LETTER TO THE EDITOR

PROPHYLACTIC STRATEGIES IN RECURRENT VULVOVAGINAL CANDIDIASIS: A 2-YEAR STUDY TESTING A PHYTONUTRIENT VS ITRACONAZOLE

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The aim of the present study was to assess the clinical efficacy of a one week/month treatment with a phytocompound with antimycotic properties (K-712, with following 100 mg composition: 10 mg of oleoresin from *Pseudowintera colorata* at 30% concentration in Polygodial together with trace amounts of *Olea europea*) in recurrent vulvo-vaginal candidiasis (RVVC), as compared to once a week treatment with an azole drug for 24 months follow up. This prospective randomized study involving 122 women (19 to 63 years old) with a history of proven episodes of RVVC in the prior 12 months. Patients were allocated in two treatment groups of 61 patients each and given A) Itraconazole 200 mg orally once a week or B) 1 tab twice a day of K-712 for one week/month. Each treatment schedule was well tolerated with 19 patients in the azole group complaining of transient mild symptoms (nausea, abdominal discomfort, unpleasant taste), while only 3 patients on K-712 reported slight dyspepsia. The number of relapses was significantly lower in the K-712-treated group as compared to the itraconazole-group (22 *vs* 39, $p<0.05$). Moreover, the former group showed a significantly decreased number of cases resistant or dose-dependent susceptible as compared to group A ($p<0.05$ *vs* itraconazole) and the same occurred for the occurrence of non-albicans species (group A 64.1% *vs* group B 31.8%, $p<0.05$). The overall mycological cure at the end of the 2-year study showed a comparable benefit between the two groups. From these data it appears that the present antifungal phytonutrient is equally effective as itraconazole in the overall treatment of RVVC over a 2-year follow-up, but yielding a significantly better prophylactic effect and also maintenance benefit with lower relapse rate, antifungal susceptibility and growth of azole-resistant species.

Vulvovaginal candidiasis (VVC) bears a significant morbidity, affecting women's health with an estimated 17% to 39% genital infections in symptomatic women (1). However, yeast colonization may also arise in up to 30% of healthy asymptomatic women harbouring a positive culture for fungi and up to 70% when this population is observed for over a 1-year period (2). In particular, recurrent vulvo-vaginal candidiasis (RVVC) is a term used when at least 3 episodes, unrelated to antibiotic use, occur in

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a year and the peculiar adhesiveness characteristics of *C. albicans* to vaginal epithelium is one of the reasons advocated for this phenomenon (3). In a study of Sobel et al., women with a history of RVVC were randomized to receive 400 mg of ketoconazole for 14 days or clotrimazole in the form of 100 mg vaginal suppositories for a week (4). One week after treatment, the clinical cure rate was reached in more than 80 percent in both groups.

However, in the absence of any maintenance therapy, two months later 53% of women in the ketoconazole treatment group and 63% of those in the clotrimazole treatment group had recurrences. Repetitive pharmacological treatments with azole drugs in women infected by *C. albicans* have been associated with a selection-driven shift from highly susceptible to less susceptible *Candida* species (5, 6). As a matter of fact, a recent survey showed that 33.5% of clinical *C. albicans* isolates from women with vaginitis were resistant to fluconazole (7). Moreover, different *Candida* species, such as *C. krusei* and *C. tropicalis* are intrinsically resistant to some azole drugs such as fluconazole (8) along with *C. tropicalis* and *C. glabrata* that are about ten times less sensitive to miconazole than *C. albicans* (9). It has to be considered that between 15 and 20 percent of women with negative cultures after treatment show positive cultures within three months (10). Thus it becomes very relevant to search for antifungal drugs with novel models of action. For this reason, natural compounds acting against *Candida* spp. have been increasingly tested in the last 10 years, and over 250 plants from almost 100 families have been used for this purpose (11). In particular, we have very recently demonstrated that a natural phytotherapeutic agent based on polygodial exerts a significant inhibitory effect on *Candida* adhesiveness to the duodenal mucosa (12) and in treating acute episodes of RVVC (13). Thus, the aim of the present study was to assess the clinical efficacy over 24 months of this compound in the maintenance treatment of RVVC as compared to conventional drug approach with an azole compound.

MATERIALS AND METHODS

This prospective randomized study involving 122 women (22-63 years), with overall general good health, presented themselves to the gynaecology outpatients clinic with a history of at least four proven episodes of VVC in the prior 12 months. Pregnant women or that were <6 weeks post-abortion or post-partum, diabetic, immunocompromised, on chronic drug therapy, such as steroids or antibiotics, pelvic inflammatory disease and diagnosed or suspected genital malignancy were excluded.

The random numbers were computer generated and patients were allocated in two treatment groups of 61 patients each. The two groups were found to be comparable for age of marriage, age at first pregnancy and number of them, types of delivery (caesarean/natural), number of possible abortions and curettage procedures. Patients were given: A) Itraconazole 200 mg orally once a week (administered as 100 mg twice daily with meals) throughout the 2-year observation period or B) 1 tab twice a day of a Polygodyal phytocompound in the form of tablets (K-712, supplied by Canova Foundation, Lesmo, Italy; where the 100mg composition is as follows: 10mg of oleoresin from *Pseudowintera colorata* at 30% concentration in Polygodial together with trace amounts of *Olea europea* for one week to a month in the same observation period. Both groups were then followed up for a further 6 months. Prior in-house studies had shown that the administration of one tablet twice a day was more effective than a single double dosage in the morning.

The primary outcome was the comparison between K-712 phytocompound and a synthetic antifungal drug in the prevention of RVVC relapse, as well as the assessment of azole-resistant strain occurrence and non-albicans species growth. The secondary outcome was the study of the rate of mycological cure at the end of the 2-year period. Tolerability was assessed as well.

Clinical follow up and biological sampling

Subjects were visited on a monthly basis or whenever they reported typical symptoms and signs of genital infection by Candida yeasts (itching, burning, vaginal curd-like discharge, urinary symptoms, dyspareunia and reddened genital mucosa. In such cases vaginal discharge was collected by cotton swab from the posterior fornix for culture of yeasts and anaerobes and samples were transferred to the laboratory within 6 h where they were dyed with Gram technique and examined microscopically to observe yeasts and pseudohyphae characteristic of Candida infection.

Culture

The vaginal swabs were inoculated on blood agar and human blood agar for growth of *Gardnerella vaginalis* and on Sabouraud's dextrose agar (SDA) for growth of yeasts. The organisms were identified according to colony morphology and standard methods. Efficacy of the treatment was assessed during follow-up based on

symptomatic relief, pelvic examination and laboratory investigations in the form of normal wet smear, pH 4.0 and lack of any growth on cultures. To assess the reliability of the agar medium at least 3 cultures were prepared from the same patient. Cultures were then reviewed by a laboratory expert. Reliability of the agar medium was determined through the uniformity of responses.

Identification of Candida

The identification of Candida species included: 1) production of germinative tube: A sample of the culture in SDS medium was inoculated in 2.5mL of human serum; this was re-suspended and incubated for 3 h at 37°C to detect the pleiomorphism by microscopy; 2) formation of chlamydoconidia, hyphae, and pseudohyphae: a sample of the same SDS medium was spread on Chlamydiospore Agar medium and was roofed with a sterile slide, the culture was then incubated at 27°C and 37°C for 48 h; hyphae and chlamydoconidia were analysed by microscopy; 3) Coloration in ChromAgar: A cellular suspension was added into a nefelometer, 5 µL were inoculated to ChromAgar Candida medium (Becton-Dickinson) and incubated for 78 h at 37°C and identified by their specific color. In particular, as for the colour code provided with the chromogenic media: *C. albicans* produces blue-green colonies, *C. tropicalis* produces dark blue-blue grey colonies, *C. guillermondii* produces bluish pink colonies, *C. parapsilosis* yields creamish to pink, *C. kefyr* produces creamish, *C. glabrata* produces pink to mauve and *T. beigelii* produces light blue on observe and dark blue on reverse; 4) the biochemical identification of the species was carried out through a API- 20C-test.

Efficacy of the treatment was assessed during follow-up based on symptomatic relief, pelvic examination and laboratory investigations in the form of normal wet smear and absence of any growth on cultures.

In case of relapse, patients of A-group were given a full week treatment with same drug while those from B-group were put on a 4-week course of same phytonutrient. At the end of each treatment for relapse, mycological cure was assessed. Those who were eradicated re-entered the follow up study whereas those who were still positive were considered as excluded and assigned to different combination treatments. These cases were also studied for systemic candidiasis by genomic analysis (see below). Each time a relapse was ascertained, a spouse mycological investigation was proposed, although this was not regarded as a pre-requisite for continuing the study.

Genomic analysis of systemic candidiasis in patients failing two consecutive treatments

DNA was extracted from blood samples using a mixture of Iris-HCL. EDIA. Sodium dodecyl sulphate (SDS) proteinase K. lysozyme and Tween 20 (Sigma Chemicals USA) as a lysis buffer. Same amounts (100µL) of the sample and the buffer were mixed and incubated for 2 h at 55°C. Proteinase K was then inactivated, by heating the mixture to 95°C for 10 min. DNA was extracted by the phenol-chloroform method and precipitated by using isopropanol. Briefly, 500 µl of saturated phenol was added with 250 µl of a chloroform/isoamyl alcohol mixture with a ratio 24:1 to the above solution and centrifuged at 14000 rpm for 15 min at 4°C. The DNA was then precipitated from the water phase with isopropanol and dissolved in 15 µL of Tris-EDTA buffer which was used as a template.

As for DNA Amplification, a 197 bp fragment of the 18S rRNA gene was amplified using the following primers: RIBIF - AGC TCT TTC TTG ATT TTG TGG and NS-6 - GCA TCA CAG ACC TGT TAT TGC C'TC. The reaction was carried out in a total volume of 50 µL with 10 mM TAPS, 1.5 mM MgCl2, 50mM KC1, 1mM of each dNTP, 0,02 µg/mL of each primer, 2,5U of Taq polymerase and 5 µL of the DNA template. The amplification reaction schedule started with an initial denaturation cycle of 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 2 min and a final extension step of 72°C for 7 min. The amplified product underwent electrophoresis on a 2% agarose gel containing 0.5 µm/mL of ethidium bromide and the product size was tested each time as compared with Hinf I digested pBR-322 marker.

Antifungal susceptibility testing

In vitro fluconazole and itraconazole susceptibility testing was performed on the banked isolates using the broth microdilution method following the guidelines outlined in Clinical and Laboratory Standards Institute (CLSI) (14). The trays were incubated at 35°C for 24 h in ambient air. The MICs were read as the lowest antifungal concentration with substantially lower turbidity (~50%) compared to growth in the antifungal-free growth control well for all agents. *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 were employed as quality controls. The MIC values were interpreted according to the CLSI interpretive breakpoints (14) as follows: the breakpoints for susceptibility (S) were ≤8µg/ml for fluconazole and ≤0.125 µg/ml for itraconazole, the breakpoints for dose dependent susceptibility (S-DD) were 16 to 32 µg/ml for fluconazole and 0.25 to 0.5 µg/ml for itraconazole, and the breakpoints for resistance R) were ≥64 µg/ml for fluconazole and ≥1µg/ml for itraconazole.

Statistical analysis

Only patients adhering to the protocol were analysed

Table I. *Overall non albicans isolated from patients with rvvc observed along 2-year period.*

species	**Itraconazole** (all relapses: 39)	**K-712** (all relapses: 22)
C. albicans	13 (33.3 %)	16 (72.7%)
C. tropicalis	3 (7.7%)	2 (9%)
C. glabrata	15 (38.4%)	3 (13.6%)
C. guilermondii	2 (5.1%)	-
C. parapsilosis	2 (5.1%)	1 (4.5%)
C. krusei	3 (7.7%)	1 (4.5%)
Overall non-albicans strains	**64.1%**	**31.8%***

** $p<0.05$ vs itraconazole-treated group.*

for efficacy; patients lost to follow-up or non-compliant with the treatment regimen were excluded from analysis; For continuous variables the groups were compared using Student's *t*-test. Significance was established by analysis of variance and the level of significance was determined by employing a Duncan's multiple-range test.

Data were expressed in the text as means (SD) and a probability value of <0.05 was set as indicating that a statistically significant difference existed between groups.

RESULTS

Patients reported no major side effects or any supervening clinical conditions necessitating to stop the treatment in either group. Each therapeutic regimen was well tolerated throughout the 2-year follow up with 19 patients (25 episodes) treated with itraconazole complaining of transitory minor symptoms (nausea, abdominal discomfort, unpleasant taste) and 3 patients (5 episodes) treated with K-712 reporting slight, self-limiting dyspepsia. All patients complied with the protocol and there was no drop out or major protocol violation besides one patient in the itraconazole group who passed away in a car accident. In few cases the timing of start-up of the medication was arbitrarily delayed or anticipated by 1-3 days but dosage and duration of each treatment was always maintained and these patients were kept in the study group.

Assessment of efficacy

There was no relationship between the age of patients, the time since the first candidiasis diagnosis, modalities of treatments and the response to each of the therapeutic regimen or the rate of relapses (data not shown). From the overall analysis it appeared that patients treated with K-712 showed a significantly lower number of relapses ($p<0.05$, fig. 1). This was not apparent at 6-month observation when a very low number was recorded but at one year it reached a statistical difference (16 cases in A group *vs* 10 cases in B group, $p<0.05$) and an increased incidence occurred during the second year of observation for group A while group B showed a comparable relapse rate (23 cases in group A *vs* 12 cases in group B, $p<0.05$), thus totalling a remarkable difference at the end of the study. Two cases in the first year and 7 during the second year in group A while no case during the first year and 5 cases during the second year in group B were not mycologically cured after the second re-treatment following a relapse and were assigned to a combined therapeutic regimen and excluded from the final analysis. Overall 51 patients from group A and 56 from group B completed the whole prophylactic/

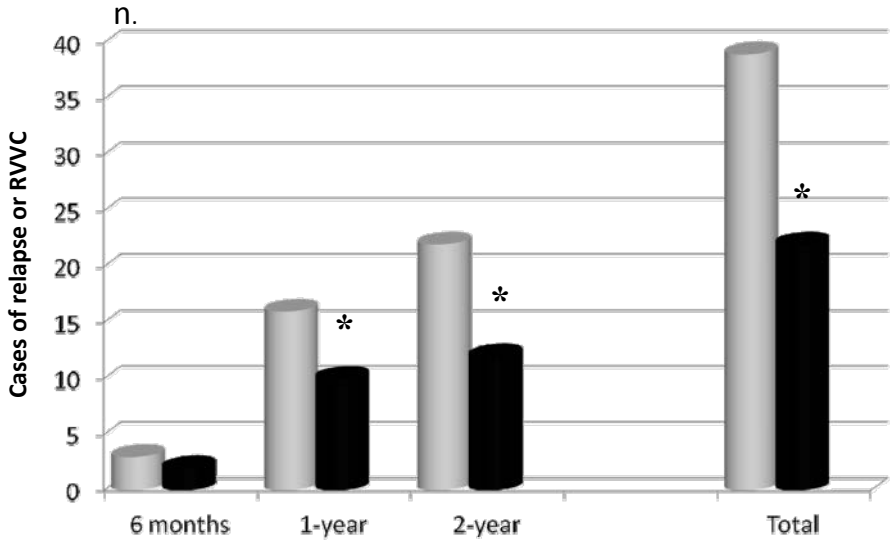

Fig. 1. *Number of patients reporting relapses during the 2-year prophylactic treatment study. Grey bars: itraconazole-treated group; black bars: K-712-treated group. * p<0.05 vs itraconazole-treated group.*

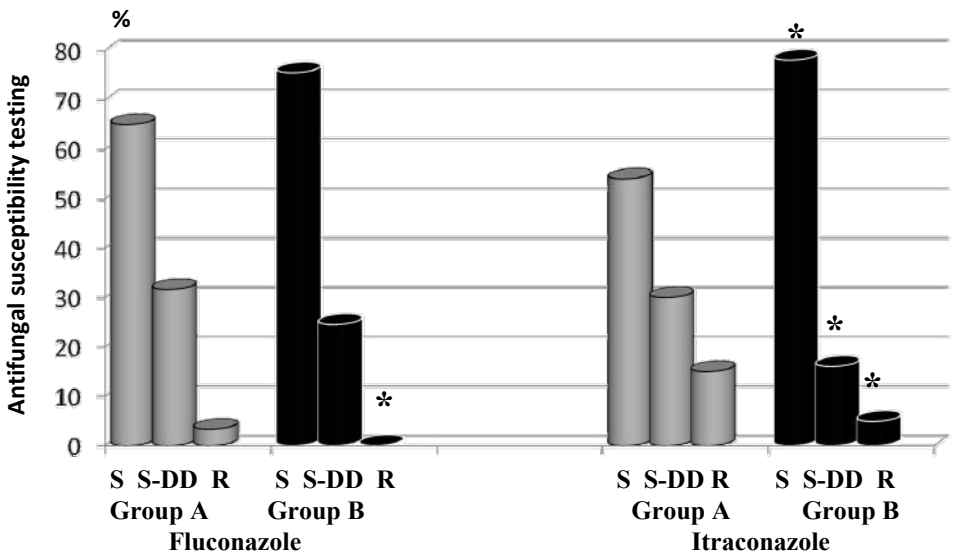

Fig. 2. *Antifungal susceptibility test against fluconazole and itraconazole: different profiles during itraconazole or k-712 treatment. White bars: Itraconazole group; Grey bars: K-712-treated group. The MIC values were interpreted according to the CLSI interpretive breakpoints (14) as follows: the breakpoints for susceptibility (S) were ≤8μg/ml for fluconazole and ≤0.125 μg/ml for itraconazole, the breakpoints for dose dependent susceptibility (S-DD) were 16 to 32 μg/ml for fluconazole and 0.25 to 0.5 μg/ml for itraconazole, and the breakpoints for resistance R) were ≥64 μg/ml for fluconazole and ≥1μg/ml for itraconazole. * p<0.05 vs itraconazole-treated group.*

maintenance course. These 14 patients showed positive culture for *C. albicans* (6), *C. glabrata* (4), *C. krusei* (2) and *C. tropicalis* (2) but none showed a positive genomic test for systemic candidiasis. In almost half (6 patients, 4 from itraconazole group and 2 from K-712 group) of these excluded cases was it possible to carry out a mycological screening of the spouse and all were found positive for *C. glabrata*. As for cumulative evaluation of the growth of non-albicans species, patients treated with K-712 showed

an 18% incidence of such species whereas the ones maintained with itraconazole showed a significant growth increase (69%, p< 0.01 *vs* group B, table I).

When carrying out susceptibility testing on yeast isolates during a relapse, resistance to fluconazole was observed only in a minority of cases (3.3%) in group A, whereas 15,5% of isolates were resistant to itraconazole (MIC>1 μg/ml). Significantly lower values were observed in group B for itraconazole (4.9%) and in no case was there resistance for fluconazole observed (p<0.5, fig 2). Moreover, susceptible dose-dependent cases for itraconazole were significantly lower in the group treated with K-712 (16% *vs* 30%, p<0.05, fig. 2) which also displayed a significantly higher number of antimycotic susceptible cases (78% *vs* 54%, p<0.05, fig 2).

Overall, the cumulative mycological cure at the end of the 2-year maintenance treatment period was comparable among the two groups (85% *vs* 91% in group A and group B, respectively, (data not shown).

DISCUSSION

Seventy-five percent of women suffer from vulvovaginal candidiasis at least once during their lives, almost 45% of women experience the disease twice or more annually and approximately 5% of women are diagnosed with chronic and recurrent infections (15, 16). In all cases, but more significantly in recurrent ones, up to 30% of *C. albicans* isolates from women with recurrent vaginitis may be resistant to fluconazole (17, 18). As a matter of fact, seventy percent of women who have RVVC and are treated with a conventional antifungal are likely to suffer from a further episode within 6 months (19). Given the unpredictability of an effective prevention after a first successful eradication of *C. albicans*-related vulvovaginitis against further attacks, the most valuable approach seems to be a tentative prophylactic antifungal strategy. Besides the more toxic ketoconazole, the molecules most commonly prescribed range from clotrimazole (500 mg suppositories weekly) (20) to itraconazole and fluconazole (200mg or 150 mg orally once weekly, respectively) (21). The latter two agents, especially fluconazole, are now preferred as maintenance regimens although this drug is considerably more expensive. Thus, in our study it was more feasible the use of itraconazole which constituted our available reference drug. By looking at the first year analysis of the data, the K-712-treated group already showed a significantly lower number of relapses and a comparable gap took place during the second year. Moreover, during the first year no case of exclusion from the study for repeated failure to treatment occurred in this group. On the contrary, although the limitation of number did not allow a time-course study of significance of the data, the itraconazole group showed a progressive increase of repeated treatment failures (2 in the first year and 7 in the second). Certainly, the relapse cases in the itraconazole group showed an overall decreased susceptibility to antifungal and an increased growth of non-albicans species.

These two phenomena are understandably linked to one another in that these non-*albicans* species are generally more resistant to imidazole therapy than *C. albicans* and that the therapy itself may create the non-*albicans* selection (17). Surveillance programs performed over the past few decades have demonstrated that although azole resistance is infrequent in *Candida albicans* isolates (<1%), it is becoming very common among isolates of *C. glabrata* (up to 15%) and other non-*albicans* species (22). Indeed, although C. albicans is known to be the main species of pathogenic yeast isolated from women attending gynaecology clinics, as expected in such recurrent cases, a species shift occurs (22) and this has been very recently confirmed also in adolescent (23). As a whole, when considering our prior work that compared itraconazole and K-712 employed as treatment on demand for relapse (24), the present prophylactic strategy proves that, unlike the former, the latter remarkably reduces the non-albicans strains growth.

Limitations in our study include the rather wide range of age with likely different hormonal asset, the lack of a thorough control of spouse co-infection and the unfeasibility of a conjunct couple eradication as well as different modalities of contraception. Moreover, although not openly stated by the patients, one cannot rule out the concomitant use of some topical remedies. A future Candida genotyping analysis could bring further understanding in the phenomenon of relapse and treatment failures.

Moreover, recent works suggest that concomitant treatment with probiotics may further increase candida eradication (25). This is likely to act also by limiting local epithelium permeability and in this regard our group has recently shown that probiotic lysate is efficient enough to beneficially modify the structure and function of stress-induced hyper-permeable gut epithelia (26).

Nonetheless, from our study it would appear that a natural antifungal phytocompound proves to be as good as itraconazole in the preventive strategy of RVVC, while offering a better maintenance via a more efficient control of relapses, reduction of resistant or poorly susceptible and azole-resistant species growth. Future azole-like compounds may prove to limit their current limitations.

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