Immune Deviation in Recurrent Vulvovaginal Candidiasis: Correlation with Iron Deficiency Anemia

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ABSTRACT

Background: Iron Deficiency Anemia (IDA) has been controversially linked to IL-4 production in previous studies. A predominant Th1 response leads to resistance against recurrent vulvovaginal candidiasis (RVVC), whereas a Th2 response exacerbates the disease. **Objective:** To investigate the possible effect of iron deficiency on the host's susceptibility to RVVC as a result of the Th1/Th2 cytokine polarization. **Methods:** We conducted a case-control study of 92 women in 4 groups based on strict inclusion and exclusion criteria: RVVC+IDA+ group consisted of 23 women with RVVC and IDA; RVVC+ IDA- group consisted of 23 women with RVVC without IDA; RVVC-IDA+ group consisted of 23 women without RVVC and with IDA and RVVC- IDA- group consisted of 23 healthy women. The iron parameters and key cytokines (IFN-γ, IL-10, IL-12, IL-4) were measured in blood samples. **Results:** Comparison of IL-4 production between RVVC+ IDA+ (12.2 ± 1.3 pg/ml) and RVVC+ IDA- (2.4 ± 4.0 pg/ml) groups (p=0.044), between RVVC- IDA+ (14.6 ± 1.7 pg/ml) and RVVC- IDA- (1.28 ± 3.6 pg/ml) groups (p=0.006), between RVVC- IDA+ (14.6 ± 1.7 pg/ml) and RVVC+ IDA-) 2.4 ± 4.0 pg/ml) groups (p=0.009) and also between RVVC+ IDA+ and RVVC- IDA- (1.28 ± 3.6 pg/ml) groups (p=0.03) showed significant differences. We found a significant positive correlation between IL-4 and total iron binding capacity (TIBC, p=0.046) and between serum IL-10 and Hb levels (p=0.041) in the RVVC+ IDA- group. There was also a significant negative correlation between serum IL-4 and levels of serum iron (SI, p=0.041) in the RVVC- IDA- group. **Conclusion:** It seems that IDA determines the balance between and the intensity of Th1 and Th2 arms of the immune response and leads to a deviation toward Th2 response which could contribute to recurrence of candidiasis.

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Keywords: Cytokine, Iron Deficiency, Recurrent Vulvovaginal Candidiasis, Th1/Th2

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INTRODUCTION

Recurrent Vulvovaginal Candidiasis (RVVC) is a prevalent opportunistic mucosal infection, caused predominantly by *Candida albicans*, which affects a significant number of otherwise healthy women of childbearing age. In spite of continuing research on RVVC, factors governing its etiopathogenesis are not fully understood (1). Because of the high incidence of recurrent mucosal *Candida* infections in individuals with impaired cell-mediated immunity (CMI), e.g., post-transplantation (2), patients undergoing corticosteroid therapy (3), or patients with AIDS (4,5), it has been postulated that deficiencies in *Candida*-specific CMI play an important role in susceptibility to RVVC (6). On the other hand, it is well recognized that iron deficiency anemia (IDA) is one of the causes of dysfunction of CMI and humoral immunity, which in turn predispose the host to certain clinical infections (7,8). Despite proven reversible functional immunological defects, a clinically significant relationship between states of iron deficiency and susceptibility to infections remains controversial (7). Iron deficiency effects are not necessarily counteracted by iron supplementation during acute illness. In fact, some studies showed reduced infectious outcome after oral iron supplementation (9,10,11,12) and some showed iron treatment association with acute exacerbations of infection (13,14,15,16,17). Cytokines are important mediators of cellular immune activity and their micro-environmental profile determine the balance between, and the intensity of, Th1 and Th2 forms of immune response. IDA, leading to various levels of reduction in the performance and activation of immune cells, induces significant changes in the cytokines production *(*7,13,18). On the other hand, an appropriate balance between Th1 and Th2 cytokine responses is required for effective protection against human candidiasis and to avoid its immunopathology. A predominant Th1 response leads to resistance and the onset of protective immunity through IL-2, IFN-γ production, and macrophage activation, whereas a Th2 response (IL-4, IL-10, and TGF-β) exacerbates the disease through the inactivation of fungicidal effector cells (19,20,21,22). The existance of a relationship between the elevated median concentration of vaginal IL-4 with RVVC has been shown in patients (23). Babula et al. found that the possession of a C-->T substitution at position -589 in the IL-4 gene predisposes women to RVVC through increasing IL-4 production (24).

Due to the complex interactions among micronutrients deficiencies, the immune system, and infectious agents, the root cause of defective cytokine production in infectious diseases remains unknown. There exists a dispute whether deficiencies of specific micronutrients including iron which modify immune responses to antigens, account for a large proportion of common infectious diseases in the population. A better understanding of the different, sometimes unexpected, changes of cytokines is required for their use in prophylaxis and therapy of fungal infections in malnourished individuals. The objective of the present study was to analyze the relationship between Th1/Th2 cytokine balance and iron parameters in women with and without RVVC in accordance to iron deficiency status of each group.

MATERIALS AND METHODS

To accomplish the purpose of the study a case-control design was utilized. The study was conducted on 92 age-matched women (19-50 years) in 4 groups based on strict

inclusion and exclusion criteria: The first group, labeled ($RVVC^+$ IDA^+), consisted of 23 women with RVVC and IDA; the second group, labeled ($RVVC^+$ IDA^-), consisted of 23 women with RVVC and without IDA; the third group, labeled ($RVVC^-$ IDA^+), consisted of 23 women without RVVC and with IDA; and the fourth group, labeled ($RVVC^-$ IDA^-), consisted of 23 healthy women without RVVC or IDA.

Inclusion Criteria. RVVC is defined as four or more episodes of symptomatic vaginal candidiasis per annum confirmed by a gynecologist (include itching, burning, soreness, an abnormal vaginal discharge, dyspareunia and vaginal and vulvar erythema and edema) and positive Pap smear Results (25). IDA was defined with three criteria: hemoglobin concentration of less than 12 gr/dL, iron serum less than 50 µg/dL, and TIBC level of more than 370 µg/dL (26). Since serum ferritin is an acute-phase reactant, ferritin level was not used for iron deficiency detection.

Exclusion Criteria. Women with symptoms and signs of diabetes, autoimmune diseases, immunodeficiencies such as AIDS, other types of anemia and infectious diseases, and patients who had a history of pregnancy, breastfeeding, immunosuppressive therapy, hormone replacement therapy, and prolonged antibiotic use were excluded. We recruited study participants using convenient sampling from the patients referred to the Gynecology and Obstetrics ward of Dr. Shariati hospital of Bandar Abbas, Iran, between 2009 and 2012. The RVVC- IDA- group consisted of 23 age-matched healthy women who had no current gynecologic complaints, no history of RVVC infection, hemoglobin levels less than 12 gr/dL (not anemic), and not pregnant or lactating. After obtaining written consent, a questionnaire was filled with demographic information and medical histories for each patient. For each patient, a 10 mL blood sample was obtained and a Pap smear test was conducted for vaginal yeast infections. Blood samples were tested for blood sugar levels and HIV infection. Parameters of iron status were also determined and sera were stored at -70°C. HIV antibody testing was performed by using enzyme-linked immunosorbent assay (ELISA) test kits (Murex Diagnostics Inc., Norcross, GA). Samples were excluded if insufficient residual serum remained or if they met one or more of the exclusion criteria. Red cell indices including hemoglobin (Hb) levels, hematocrit (HCT), mean corpuscular volume (MCV), red blood cell distribution width (RDW), mean corpuscular hemoglobin (MCH), MCHC (mean corpuscular hemoglobin concentration), Serum Iron (SI), ferritin, total iron binding capacity (TIBC), and cytokine levels were determined in the remaining 92 stored serums. Serum levels of IL-4, IL-10, IL-12, and IFN-γ concentrations were measured using ELISA (Awareness Statfax-2100, USA) and kits from Bender Med Systems (Austria). All cytokines levels were measured according to manufacturers' protocols. The reference ranges for the intra- and inter-assay coefficients of variation as provided by the manufacturers were above 7.5-500 pg/mL, 6.3% and 7.1% for IL-4, 3.2-200 pg/mL, 6.1% and 9.1% for IL-10, 0.16-10 pg/mL,7.1% and 9.6%, for IL-12 and 1.6-100 pg/mL, 5.3% and 8.8% for IFN-γ. The lower limits of sensitivity for the kits were 0.6 pg/mL, 0.66 pg/mL, 0.1 pg/mL, and 0.99 pg/mL, respectively.

All results were stored in a computer database. Data was analyzed using One way ANOVA followed by Tukey's post hoc test and the Pearson's correlation test. Significance was established at the 0.05 level.

RESULTS

Ninety-two women were categorized in 4 groups according to the presence or absence of RVVC and IDA: $RVVC^+$ IDA^+ (n=23), $RVVC^+$ IDA^- (n=23), $RVVC^-$ IDA^+ (n=23) and $RVVC^-$ IDA^- (n=23). Women's ages were between 19 and 50 years and age distribution was similar between comparison groups. Table 1 exhibits the means and standard deviations (SD) of age and different hematologic factors in our study participants.

Table 1. Baseline characteristics of participants in defined groups.

Mean ± SD (median)	RVVC+ IDA- (n=23)	RVVC+ IDA+ (n=23)	RVVC- IDA+ (n=23)	RVVC- IDA- (n=23)
Age (years)	30.1± 6.4	30.8± 8.5	33.5± 9.0	33 ± 7.4
Hb (g/dL)	13 ± 0.7	0.9 ±10.7	1 ± 10.3	0.7 ± 13.2
HCT (%)	40 ± 1.9	1.9 ± 35	2.4 ± 34.5	2.4 ± 39.8
MCV (fL)	5.4 ± 85.1	8.3 ± 73.2	7.4 ± 72.9	57 ± 84.6
MCH (pg)	2.2 ± 28.7	3.9 ±23	3.3 ± 23.1	22 ± 29
MCHC(g/dl)	1 ± 32.5	1.5 ± 31	1.3 ± 31.7	1.2 ± 33.1
Serum iron (µg/dL)	30.2 ± 102.8	8.2 ± 39.5	9.4 ± 37.3	36.5 ± 111.2
TIBC (µg/dL)	39.9 ± 373	59.3 ± 503.3	56.8 ± 404.3	77.5 ± 364.7
Ferritin (ng/mL)	12.6 ± 59.7	7.3 ± 26.5	8.4 ± 24	15 ± 69.8
RDW (%)	1.3 ± 12.9	1.9 ± 14.9	3.3 ± 16.3	1 ± 13.1

*Hb ,hemoglobin ;HCT, hematocrit; MCV, mean corpuscular volume ; RDW, red blood cell distribution width; MCH ,mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration ; SI ,Serum Iron ; TIBC, total iron binding capacity .

*$RVVC^+$ IDA^-: women with recurrent vulvovaginal candidiasis and without iron deficiency anemia; $RVVC^+$ IDA^+: women with recurrent vulvovaginal candidiasis and iron deficiency anemia; $RVVC^-$ IDA^+: women without recurrent vulvovaginal candidiasis and with iron deficiency anemia; $RVVC^-$ IDA^-: women without recurrent vulvovaginal candidiasis and iron deficiency anemia (healthy women).

Comparison of Cytokines Levels in Serum. Comparison of IL-4 production in $RVVC^+$ IDA^+ (12.2 ± 1.3 pg/mL) and $RVVC^+$ IDA^- (2.4 ± 4.0 pg/mL) groups showed a significant difference (p=0.044). Similar increase of IL-4 production was observed in $RVVC^-$ IDA^+ (14.6 ± 1.7 pg/mL) group compared with $RVVC^-$ IDA^- (1.28 ± 3.6 pg/mL) group (p=0.006) , $RVVC^+$ IDA^+ (12.2 ± 1.3 pg/mL) group compared with $RVVC^-$ IDA^- (1.28 ± 3.6 pg/mL) group (p=0.03) and $RVVC^-$ IDA^+ (14.6 ± 1.7 pg/mL) group compared with $RVVC^+$ IDA^- (2.4 ± 4.0 pg/mL) group (p=0.0 09) (Table 2). There were no significant differences between other groups, neither in IL-10 and IFN-γ levels, nor in IL-12 serum levels.

Table 2. Comparison of plasma levels of measured cytokines in defined groups.

Index (unit)	RVVC+ IDA- (n=23)	RVVC+ IDA+ (n=23)	RVVC-IDA+ (n=23)	RVVC-IDA- (n=23)
IFN-γ Pg/ml	4.42 ± 4	2.08 ± 1.6	3.2 ± 1.9	2.08 ± 1.6
IL-12 Pg/ml	1.3 ± 1.2	1.13 ± 1.8	1.14 ± 7.4	1.3 ± 7.8
IL-10 Pg/ml	5.3 ± 2.3	3.87 ± 1.92	4.1 ± 1.3	4.05 ± 2.11
IL-4 Pg/ml	2.4 ± 4.02 [a,d]	12.2 ± 1.3[a,c]	14.6 ± 1.7[b,d]	1.28 ± 3.6[b,c]

[a,b,c,d,] Groups with same letters have significant differences (Student *t*-test, P < 0.05).
*RVVC$^+$ IDA$^-$: women with recurrent vulvovaginal candidiasis and without iron deficiency anemia; RVVC$^+$ IDA$^+$: women with recurrent vulvovaginal candidiasis and iron deficiency anemia; RVVC$^-$ IDA$^+$: women without recurrent vulvovaginal candidiasis and with iron deficiency anemia; RVVC$^-$ IDA$^-$: women without recurrent vulvovaginal candidiasis and iron deficiency anemia (healthy women). Data presented as mean ± SD.

Relationships between Iron Parameters and Serum Cytokine Levels. Regardless of grouping, we found a significant negative association between serum IL-4 and levels of Hb (r=0.23, p=0.034) and between IL-4 and level of SI (r=0.36, p=0.001) in all 92 participants (Figure 1).

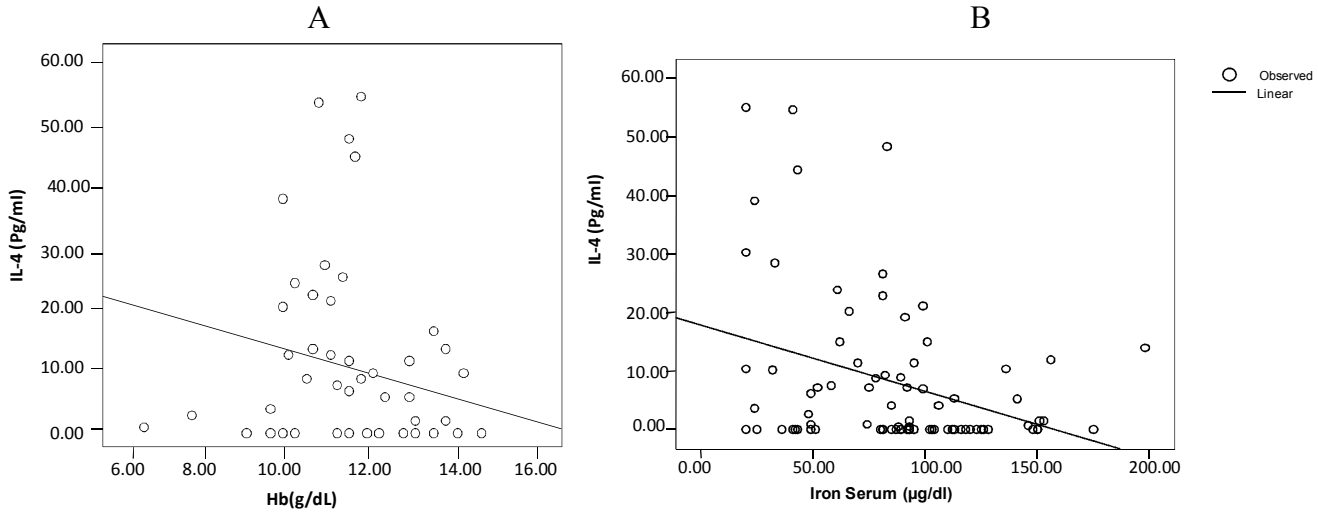

Figure 1. Correlations between serum cytokines levels and iron parameters in all 92 subjects, regardless of grouping. A) Serum IL-4 levels were inversely correlated with Hemoglobin levels (r=0.23, p=0.034). B) Serum IL-4 levels were inversely correlated with Iron serum level (r=0.36, p=0.001).

Considering the grouping, we also found a significant positive association between IL-4 and TIBC (r=0.44, p=0.046) and a significant positive correlation between serum IL-10 and Hb levels, (r=0 .45, p=0.041) in the RVVC$^+$ IDA$^-$ group (Figure 2). There was also a significant negative association between serum IL-4 and levels of SI (r=0.46, p=0.041) in RVVC$^-$ IDA$^-$ group (Figure 3). No significant relations were noted between other iron parameters and serum cytokine levels.

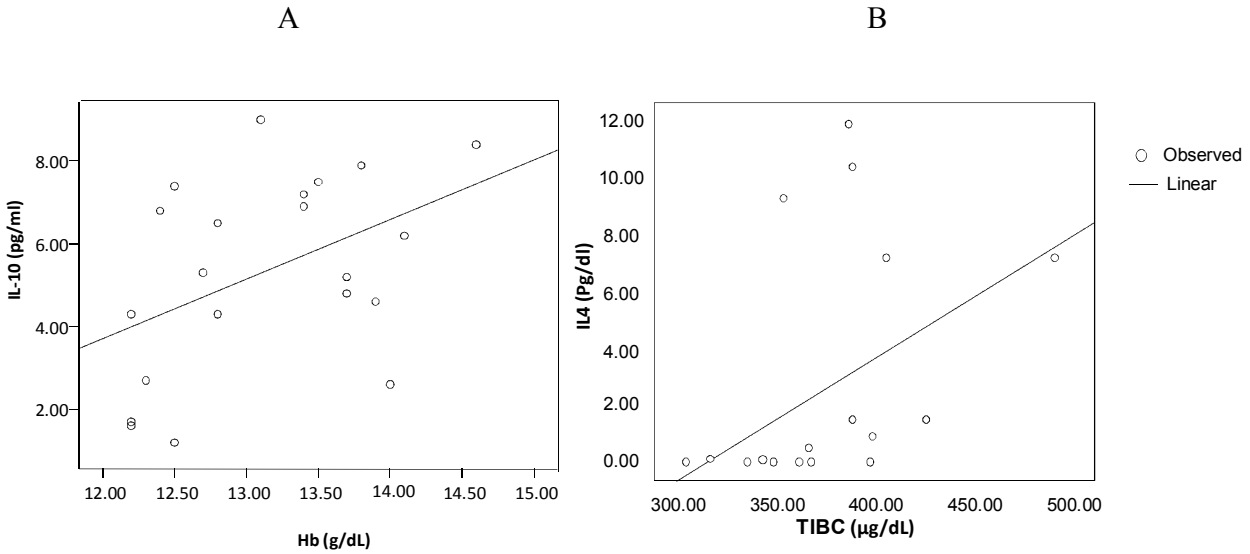

Figure 2. Correlations between serum cytokines levels and iron parameters in the $RVVC^{+}$ IDA^{-} group. A) Serum IL-10 levels were positively correlated with Hb levels(r=0.45, p=0.041) B) Serum IL-4 levels were positively correlated with TIBC (r=0.46, p=0.044).

DISCUSSION

Studies done on humans and animals have shown that iron deficiency is associated with host susceptibility or resistance to certain infections (7,18), but the ethiopathogenic mechanism have not been known due to lack of sufficient studies about the complex interactions among micronutrients deficiencies, the immune system, and infections. However, to our knowledge, the impact of iron status on immune response in women with RVVC has not been well investigated.

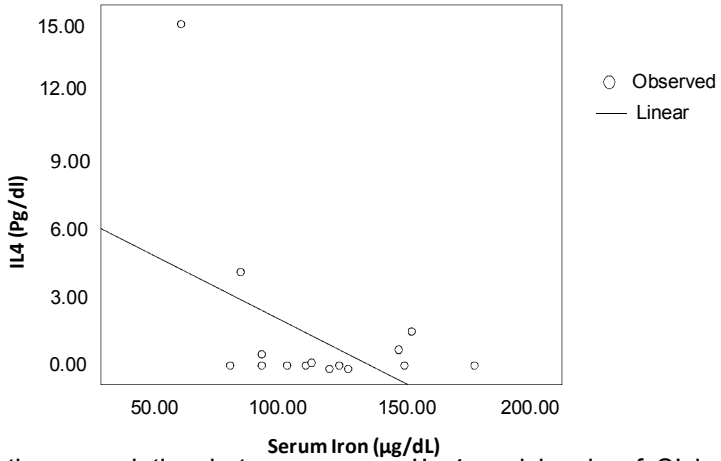

Figure 3. The negative association between serum IL-4 and levels of SI in $RVVC^{-}$ IDA^{-} group(r=0.46, p=0.041).

We found several cytokine patterns associated with iron deficiency, some of which are consistent with previously reported data. First and most straight forward, we found a significant negative association between serum IL-4 and Hb levels (r=0.23, p=0.034) as

well as between IL-4 and level of SI (r=0.36, p=0.001) in all 92 participants (Figure 1). Secondly, the level of IL-4 production was higher in $RVVC^+$ IDA^+ (12.2 ± 1.3 pg/mL) group than in $RVVC^+$ IDA^- (2.4 ± 4.0 pg/mL) group (p=0.044), higher in $RVVC^-$ IDA^+ (14.6 ± 1.7 pg/mL) group than in $RVVC^-$ IDA^- (1.28 ± 3.6 pg/mL) group (p=0.006) , higher in $RVVC^+$ IDA^+ (12.2 ± 1.3 pg/mL) group than in $RVVC^-$ IDA^- (1.28 ± 3.6 pg/mL) group (p=0.03) and higher in $RVVC^-$ IDA^+ (14.6 ± 1.7 pg/mL) group than in $RVVC^+$ IDA^- (2.4 ± 4.0 pg/mL) group (p=0.009) (Table 2).

These findings are consistent with those of Jason et al. in hospitalized Malawian children, who showed only the IL-4 parameter was related to the most severe levels of iron deficiency (27) and against findings of Kuvibidila et al. who showed iron deficiency reduces plasma IL-4 levels in mice and iron repletion improve IL-4 measurements (28). Relation between the elevated median concentration of vaginal IL-4 and RVVC was shown before (24,29). Puccetti et al. showed that neutralization of IL-4 using recombinant soluble IL-4 receptor treatment is associated with a cure rate of > 90% in otherwise lethally *Candida*-infected mice, the onset of durable protection, and a shift from a predominant Th2 to a Th1 pattern of reactivity (30,31). Our third notable finding was unchanged level of the IL-10, IL-12 and IFN-γ between groups. Regarding IFN-γ and IL-12, these findings are against evidence that showed IDA contribute to dampen/reduce the Th1 related cytokines in human PBMCs (32,33,34) and serum (35). Apparently complex interaction in cytokine networks in infectious diseases and IDA hinder the prediction of cytokine alteration as seen in coincidence of malaria and IDA (27) or coincidence of leishmaniasis and IDA (36). Our finding about IL-10 is consistent with Bergman's study on production of IL-1β, IL-2, IL-6, IL-10, and tumor necrosis factor alpha (TNF-α) by peripheral blood mononuclear cells (PBMC) from 20 patients with IDA showed that IL-6, IL-10, and TNF-alpha production in IDA patients did not differ from that of controls but addition of iron to the culture medium did not affect the secretion of IL-2 and IL-1β, but caused an increase in IL-6, IL-10, and TNF-α (37). In contrast to our findings, study of Kuvibidila group showed that mean baseline IL-10 levels in supernatant of spleen cell cultures of iron deficient mice (38) tended to be higher than those of other groups and IL-10 levels negatively correlated with indicators of iron status and lymphocyte proliferation, but positively correlated with IFN-γ levels. From another point of view, searching for relationship between RVVC and IL-10, Weissenbacher et al. found higher levels of IL-10 in RVVC patients (39). The discrepancy between the expected result and the actual results in our research could be attributed to coincidence of IDA and RVVC which both influence the immune system and cytokine networks. Altogether, these findings reveal that IDA could evoke a deviant immune response characterized by a predominant Th2 response which prevents the development of protective Th1 response, exacerbates infections with *Candida albican*s, and explains greater incidence of persistent infection that has been reported in anemic rats (40). It seems that the effect of iron deficiency on the incidence and persistence of infections cannot be fully explained without taking into account both the type of invading microorganism and the impact of iron deficiency anemia on the appropriate immune response needed to eradicate a specific organism.

Overally, although *Candida albicans* is normally a harmless commensal fungus of humans, nutritional disorders such as iron deficiency could result in disruption of a finely regulated balance of directive Th1/Th2 cytokines and subsequently contribute to RVVC incidence. We cannot be certain that the immune relationships we found were related solely to iron deficiency, rather than other unexamined but associated nutritional

deficiencies. However, if any of these findings are related to unexamined deficiencies, the levels and effects of these additional deficiencies would have to be closely correlated with those of iron, for example, a deficiency of another mineral requiring iron for its absorption. Thus, separation of these effects in a clinical setting would be extremely difficult.

This study provides new insights towards iron therapy that could prove beneficial in the management of RVVC. However, the most useful pieces of information could be derived from specific controlled interventions in volunteers and patients, and could be exploited in the development of therapeutic interventions for RVVC and co-infections.

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