCC 24433 and five isolates from the collection of the Department of Biology and Parasitology, Medical University of Lodz were tested. Susceptibility of all strains for FCZ (Pol-Aura, 894BNE), VCZ (Pol-Aura, 544IED), and ICZ (Pol-Aura, 943YVR) was evaluated, and the minimum inhibitory concentrations (MICs) were determined in triple repetition before and after stimulation with MTX (Pol-Aura, 269QEF) using the EUCAST microdilution method (Arendrup et al., 2020; European Society of Clinical Microbiology and Infectious Diseases, 2020). The test range was as follows: FCZ, 0.125 to 64 mg/L; VCZ, 8 mg/L; and ICZ, 8 mg/L (European Society of Clinical Microbiology and Infectious Diseases, 2020). MIC values were interpreted according to EUCAST 2020 and CLSI M27 (Clinical and Laboratory Standards Institute, 2017; European Society of Clinical Microbiology and Infectious Diseases, 2020; Tseng et al., 2018). EUCAST 2020 did not provide any breakpoints for *M. guilliermondii*; therefore, based on Tseng et al. (2018), we determined the epidemiological cutoff values for non-wild isolates (resistant) as >8, >0.5, and >1 mg/L for FCZ, VCZ, and ICZ, respectively. For *C. albicans*, the following MIC breakpoints were applied: >4, >0.25, and >0.06 mg/L for isolates resistant to FCZ, VCZ, and ICZ, respectively

(European Society of Clinical Microbiology and Infectious Diseases, 2020).

Double stimulation with MTX was performed using the microdilution method in the test range of 0.0088 to 45 mg/L, which was developed based on the bacterial sensitivity to MTX (Nayak et al., 2019; Sulavik et al., 2001). Fungal cells of each strain that had been exposed to the highest non-inhibitory concentration of MTX were collected, multiplied, and used in further experiments. The data are presented as the average of three repeated measurements and their standard deviations. The median for each strain was determined using the interquartile range (IQR). Differences between MIC values before and after stimulation with MTX were assessed using the Wilcoxon signed-rank test, while the differences in MIC change between the species were assessed using the Kruskal–Wallis and Mann–Whitney *U* tests. Statistical analysis was performed using STATISTICA 13.1 software (StatSoft), and statistical significance was set at $p < 0.05$.

3. Results

3.1. *Meyerozyma guilliermondii*

The studied strains showed various initial azole susceptibilities (Table 1). Stimulation of *M. guilliermondii* strains with MTX led to a significant increase in the median MICs for both FCZ and ICZ. The median MICs before and after stimulation were 9.333 (IQR: 0.667–64) and 64 mg/L (IQR: 64–64; $p = 0.005$) for FCZ; 0.917 (IQR: 0.583–1.333) and 1.667 mg/L (IQR: 1.333–2.667; $p = 0.001$) for ICZ, respectively. For VCZ, the median MICs before and after stimulation were 3.5 (IQR: 1.008–6.333) and 3.167 mg/L (IQR: 1.359–4.167; $p = 0.218$), respectively.

The differences in the MIC values before and after stimulation were statistically significant for FCZ and ICZ ($p = 0.005$ and 0.001 , respectively) but not for VCZ ($p > 0.05$) (Figure 2). According to the epidemiologic cutoff values, 100%, 0%, and 62.5% of the previously susceptible strains became resistant to FCZ, VCZ, and ICZ, respectively, following stimulation with MTX.

3.2. *Candida albicans*

In the case of *C. albicans* strains, MTX stimulation led to a significant increase in the median MIC values for FCZ that showed a value of 0.917 mg/L (IQR: 0.667–1.083) before stimulation and 64 mg/L (IQR: 64–64 mg/L; $p = 0.012$) after stimulation and VCZ, as evident from a value of 0.344 mg/L (IQR: 0.122–1.208) before stimulation and 1.135 mg/L (IQR: 0.216–2.333; $p = 0.018$) after stimulation but not for ICZ. The MIC values for ICZ before and after stimulation were 4.333 (IQR: 2.333–6.002) and 4.667 mg/L (IQR: 2.667–6; $p = 0.89$), respectively.

The differences in the MIC values before and after stimulation were statistically significant for FCZ and VCZ ($p = 0.012$ and 0.018 , respectively) but not for ICZ ($p = 0.89$) (Figure 3). According to the EUCAST breakpoints, 100%, 25%, and 0% of the previously susceptible strains were resistant to FCZ, VCZ, and ICZ, respectively, after stimulation with MTX.

3.3. Comparison of MIC Value Between the Species

No statistically significant difference was observed in the change in the MICs of each azole for *M. guilliermondii* and *C. albicans* (all $p > 0.05$).

4. Discussion

Numerous studies have indicated resistance (including multi-drug resistance) or reduced sensitivity to antimycotics in species other than *C. albicans* (Berkow & Lockhart, 2017; Cretella et al., 2016). A typical example of a multidrug-resistant species is *Candida auris*. This species was first identified in 2009 in Japan and has been found in over 30 countries worldwide. *Candida auris* strains are characterized with high resistance to polyenes, azoles, nucleoside analogs, and echinocandins.

Table 1 MIC values of the examined strains.

Strain\MIC value (mg/L)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
	FCZ BS	FCZ BS	FCZ AS	FCZ AS	VCZ BS	VCZ BS	VCZ AS	VCZ AS	ICZ BS	ICZ BS	ICZ AS	ICZ AS
<i>Meyerozyma guilliermondii</i>												
ATCC6260	0.375	0.217	64.000	0.000	6.667	2.309	8.000	0.000	1.167	0.764	2.667	1.155
93	1.000	0.000	64.000	0.000	8.000	0.000	8.000	0.000	0.417	0.144	0.583	0.382
22	0.667	0.289	64.000	0.000	6.000	3.464	4.667	3.055	2.667	1.155	3.333	1.155
10	0.833	0.289	64.000	0.000	2.000	0.000	2.667	1.155	1.167	0.764	1.333	0.577
5	0.667	0.289	64.000	0.000	0.016	0.000	0.042	0.018	0.917	0.946	0.917	0.946
3	0.667	0.289	64.000	0.000	0.016	0.000	0.036	0.024	0.211	0.257	1.667	0.577
8	0.417	0.144	64.000	0.000	0.016	0.000	0.052	0.018	0.333	0.144	0.333	0.144
14	9.333	6.110	64.000	0.000	0.016	0.000	0.021	0.009	0.667	0.289	1.667	0.577
12	42.667	18.475	64.000	0.000	6.667	2.309	8.000	0.000	2.333	1.528	3.333	1.155
94	64.000	0.000	64.000	0.000	3.167	4.193	3.000	1.732	1.500	0.866	2.333	1.528
24	64.000	0.000	64.000	0.000	8.000	0.000	3.667	3.786	1.333	0.577	1.833	1.893
7	64.000	0.000	64.000	0.000	4.000	3.464	2.833	2.021	0.667	0.289	1.333	0.577
47	53.333	18.475	64.000	0.000	3.500	3.969	3.667	3.786	1.000	0.000	2.667	1.155
11	64.000	0.000	64.000	0.000	4.333	3.512	3.333	1.155	0.583	0.382	1.333	0.577
9	64.000	0.000	64.000	0.000	2.333	1.528	3.167	4.193	0.667	0.289	1.667	0.577
Median of all strains	9.333	0.217	64.000	0.000	3.500	1.528	3.167	1.155	0.917	0.382	1.667	0.577
Mean of all strains	28.664	2.972	64.000	0.000	3.649	1.650	3.410	1.397	1.042	0.560	1.800	0.865
<i>Candida albicans</i>												
ATCC24433	2.000	0.000	64.000	0.000	0.125	0.108	0.229	0.237	4.000	0.000	5.333	2.309
L-45	0.667	0.289	64.000	0.000	0.031	0.027	0.177	0.126	4.667	3.055	4.667	3.055
CBS2312	1.000	0.000	64.000	0.000	0.147	0.094	0.271	0.219	8.000	0.000	8.000	0.000
23	1.000	0.000	64.000	0.000	1.167	0.764	2.333	1.528	2.333	1.528	2.667	1.155
20	1.333	0.577	64.000	0.000	1.333	0.577	2.000	0.000	8.000	0.000	8.000	0.000
34	0.833	0.289	64.000	0.000	0.542	0.439	2.333	1.528	5.336	4.614	4.667	3.055
17	0.125	0.000	64.000	0.000	8.000	0.000	8.000	0.000	2.333	1.528	1.333	0.577
x	0.667	0.289	64.000	0.000	0.115	0.118	0.146	0.095	2.000	0.000	2.667	1.155
Median of all strains	0.917	0.144	64.000	0.000	0.344	0.113	1.135	0.173	4.333	0.764	4.667	1.155
Mean of all strains	0.953	0.180	64.000	0.000	1.432	0.266	1.936	0.467	4.584	1.340	4.667	1.413

MIC was calculated as a mean of triplicate values. Values used to determine the median are shown in bold. FCZ – fluconazole; VCZ – voriconazole; ICZ – itraconazole; BS – before stimulation; AS – after stimulation; SD – standard deviation; MIC – minimum inhibitory concentration.

Therefore, some *C. auris* strains can be considered pan-resistant (Ademe & Girma, 2020).

Increased resistance is primarily related to FCZ, as this drug has been most frequently used to treat candidiasis. Based on the data from Centers for Disease Control and Prevention (CDC), the prevalence of FCZ resistance is around 2% for *C. albicans* and 9%–12% for other *Candida* species. In the case of the echinocandin resistance, it is around 1% for *C. albicans* and 1%–2% for other *Candida* species (Cleveland et al., 2012).

Meyerozyma guilliermondii exhibits reduced susceptibility to antimycotics, especially FCZ. Marcos-Zambrano et al. (2017) reported about 10% prevalence of azole-resistant strains and Deorukhkar et al. (2014) observed that 18.2% and 27.3% *M. guilliermondii* were resistant to FCZ and ICZ, respectively. However, in patients with fungemia, 22.7%–36.4% of *M. guilliermondii* strains were found to be resistant to FCZ, ICZ, and VCZ (Deorukhkar et al., 2014; Marcos-Zambrano et al., 2017;

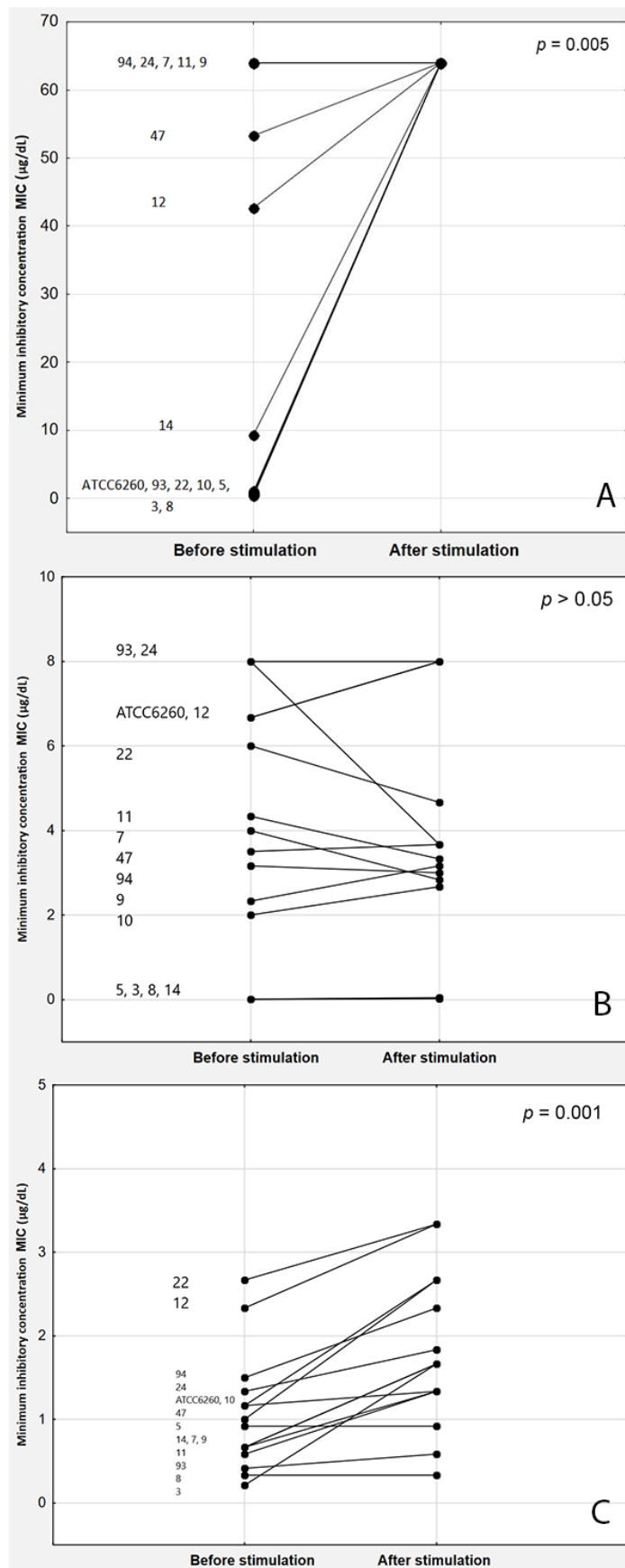


Figure 2 Median minimum inhibitory concentration (MIC) value of each *Meyerozyma guilliermondii* strain before and after methotrexate stimulation for fluconazole (A), voriconazole (B), and itraconazole (C).

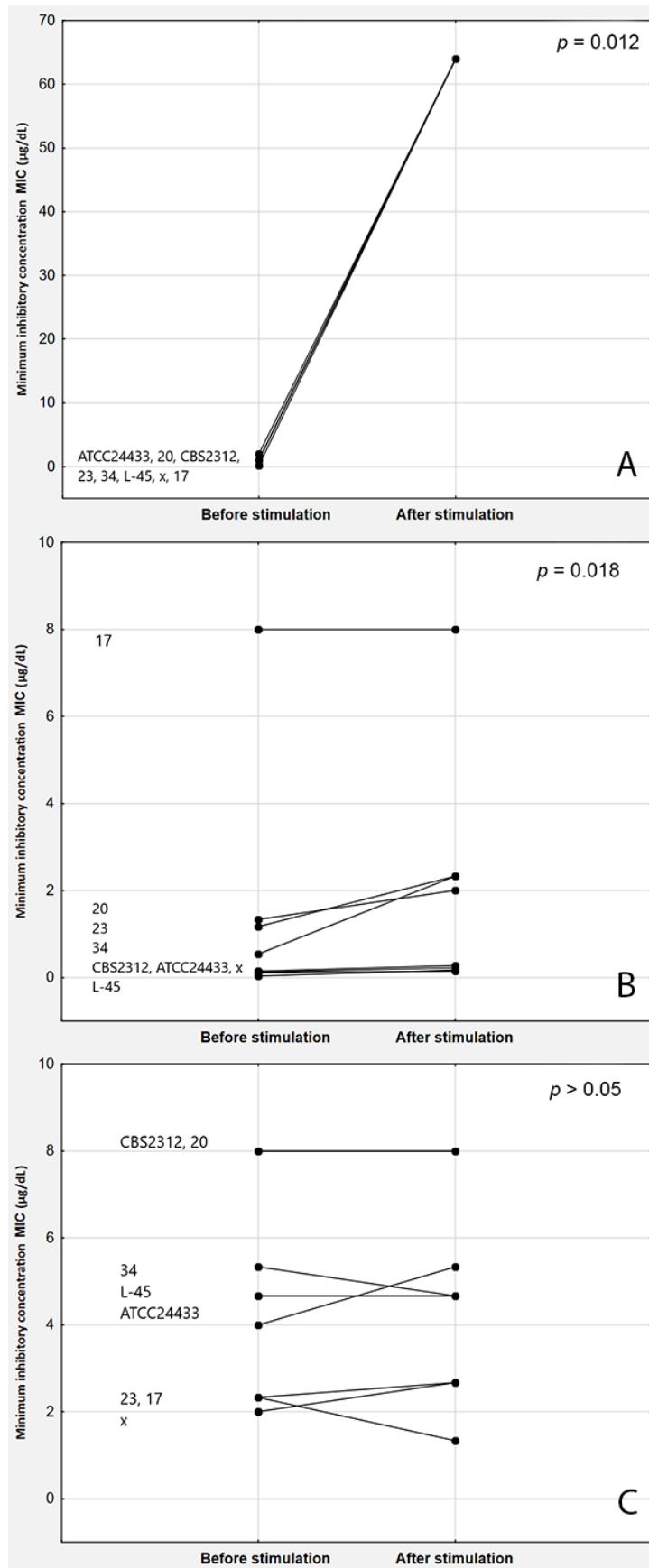


Figure 3 Median minimum inhibitory concentration (MIC) value of each *Candida albicans* strain before and after methotrexate stimulation for fluconazole (A), voriconazole (B), and itraconazole (C).

Tseng et al., 2018). Among the strains we examined, 53.3% were resistant to FCZ before stimulation with MTX but all strains acquired resistance after MTX stimulation. This observation was not reported for ICZ and VCZ. Unfortunately, there were no breakpoint values for this species. Classification of sensitive and resistant isolates is based on epidemiological cutoff values. Therefore, it is difficult to determine the actual incidence of resistance in *M. guilliermondii* and compare strains of different origins (Ahangarkani et al., 2019; European Society of Clinical Microbiology and Infectious Diseases, 2020; Marcos-Zambrano et al., 2017; Tseng et al., 2018).

According to the published data, the frequency of azole-resistant *C. albicans* strains is approximately 0.5%–2% (Berkow & Lockhart, 2017; Jensen et al., 2016).

The analysis of isolates from hospitals from 29 countries showed that about 0.4% *C. albicans* isolates were resistant to FCZ and less than 0.1% were resistant to VCZ (Castanheira et al., 2017). However, Deorukhkar et al. (2014) found that the prevalence of resistance to FCZ and ICZ was 33.8% and 38.5%, respectively. In our study, all *C. albicans* isolates were sensitive to FCZ and 50% were sensitive to VCZ before exposure to MTX. However, all strains became resistant to FCZ and there was no increase in VCZ resistance following MTX stimulation.

The mechanism underlying drug resistance is not fully understood. The most important mechanisms include drug target overexpression, drug target alteration, bypass pathways, efflux pump overexpression, aneuploidy, or loss of heterozygosity (Berkow & Lockhart, 2017). In addition, microorganisms also exhibit the ability to acquire resistance to a drug after contact with another drug, a phenomenon termed as cross-resistance. Cross-resistance has been observed not only in the field of infectious diseases but also during oncological treatment. In a study by van Soest et al. (2015), it was proved that prostate cancer treatment with docetaxel was less effective in patients treated with a novel androgen receptor-targeted agent, enzalutamide. However, this phenomenon was not observed for cabazitaxel, which belongs to the same group of drugs as docetaxel (taxanes) (Nakazawa et al., 2017; van Soest et al., 2015).

In the case of cross-resistance in fungi, cell membrane transporters are very important because they have the ability to actively eject drug molecules used in the therapy of other diseases, for instance cancer, out from the host pathogen cell. It has been established that in *C. albicans*, the CaMdr1p transporter can actively remove FCZ and MTX from cells (Kohli et al., 2001). MTX is used to treat many skin diseases, particularly severe psoriasis and psoriatic arthritis, as well as pityriasis rubra pilaris, sarcoidosis, skin lymphomas, lichen planus, atopic dermatitis, bullous pemphigoid, dermatomyositis, cutaneous lupus erythematosus, and localized scleroderma (morphea) (Polańska et al., 2016). MTX, one of the earliest cancer chemotherapy agents, continues to be extensively used in the management of acute leukemia and a variety of other solid tumors, including breast cancer, osteosarcoma, primary central nervous system, and head and neck cancers (Abolmaali et al., 2013; Huennekens, 1994). Therefore, MTX is mainly used in patients with an increased risk of invasive candidiasis. The awareness of cross-resistance between FCZ and MTX may be of great significance in the field of medicine where the use of both FCZ and MTX is widespread. To date, no correlation has been described between MTX and other azoles, including VCZ and ICZ. However, our research has demonstrated the possibility of cross-resistance between MTX and FCZ or ICZ in *M. guilliermondii* and MTX and FCZ or VCZ in *C. albicans*.

Yang et al. (2019) showed that MTX exhibits antifungal properties and can synergistically act with azoles, including FCZ, ICZ, and VCZ. This phenomenon may be associated with the affinity for MDR1 transporters, which remove MTX from *Candida* cells instead of azoles (Yang et al., 2019). This confirms the possibility of cross-resistance between these drugs. Our in vitro studies indicate that such a relationship between both drugs may occur in *Candida* spp. Unfortunately, the available literature lacks information on the relationship between the frequency of FCZ resistance in fungi and the use of MTX in patients.

By comparing the sensitivity of *C. glabrata* to FCZ in patients with candidiasis before and after FCZ therapy, Jensen et al. (2016) showed a significant increase in the

percentage of resistant isolates. The initial 4.8% isolates increased to 29.4% after 7 days. These authors also observed an increase in the number of resistant isolates belonging to *C. dubliniensis* and *C. parapsilosis*, while no increase in the prevalence of resistance was noted in *C. albicans* (Jensen et al., 2016). In our study, the exposure to MTX caused a rapid increase in resistance to FCZ in the studied strains. After exposure, all the tested strains of *C. albicans* and *M. guilliermondii* were resistant to FCZ. Studies on the occurrence of cross-resistance between azoles used as fungicides in agriculture and those used as medicines in humans may also reveal interesting information about this phenomenon. The use of tetraconazole in agriculture has been shown to result in the acquisition of FCZ resistance in *C. parapsilosis* (Rocha et al., 2016).

The gene *CaMDR1* is responsible for the cross-resistance between FCZ and MTX in *C. albicans* strains, whereas other species of *Candida* genus have homologous genes encoding membrane transporters (for example, *CpMDR1* in *C. parapsilosis*) whose overexpression may also be associated with resistance to FCZ (Arastehfar et al., 2020; Ben-Yaacov et al., 1994; Kohli et al., 2001). In addition, azole resistance in *Candida* species has been associated with overexpression or mutations of *ERG11* or *CDR1* (Arastehfar et al., 2020; de Oliveira Santos et al., 2018). Further studies investigating the molecular mechanism of cross-resistance between azoles and MTX showed that these genes and their expression products (proteins) should be taken into consideration. Aside from *MDR1*, other transport systems such as multidrug resistance-associated proteins (*MRP1–5*) and breast cancer resistance proteins (*BCRP* or *ABCG2*) have also been shown to be involved in the efflux of MTX from cancer cells (Hagner & Joerger, 2010; Wielinga et al., 2005). For this reason, the interaction between azoles and cancer cells should be assessed to detect their azole-induced resistance to MTX chemotherapy.

Appropriate antifungal agent selection and avoiding FCZ may result in an increase in the effectiveness of antifungal empiric therapy and mycosis treatment. In particular, patients with comorbidities who have been previously exposed to antifolates have high probability of cross-resistance to azoles. The prediction of possible drug resistance profiles and selection of a more suitable agent may be crucial for the survival of patients with sepsis of fungal origin (Sipsas & Kontoyiannis, 2012). More targeted therapy would reduce the speed of MDR (Holmes et al., 2016).

Although FCZ continues to be a first-line treatment for candidiasis, the ESCMID and IDSA guidelines suggest echinocandins for unstable patients and infections caused by *C. glabrata*, which is an azole-resistant strain (Matthaiou et al., 2015). Further, IDSA guidelines do not recommend FCZ as first-line treatment for patients recently exposed to FCZ (Delaloye & Calandra, 2014; Matthaiou et al., 2015). In the context of the cross-resistance phenomenon observed in our study, recent treatment with MTX can also serve as an indication for the use of echinocandins as the first-line treatment for sepsis. However, other aspects such as drug interactions and contraindications for echinocandins should also be considered (Yeoh et al., 2018). Empiric therapy with azoles for fungemia caused by *Candida* spp. should be chosen according to the fungal species and the patient's medical history. This may help achieve a higher possibility of successful treatment.

References

- Abolmaali, S. S., Tamaddon, A. M., & Dinarvand, R. (2013). A review of therapeutic challenges and achievements of methotrexate delivery systems for treatment of cancer and rheumatoid arthritis. *Cancer Chemotherapy and Pharmacology*, 71(5), 1115–1130. <https://doi.org/10.1007/s00280-012-2062-0>
- Ademe, M., & Girma, F. (2020). *Candida auris*: From multidrug resistance to pan-resistant strains. *Infection and Drug Resistance*, 13, 1287–1294. <https://doi.org/10.2147/IDR.S249864>
- Ahangarkani, F., Badali, H., Rezai, M. S., Shokohi, T., Abtahian, Z., Nesheli, H. M., Karami, H., Roilides, E., & Tamaddon, A. (2019). Candidemia due to *Candida guilliermondii* in an immunocompromised infant: A case report and review of literature. *Current Medical Mycology*, 5(1), 32–36. <https://doi.org/10.18502/cmm.5.1.535>

- Antonovics, J., Abbate, J. L., Baker, C. H., Daley, D., Hood, M. E., Jenkins, C. E., Johnson, L. J., Murray, J. J., Panjeti, V., Rudolf, V. H. W., Sloan, D., & Vondrasek, J. (2007). Evolution by any other name: Antibiotic resistance and avoidance of the e-word. *PLoS Biology*, 5(2), Article e30. <https://doi.org/10.1371/journal.pbio.0050030>
- Arastehfar, A., Daneshnia, F., Najafzadeh, M. J., Hagen, E., Mahmoudi, S., Salehi, M., Zarrinfar, H., Namvar, Z., Zareshahrabadi, Z., Khodavaisy, S., Zomorodian, K., Pan, W., Theelen, B., Kostrzewa, M., Boekhout, T., & Lass-Flörl, C. (2020). Evaluation of molecular epidemiology, clinical characteristics, antifungal susceptibility profiles, and molecular mechanisms of antifungal resistance of Iranian *Candida parapsilosis* species complex blood isolates. *Frontiers in Cellular and Infection Microbiology*, 10, Article 206. <https://doi.org/10.3389/fcimb.2020.00206>
- Arendrup, M. C., Meletiadis, J., Mouton, J. W., Lagrou, K., Hamal, P., & Guinea, J. (2020). EUCAST DEFINITIVE DOCUMENT E.DEF 7.3.2. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_7.3.2_Yeast_testing_definitive_revised_2020.pdf
- Bello, A. E., Perkins, E. L., Jay, R., & Efthimiou, P. (2017). Recommendations for optimizing methotrexate treatment for patients with rheumatoid arthritis. *Open Access Rheumatology: Research and Reviews*, 9, 67–79. <https://doi.org/10.2147/OARRR.S131668>
- Ben-Ami, R. (2018). Treatment of invasive candidiasis: A narrative review. *Journal of Fungi*, 4(3), Article 97. <https://doi.org/10.3390/jof4030097>
- Ben-Yaacov, R., Knoller, S., Caldwell, G. A., Becker, J. M., & Koltin, Y. (1994). *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. *Antimicrobial Agents and Chemotherapy*, 38(4), 648–652. <https://doi.org/10.1128/aac.38.4.648>
- Berkow, E. L., & Lockhart, S. R. (2017). Fluconazole resistance in *Candida* species: A current perspective. *Infection and Drug Resistance*, 10, 237–245. <https://doi.org/10.2147/IDR.S118892>
- Bongomin, F., Gago, S., Oladele, R. O., & Denning, D. W. (2017). Global and multi-national prevalence of fungal diseases – Estimate precision. *Journal of Fungi*, 3(4), Article 57. <https://doi.org/10.3390/jof3040057>
- Brody, T. (2016). Inclusion/exclusion criteria, stratification, and subgroups – Part I. In *Clinical trials* (2nd ed., pp. 83–113). Academic Press. <https://doi.org/10.1016/b978-0-12-804217-5.00004-7>
- Castanheira, M., Deshpande, L. M., Davis, A. P., Rhomberg, P. R., & Pfaller, M. A. (2017). Monitoring antifungal resistance in a global collection of invasive yeasts and molds: Application of CLSI epidemiological cutoff values and whole-genome sequencing analysis for detection of azole resistance in *Candida albicans*. *Antimicrobial Agents and Chemotherapy*, 61(10), Article e00906-17. <https://doi.org/10.1128/AAC.00906-17>
- Castillo-Bejarano, J. I., Tamez-Rivera, O., Mirabal-García, M., Luengas-Bautista, M., Montes-Figueroa, A. G., Fortes-Gutiérrez, S., & González-Saldaña, N. (2020). Invasive candidiasis due to *Candida guilliermondii* complex: Epidemiology and antifungal susceptibility testing from a third-level pediatric center in Mexico. *Journal of the Pediatric Infectious Diseases Society*, 9(3), 404–406. <https://doi.org/10.1093/JPIDS/PIAA043>
- Choi, M. J., Won, E. J., Shin, J. H., Kim, S. H., Lee, W. G., Kim, M. N., Lee, K., Shin, M. G., Suh, S. P., Ryang, D. W., & Im, Y. J. (2016). Resistance mechanisms and clinical features of fluconazole-nonsusceptible *Candida tropicalis* isolates compared with fluconazole-less-susceptible isolates. *Antimicrobial Agents and Chemotherapy*, 60(6), 3653–3661. <https://doi.org/10.1128/AAC.02652-15>
- Cleveland, A. A., Farley, M. M., Harrison, L. H., Stein, B., Hollick, R., Lockhart, S. R., Magill, S. S., Derado, G., Park, B. J., & Chiller, T. M. (2012). Changes in incidence and antifungal drug resistance in candidemia: Results from population-based laboratory surveillance in Atlanta and Baltimore, 2008–2011. *Clinical Infectious Diseases*, 55(10), 1352–1361. <https://doi.org/10.1093/cid/cis697>
- Clinical and Laboratory Standards Institute. (2017). *M27Ed4: Reference method for broth dilution antifungal susceptibility testing of yeasts, 4th edition*. <https://clsi.org/standards/products/microbiology/documents/m27/>
- Cowen, L. E., Sanglard, D., Howard, S. J., Rogers, P. D., & Perlin, D. S. (2015). Mechanisms of antifungal drug resistance. *Cold Spring Harbor Perspectives in Medicine*, 5(7). <https://doi.org/10.1101/cshperspect.a019752>
- Cretella, D., Barber, K. E., King, S. T., & Stover, K. R. (2016). Comparison of susceptibility patterns using commercially available susceptibility testing methods performed on prevalent *Candida* spp. *Journal of Medical Microbiology*, 65(12), 1445–1451. <https://doi.org/10.1099/jmm.0.000383>

- Delaloye, J., & Calandra, T. (2014). Invasive candidiasis as a cause of sepsis in the critically ill patient. *Virulence*, 5(1), 154–162. <https://doi.org/10.4161/viru.26187>
- de Oliveira Santos, G. C., Vasconcelos, C. C., Lopes, A. J. O., de Sousa Cartágenes, M. d. S., Filho, A. K. D. B., do Nascimento, F. R. F., Ramos, R. M., Pires, E. R. R. B., de Andrade, M. S., Rocha, F. M. G., & de Andrade Monteiro, C. (2018). *Candida* infections and therapeutic strategies: Mechanisms of action for traditional and alternative agents. *Frontiers in Microbiology*, 9, Article 1351. <https://doi.org/10.3389/fmicb.2018.01351>
- Deorukhkar, S. C., Saini, S., & Mathew, S. (2014). Non-*albicans* *Candida* infection: An emerging threat. *Interdisciplinary Perspectives on Infectious Diseases*, 2014, Article 615958. <https://doi.org/10.1155/2014/615958>
- European Society of Clinical Microbiology and Infectious Diseases. (2020). *EUCAST: Clinical breakpoints for antifungals*. <https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/>
- Guinea, J. (2014). Global trends in the distribution of *Candida* species causing candidemia. *Clinical Microbiology and Infection*, 20(6), 5–10. <https://doi.org/10.1111/1469-0691.12539>
- Hagner, N., & Joerger, M. (2010). Cancer chemotherapy: Targeting folic acid synthesis. *Cancer Management and Research*, 2, 293–301. <https://doi.org/10.2147/CMAR.S10043>
- Holmes, A. R., Cardno, T. S., Strouse, J. J., Ivnitiski-Steele, I., Keniya, M. V., Lackovic, K., Monk, B. C., Sklar, L. A., & Cannon, R. D. (2016). Targeting efflux pumps to overcome antifungal drug resistance. *Future Medicinal Chemistry*, 8(12), 1485–1501. <https://doi.org/10.4155/fmc-2016-0050>
- Huennekens, F. M. (1994). The methotrexate story: A paradigm for development of cancer chemotherapeutic agents. *Advances in Enzyme Regulation*, 34(C). [https://doi.org/10.1016/0065-2571\(94\)90025-6](https://doi.org/10.1016/0065-2571(94)90025-6)
- Jensen, R. H. (2016). Resistance in human pathogenic yeasts and filamentous fungi: Prevalence, underlying molecular mechanisms and link to the use of antifungals in humans and the environment. *Danish Medical Journal*, 63(10), Article B5288.
- Jensen, R. H., Johansen, H. K., Søes, L. M., Lemming, L. E., Rosenvinge, F. S., Nielsen, L., Olesen, B., Kristensen, L., Dzajic, E., Astvad, K. M. T., & Arendrup, M. C. (2016). Posttreatment antifungal resistance among colonizing *Candida* isolates in candidemia patients: Results from a systematic multicenter study. *Antimicrobial Agents and Chemotherapy*, 60(3), 1500–1508. <https://doi.org/10.1128/AAC.01763-15>
- Kohli, A., Gupta, V., Krishnamurthy, S., Hasnain, E., & Prasad, R. (2001). Specificity of drug transport mediated by *CaMDR1*: A major facilitator of *Candida albicans*. *Journal of Biosciences*, 26(3), 333–339. <https://doi.org/10.1007/bf02703742>
- Marcos-Zambrano, L. J., Puig-Asensio, M., Pérez-García, F., Escribano, P., Sánchez-Carrillo, C., Zaragoza, O., Padilla, B., Cuenca-Estrella, M., Almirante, B., Martín-Gómez, M. T., Muñoz, P., Bouza, E., & Guinea, J. (2017). *Candida guilliermondii* complex is characterized by high antifungal resistance but low mortality in 22 cases of candidemia. *Antimicrobial Agents and Chemotherapy*, 61(7), Article e00099-17. <https://doi.org/10.1128/AAC.00099-17>
- Matthaiou, D. K., Christodouloupoulou, T., & Dimopoulos, G. (2015). How to treat fungal infections in ICU patients. *BMC Infectious Diseases*, 15, Article 205. <https://doi.org/10.1186/s12879-015-0934-8>
- Nakazawa, M., Paller, C., & Kyprianou, N. (2017). Mechanisms of therapeutic resistance in prostate cancer. *Current Oncology Reports*, 19(2), Article 13. <https://doi.org/10.1007/s11912-017-0568-7>
- Nayak, R. R., Alexander, M., Stapleton-Grey, K., Ubeda, C., Scher, J. U., & Turnbaugh, P. J. (2019). *Perturbation of the human gut microbiome by a non-antibiotic drug contributes to the resolution of autoimmune disease*. BioRxiv. <https://doi.org/10.1101/600155>
- Pappas, P. G., Kauffman, C. A., Andes, D. R., Clancy, C. J., Marr, K. A., Ostrosky-Zeichner, L., Reboli, A. C., Schuster, M. G., Vazquez, J. A., Walsh, T. J., Zaoutis, T. E., & Sobel, J. D. (2016). Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. *Clinical Infectious Diseases*, 62(4), e1–e50. <https://doi.org/10.1093/cid/civ933>
- Polańska, A., Dańczak-Pazdrowska, A., Żaba, R., & Adamski, Z. (2016). The use of methotrexate in the treatment of selected autoimmune connective tissue diseases—our own experience and review of the literature data. *Forum Dermatologicum*, 4(2), 165–170.
- Rocha, M. F. G., Alencar, L. P., Paiva, M. A. N., Melo, L. M., Bandeira, S. P., Ponte, Y. B., Sales, J. A., Guedes, G. M. M., Castelo-Branco, D. S. C. M., Bandeira, T. J. P. G., Cordeiro, R. A., Pereira-Neto, W. A., Brandine, G. S., Moreira, J. L. B., Sidrim, J. J. C., & Brilhante, R. S. N. (2016). Cross-resistance to fluconazole induced by exposure to the agricultural

- azole tetraconazole: An environmental resistance school? *Mycoses*, 59(5), 281–290. <https://doi.org/10.1111/myc.12457>
- Roszkiewicz, J., Michałek, D., Ryk, A., Swacha, Z., Szmyd, B., & Smolewska, E. (2020). The impact of single nucleotide polymorphisms in *ADORA2A* and *ADORA3* genes on the early response to methotrexate and presence of therapy side effects in children with juvenile idiopathic arthritis: Results of a preliminary study. *International Journal of Rheumatic Diseases*, 23(11), 1505–1513. <https://doi.org/10.1111/1756-185X.13972>
- Roszkiewicz, J., Michałek, D., Ryk, A., Swacha, Z., Szmyd, B., & Smolewska, E. (2021). *SLCO1B1* variants as predictors of methotrexate-related toxicity in children with juvenile idiopathic arthritis. *Scandinavian Journal of Rheumatology*, 50(3), 213–217. <https://doi.org/10.1080/03009742.2020.1818821>
- Scorzoni, L., de Paula e Silva, A. C. A., Marcos, C. M., Assato, P. A., de Melo, W. C. M. A., de Oliveira, H. C., Costa-Orlandi, C. B., Mendes-Giannini, M. J. S., & Fusco-Almeida, A. M. (2017). Antifungal therapy: New advances in the understanding and treatment of mycosis. *Frontiers in Microbiology*, 8, Article 36. <https://doi.org/10.3389/fmicb.2017.00036>
- Sharafutdinov, I. S., Ozhegov, G. D., Sabirova, A. E., Novikova, V. V., Lisovskaya, S. A., Khabibrakhmanova, A. M., Kurbangalieva, A. R., Bogachev, M. I., & Kayumov, A. R. (2020). Increasing susceptibility of drug-resistant *Candida albicans* to fluconazole and terbinafine by 2(5H)-furanone derivative. *Molecules*, 25(3), Article 642. <https://doi.org/10.3390/molecules25030642>
- Sipsas, N. V., & Kontoyiannis, D. P. (2012). Invasive fungal infections in patients with cancer in the intensive care unit. *International Journal of Antimicrobial Agents*, 39(6), 464–471. <https://doi.org/10.1016/j.ijantimicag.2011.11.017>
- Sulavik, M. C., Houseweart, C., Cramer, C., Jiwani, N., Murgolo, N., Greene, J., Didomenico, B., Shaw, K. J., Miller, G. H., Hare, R., & Shimer, G. (2001). Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimicrobial Agents and Chemotherapy*, 45(4), 1126–1136. <https://doi.org/10.1128/AAC.45.4.1126-1136.2001>
- Tseng, T.-Y., Chen, T.-C., Ho, C.-M., Lin, P.-C., Chou, C.-H., Tsai, C.-T., Wang, J.-H., Chi, C.-Y., & Ho, M.-W. (2018). Clinical features, antifungal susceptibility, and outcome of *Candida guilliermondii* fungemia: An experience in a tertiary hospital in mid-Taiwan. *Journal of Microbiology, Immunology and Infection*, 51(4), 552–558. <https://doi.org/10.1016/j.jmii.2016.08.015>
- van Soest, R. J., De Morrée, E. S., Kweldam, C. F., de Ridder, C. M. A., Wiemer, E. A. C., Mathijssen, R. H. J., de Wit, R., & van Weerden, W. M. (2015). Targeting the androgen receptor confers in vivo cross-resistance between enzalutamide and docetaxel, but not cabazitaxel, in castration-resistant prostate cancer. *European Urology*, 67(6), 981–985. <https://doi.org/10.1016/j.eururo.2014.11.033>
- Wiederhold, N. P. (2017). Antifungal resistance: Current trends and future strategies to combat. *Infection and Drug Resistance*, 10, 249–259. <https://doi.org/10.2147/IDR.S124918>
- Wielinga, P., Hooijberg, J. H., Gunnarsdottir, S., Kathmann, I., Reid, G., Zelcer, N., van der Born, K., de Haas, M., van der Heijden, I., Kaspers, G., Wijnholds, J., Jansen, G., Peters, G., & Borst, P. (2005). The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates. *Cancer Research*, 65(10), 4425–4430. <https://doi.org/10.1158/0008-5472.CAN-04-2810>
- Yang, J., Gao, L., Yu, P., Kosgey, J. C., Jia, L., Fang, Y., Xiong, J., & Zhang, F. (2019). In vitro synergy of azole antifungals and methotrexate against *Candida albicans*. *Life Sciences*, 235, Article 116827. <https://doi.org/10.1016/j.lfs.2019.116827>
- Yapar, N. (2014). Epidemiology and risk factors for invasive candidiasis. *Therapeutics and Clinical Risk Management*, 10, 95–105. <https://doi.org/10.2147/TCRM.S40160>
- Yeoh, S. F., Lee, T. J., Chew, K. L., Lin, S., Yeo, D., & Setia, S. (2018). Echinocandins for management of invasive candidiasis in patients with liver disease and liver transplantation. *Infection and Drug Resistance*, 11, 805–819. <https://doi.org/10.2147/IDR.S165676>