SUBCLINICAL CANDIDURIA IN PATIENTS WITH GASTROINTESTINAL MALIGNANCIES: A PRELIMINARY STUDY ON THE PROTECTIVE EFFECT OF A NATURAL PHYTOCOMPOUND

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There is a great concern for the increasing incidence of candidiasis in cancer patients following immune-suppressive, cytostatic or antibiotic treatment. There are cancer patients with repeat asymptomatic recovery of candida in the urine in whom the choice of treatment, if any, is still a matter of debate. The aim of the study is to test the efficacy and tolerability of a natural anti-fungal phytocompound in patients with tumors of the gastrointestinal tract with prior or ongoing candiduria. Thirty-nine patients with operated gastrointestinal malignancies (18 still under current chemotherapy) with a history of repeated candiduria were enrolled. Eleven patients showed candiduria on enrolment and were treated with K-712, a natural antifungal phytocompound. Genomic analysis was carried out on blood samples of all patients on a monthly basis for 6 months. Within 3 weeks all 11 treated patients had negative cultures in the urine (10 patients after 2 weeks), 7 patients remained free of candiduria throughout the study period while 4 required a new treatment course. Three patients had positive genomic tests for systemic candidiasis and were treated with fluconazole. Eighteen (64%) out of the 28 patients who were free of candiduria on enrolment, developed a urinary candida infection during the 6-month follow-up and all cases were successfully treated with K-712. Seven (38%) of these cases presented a further recurrence at a later stage and all responded to a new course of K-172. No positive genomic tests were observed during the follow-up period. These data suggest that a consistent part of patients, mostly with gastrointestinal malignancies develop urinary candida infection when following chemotherapy treatment. A therapeutic approach with a natural antifungal phytocompound seems a safe and effective measure and a tentative prophylactic approach might also be envisaged.

The incidence of candidiasis has been steadily increasing over the past decade (1). Although candiduria is a frequently reported phenomenon in hospitalized patients (2-3), its overall clinical relevance is still uncertain and the question remains whether this event goes beyond mere non- virulent

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colonization (4). Nonetheless, there are a number of well-defined risk factors and/or predisposing conditions which suggest the pathological significance of candiduria even when detected in asymptomatic patients (5). These are: diabetes mellitus, antibiotic or immune suppressive therapy and malignancies (6-7). For instance, about 20% of cancer patients are found to have fungal lesions in the small and large intestine (8). Even if the association between candida colonization and cancer has been well documented, only recently it was further clarified that, among cancer patients, candidiasis is mostly present in those with solid tumors of the gastrointestinal tract, especially if metastasis is present (9). Nonetheless, while overt candida infection with pyrexia and/or severe neutropenia meets a general consensus for the need of medical treatment, the medical conduct still remains to be defined in asymptomatic patients without major derangements of the immune system. A further caution in such cases is represented by the possible side effects of pharmacological antifungal treatment and drug resistance (10-11). Indeed, several chemically synthesized antifungal molecules have proven their efficacy in clinical practice but also pose the threat of toxic effects and opportunistic bacterial overgrowth (12). During the past decade, fluconazole has been mainly used in high-risk populations, such as neutropenic patients and, together with its unquestionable benefits against systemic candidiasis, this has also led to a selection-driven shift from highly susceptible to less susceptible Candida species. We have recently shown that a phytocompound containing polygodyal and anethole could significantly inhibit candida colonization of the gut as well its systemic translocation in experimental animals (13-14). Polygodial is a component of *Pseudowintera colorata* and it has long been used as a phytotherapeutic agent for its antibacterial and antifungal properties (15). In recent years an anethole/polygodial compound has been used clinically (16) since it has been found that anethole, a natural compound isolated from *Pimpinella anisum*, enables a more than 30-fold increase in the antifungal activity of polygodial against *Candida Albicans* in *in vitro* studies (17). The aim of the present study is to test the efficacy and tolerability of this phytocompound in patients with tumors of the gastrointestinal tract with prior or ongoing candiduria and no other known infectious complications.

MATERIALS AND METHODS

The study population consisted of thirty-nine subjects (male/female: 22/17, mean age: 68 yrs age range: 61-77) (Table I). All subjects had a history of surgical resection and chemotherapy for gastrointestinal tumor (21 colon cancer, 13 gastric cancer, 5 oesophageal cancer) and repeat asymptomatic/subclinical candiduria in the last year (four or more episodes). Eighteen subjects were being treated with chemotherapy while the remaining had completed their therapeutic protocol within the previous year. Seven had also been subjected to radiotherapy. Ten patients had been found to have distant metastasis (11 liver, 2 lungs, 1 peritoneal, 1 ovary, 1 bone). Twelve patients had a history of associated major diseases (4 myocardial infarct, 2 stroke, 10 diabetes) (Table I). Eight patients were underweight, nine were anaemic (Hb < 11.0g/dl) and seven patients (all under current chemotherapy) were neutropenic (defined if the absolute neutrophilic count in the peripheral circulation was <500 cells/mm3). All patients gave their informed consent at entry and were clinically stable without fever or clinical/laboratory signs of infection. Patients were instructed to avoid taking probiotics or any food supplement and to inform the investigator if prescribed any antibiotics by their referring oncologist. After an overnight fast, patients provided a urine sample for bacterial and candida infection determination. The success was defined as a proven eradication of candida species at the first control after having started the treatment,

Assessment of candiduria

The urine samples were spread by calibrated loop (0.01 ml) onto Sabouraud dextrose agar plates which were supplemented with 100 µg/ml of chloramphenicol. Plates were incubated in anaerobiotic condition at 37ºC and read within 24 h. The amount of 100 CFU/ml, representing a single colony of yeast on a plate was regarded as a detection limit. *Candida* species were identified by germ tube formation in fetal calf serum at 37ºC, colony morphology on cornmeal agar and sugar fermentation and assimilation tests (18). Identification was confirmed by API 20C Aux System for yeasts and *C. albicans* ATCC 10231 and *C. parapsilosis* 22019 were used as control.

Genomic analysis of systemic candidiasis

DNA was extracted from blood specimen using a lysis buffer containing Iris-HCL. EDIA. sodium dodecyl sulphate (SDS), proteinase K. lysozyme and Tween 20

(Sigma Chemicals. USA). Equal quantities (100 L) of the sample and the lysis 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 minutes. DNA was then 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 in the ratio 24:1 to the above solution and centrifuged at 14000 rpm for 15 minutes and maintained at 4°C. The DNA was then precipitated from the aqueous phase with isopropanol. The DNA was then dissolved in 15 µL of Tris-EDTA buffer to serve as a template.

As for DNA Amplification, a 197 bp fragment of the 18S rRNA gene, which is common in all medically relevant fungi was amplified. Primers RIBIF - AGC TCT TTC TTG ATT TTG TGG and NS-6 - GCA TCA CAG ACC TGT TAT TGC C'TC were used to yield appropriate amplification. The reaction was performed in a total volume of 50 µL with 10 mM TAPS, 1.5 mM MgCl2, 50 mM KC1, 1 mM of each dNTP, 0.02 µg/mL of each primer, 2.5U of Taq polymerase and 5 µL of the DNA template which had been preheated to 95°C for 2 minutes. The amplification reaction consisted in 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 was subjected to electrophoresis on a 2% agarose gel containing 0.5 µm/mL of ethidium bromide and the product size was checked each time as compared with Hinf I digested pBR-322 marker.

Treatment protocol

In the case of positive candida detection in the urine but in the absence of fever or systemic signs of infection, patients were immediately supplemented with 1 tablet three times a day for 3 weeks of a polygodial/anethole phytocompound (K-712, supplied by Named srl, Lesmo, Italy; the 100 mg composition is: *Pseudowintera colorata* 50 mg, *Pimpinella anisum* 41.5 mg, lactobacillus acidophilus 2.9 mg and vitamin C 5.6 mg). Urine cultures were carried out on a weekly rate till eradication of the fungi and thereafter monthly for 6 months. Treatment was stopped and a new clinical evaluation was required in case of a febrile illness defined as more than two successive oral readings of greater than or equal to 38°C for the 24-hour period prior to administration of a systemic antifungal therapy. The diagnosis of systemic candidaemia was based on the isolation of the yeast from one or more blood culture sets (aerobic and/or anaerobic bottles) collected from febrile patients. Blood cultures were performed using the automated blood culture system (Beckton Dickinson, USA). Presumptive identification was made by the use of germ tube reaction, colony morphology and colour reaction on chrom agar. Identification to species level was done by the commercial identification system API 20C (Analytic Products, NY, USA). Concomitantly, genomic analysis was performed in all patients on a monthly basis all during the study, irrespective of the clinical evidence of infection.

RESULTS

At the start of the study period, eleven patients (28.9% of the entire study group, male/female: 4/7) showed evidence of candiduria without any remarkable subjective changes in their clinical status. The demographic characteristics of positive patients at entry were as follows: eight (72 %) of the 11 patients were undergoing a chemotherapeutic treatment, six (54%) were underweight, five (45%) had distant metastasis, five (45%) were neutropenic and three (27%) were diabetic. As shown in Fig. 1, 63% of detected candida at entry (7 patients) was diagnosed as *Candida albicans* while the remaining were non-albicans strains. Genomic analysis revealed that 3 patients had systemic spread of fungal infection. One patient also showed a positive urine culture for candida, while the other two had no candiduria. All these patients were treated with fluconazole. Besides the one patient with candiduria who also developed systemic infection, of the other patients with candiduria at entry who were treated with K-712, 7 patients (63%) showed a repeat episode (including one case with definite symptoms but whose sample could not be examined due to technical problems). Such patients were successfully treated within 2 weeks with a new course of treatment containing the same phytocompound (Fig. 2). The same occurred with another 4 (36%) recurrent cases. The remaining 3 patients maintained a candida-free condition throughout a 6-month observation period. Eighteen (64%) of the 28 patients who were free of candiduria at entry, developed urinary candida infection during the 6-month follow-up and all cases were successfully treated with K-712. Six (33%) of these cases presented with further recurrence at a later stage (1-4 months afterwards) and all responded to a new course of K-172. No positive genomic tests for systemic candidiasis were found during the observation period. No side effects of treatment with phytocompound were reported. During the follow-

Table I. *Demographic and clinical characteristics of cancer patientsand associated diseases.*

Male: female	22: 17
Mean age (age range)	68 (61-77)
Colon cancer (non resected)	21 (1)
Without distant metastasis	15
With distant metastasis	6
Gastric cancer	13
Without distant metastasis	10
With distant metastasis	3
Oesophageal cancer	5
Without distant metastasis	4
With distant metastasis	1
Chemotherapy (currently)	39 (18)
Additional radiotherapy	7
Distant metastasis localization	
Liver	11
Lungs	2
Peritoneal	1
Ovary	1
bone	1

Associated Diseases	
Myocardial infarction	4
stroke	2
diabetes	10
malnourishment	16
anemia	9
neutropenia	7 (all under current chemotherapy)

up period 9 patients received antibiotic treatment (6 for pulmonary infection, 1 for suppurative arthritis, 1 for diverticulitis, 1 for urinary bacterial infection and 1 for suppurative post-traumatic skin lesion). Three of these patients were on K-712 treatment (1 with pulmonary infection, 1 with diverticulitis and 1 with suppurative skin lesion) which was maintained and no fungal overgrowth following the antibiotic treatment occurred at urinary culture and genomic blood analysis. Overall, *Candida albicans* accounted for 33 (70%) of all 47 positive candiduria tests while the remaining were due to *Candida glabrata* (12 cases, 25%) and *Candida parapsilosis* (2 cases, 4.2%). In all 11 episodes of repeat candiduria episodes after first treatment with K-172, there was no shift of candida strain. Overall, there did not appear to be any significant difference between diabetic and non-diabetic patients, although the limited number of diabetic patients might have precluded from a detailed analysis of this relevant further risk factor for candidiasis. Moreover, although a statistical analysis could not be applied due to the limited number of cases, malnourished patients constituted 63% (12/22) of patients presenting their first episode of candiduria during the study and 70% (12/17) of all recurrences after a successful eradication .

DISCUSSION

The incidence of hospital urinary tract infections has remarkably increased in the two past decades due to a number of predisposing or risk factors associated with the onset of candiduria such as the use of indwelling urinary catheters, antibiotic treatments, diabetes mellitus, immunosuppressive therapy, prolonged hospital stay, female sex and advanced

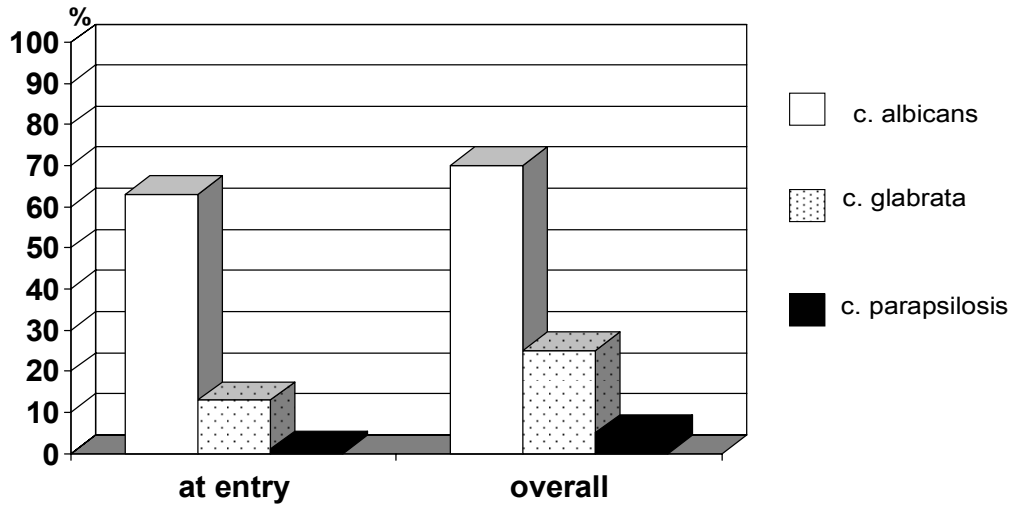

Fig. 1. *Candida infection: microbiological findings at the entry and in the overall population. Percentage distribution of different Candida species in patients who were positive for candiduria at entry (11 patients) and in the whole population (39 patients) during treatment and follow-up period.*

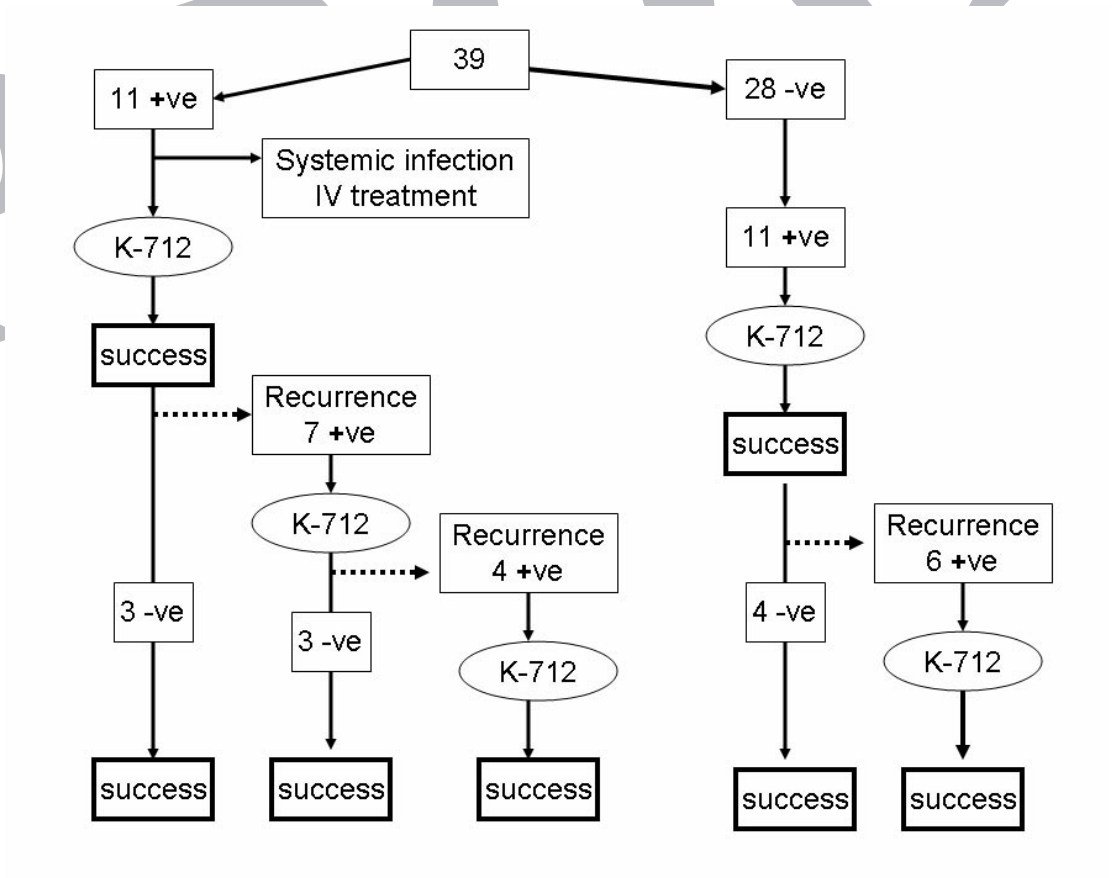

Fig. 2. *Treatment schedule and follow-up of all patients.*

age (5). Understandably, cancer patients are the most exposed subjects to such clinical complication and Candida is one of the five most frequently isolated microbes from blood cultures. Indeed, Candida species are by far the most common cause of fungal infections and they produce infections that range from non-life threatening mucocutaneous illnesses to invasive processes that may eventually involve any body organ (19).

Although previous studies suggest that asymptomatic candiduria might be uneventful and not require local or systemic antifungal therapy (20), even in compromised patients, this opportunistic overgrowth should never be dismissed, since this condition may unexpectedly trigger a systemic or invasive candidiasis (21). Accordingly, Ahmad et al. (22) observed that two *Candida albicans* isolates from overt candidemia patients were similar to those from their urine and oral isolates, when analyzed by random amplification of polymorphic DNA, thus supporting the view that prior colonization is a strong risk factor. In this context, current cytotoxic and immunosuppressive therapies are limited by the inadequate availability of non-toxic antifungal molecules. In view of minimizing further interference with the existing biological ecosystem secondary to the use of chemically synthesized antifungal molecules (23-25), a number of different approaches, such as the use of phytochemicals, are under investigation (26-27). In the present study we used a natural phytotherapeutic agent based on anethole/polygodial, which we have previously shown to significantly inhibit Candida adhesiveness to duodenal mucosa as well as limiting its luminal translocation while being devoid of toxic effects in experimental studies (13-14, 28).

Despite the increased rates in life-threatening invasive yeast infection, many of these infections either remain undetected or are detected late in clinical practice since the blood concentration of fungal cells is generally low (29) and patients with fungemia may often present with minimal clinical manifestations. Already 15 years ago, Berenguer et al. (30) showed that approximately one-half of patients with serious invasive Candida infections may repeatedly have negative blood cultures. In our study, we selected a population of cancer patients who were theoretically more liable to undergo Candida infection, due to their prior repeated exposure. Given the subtle condition, to the best of our knowledge, there is no definite data of cancer patients developing candiduria and this may be the first observation. Our data suggest that in such a situation, there was indeed a significant percentage (46%) with ongoing asymptomatic candiduria which was successfully treated with the natural K-712 intervention. Interestingly, none of these patients developed a relapse candiduria episode during treatment nor any superimposing urinary bacterial infection. To foster this data, it appeared that those who underwent an antibiotic treatment for an infection located elsewhere in the body, did not develop any fungal overgrowth, although the limitation of blood concentration testing does not allow to make any concrete inference. One patient with candiduria following the K-172 treatment showed genomic signs of systemic infection which was promptly treated with fluconazole. It is not possible to ascertain whether the systemic spread originated in his urinary tract or elsewhere, and a strict surveillance of fungemia remains a gold standard in this specific clinical setting. Interestingly, candiduria appeared to be more frequent in female patients (72%) than in males ($p < 0.05$). The higher incidence in females may reflect vaginal candidiasis since it is possible that yeasts may ascend from the genital tract to the urinary tract, as already suggested by Febré et al. (31). This author found that five of eight patients with positive vaginal secretions had the same yeast species in their urine. The vaginal microflora involvement might be a plausible explanation also in our study although we did not obtain vaginal samples to be tested. Over 40% of our candiduria patients were underweight and our previous experimental findings (13) had stressed the importance of nutritional status in maintaining host defence mechanisms against fungal invasion. Furthermore, impaired macrophage responses to Candida have also been observed in underweight patients by other authors (31). In particular, the kidneys seemed the most vulnerable extra-intestinal organ and have been reported to be particularly susceptible to *Candida albicans*. In fact, renal failure represents the major cause of death in systemic candidiasis, probably because of the lack of activation of NF-κB, which promotes the production

of anticandidal proinflammatory cytokines (33).

While candiduria surveillance should be routinely performed in hospital patients to help reduce nosocomial infections, as well as in compromised outpatients, and a prompt antifungal drug should be employed in positive cases, the present data suggest that a natural antifungal compound may be an amenable therapeutic strategy in asymptomatic and subclinical cases of candiduria with a potential for prophylactic use. Longer studies are awaited to test the efficacy of this phytocompound in maintaining a Candida-free status in these patients as well as the possible role of the mild estrogenic-like activity of anethole (34). Moreover, the concomitant existence of vaginosis cannot be excluded nor can the possible synergistic effect played by probiotic components of the polyphytocompound.

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