Relationship between the quantity of oral *Candida* and systemic condition/diseases of the host: Oral *Candida* increases with advancing age and anemia

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Running title: Relationship between oral *Candida* and medical health conditions

Abstract

Background: The impact of host systemic conditions/diseases on the prosperity of oral *Candida* colonies remains unclear. The aim of the present study was to investigate whether a relationship exists between the quantity of oral *Candida* and the systemic condition/diseases of the host.

Patients and methods: The cross-sectional relationship between *Candida* mannan concentrations and health check-up results was analyzed in consideration of local conditions that influence the prevalence of oral *Candida*.

Results: *Candida* mannan concentrations correlated with age, the number of untreated decayed teeth, number of prosthetic teeth, salivary pH, HbA1c, and the red blood cell count in a univariate analysis. In a multivariate analysis, *Candida* mannan concentrations correlated with age, the number of untreated decayed teeth, number of prosthetic teeth, salivary pH, and the red blood cell count. *Candida* mannan concentrations were higher in subjects older than 80 years, with a higher number of either untreated or prosthetic teeth, with a lower salivary pH, and with a decreased red

blood cell count. Mannan concentrations were slightly higher in subjects with elevated HbA1c.

Conclusions: The present results suggest a close relationship between the quantity of oral *Candida* and the systemic condition/diseases of the host. Oral *Candida* may increase in immunocompromised hosts.

Key words: oral *Candida*, systemic condition, systematic disease, age, anemia

Introduction

Candida species are commensal fungal organisms as well as opportunistic pathogens of mucosal tissues. *Candida* habitually resides in the oral cavity and a number of systemic and local factors may cause an increase in *Candida* species.

Previous studies reported that the amount of oral *Candida* increased under specific oral conditions, including denture wearing, hyposalivation, low salivary pH, and the presence of dental carious lesions [1-6]. However, an increase in oral *Candida* has also been suggested to be closely related to host immunity. While HIV-infected individuals were previously found to have a similar prevalence of oral *Candida* carriage to that of a control group, the immune status of the host (CD4-positive cell count) was suggested to influence the oral colonization of *Candida* [7]. Furthermore, the prevalence of *Candida* in the oral cavity was shown to be higher in transplant recipients than in immunocompetent control subjects. Kidney or liver transplantation predisposes patients to the development of an increased density of *Candida* colonies [8]. We previously demonstrated that the amount of oral *Candida* was greater in hosts with a lower immunological status [9]. Correlations have also been reported between the amount of oral *Candida* and the numbers of T cells, naïve T cells, and Natural Killer (NK) cells [9]. These findings suggest that oral *Candida* increases in immunocompromised individuals, including HIV-positive and AIDS patients, organ transplant recipients, and patients who undergo immunosuppressive and/or cytotoxic anticancer chemotherapy [10-16]. On the other hand, other systemic conditions or diseases have been suggested to impair immunity, such as aging, malnutrition, obesity, malignancy, diabetes mellitus (DM), and anemia. However, limited information is currently available on the impact of these conditions/diseases on the prosperity of oral *Candida* colonies.

Although the conventional detection method for oral *Candida* is a culture of oral samples (i.e., smear, swab, or imprint specimen; whole saliva; and oral rinse solution), previous studies demonstrated the low positivity of cultures [17-19]. On the hand, *Candida* mannan antigen detection is a reliable and sensitive method for the serological diagnosis of systemic candidiasis, and an assay kit is commercially available [20-22]. The Unimedi *Candida* kit is a microplate enzyme-linked immunosorbent assay (ELISA) using affinity-purified polyclonal antibodies against *Candida albicans* mannan. Although ELISA methods has generally time-consuming nature, the Unimedi Candida test has been reported to be more sensitive and specific than other commercially available mannan antigen detection kits using a monoclonal antibody against *C. albicans* mannan [21,23]. This assay was reported to have high sensitivity for mannanemia with *C. albicans*, *C. tropical*, *C. guilliermondii*, and C. lusitanite and low sensitivity with *C. glabrata*, *C. parasilosis*, and *C. krusei* [21,23].

Therefore, the aim of the present study was to investigate whether a relationship exists between the quantity of oral *Candida* and systemic condition/diseases of the host. We examined the quantity of oral *Candida* using the *Candida* mannan antigen in patients who underwent specific health check-ups [24]. We also examined the cross-sectional relationship between *Candida* mannan concentrations and health check-up results while considering local conditions that influence the prevalence of oral *Candida*.

Materials and methods

This study protocol was approved by the Committees on Medical Research of Shinshu University (#2795 and #3683) and Aizawa Hospital (#2012-091).

1. Candida *mannan antigen and* Candida *colonies in cultures*

Oral rinse solutions obtained from 32 subjects (18 patients and 14 healthy volunteers) were used in the present study. Informed consent was obtained from all subjects. They included 13 men and 19 women, with a mean age of 53.4 years (range: 26 – 88 years) (Table 1). Oral rinse solution was collected by rinsing the mouth with 5 mL of sterile saline, which was held in the mouth for 30 seconds before collection in a sterile container. Half of the solution was used for cultivation and the remaining was submitted for the detection of the *Candida* mannan antigen.

Concentrated oral rinse solution was prepared by centrifuging the rinse solution at 3,200×*g* for 20 minutes. After the supernatant was removed, the precipitate was resuspended in 250 μL saline and 50 μL of the sample was inoculated onto Chromagar

Candida agar (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan). *Candida* colonies on culture agar were counted after an incubation at 37 °C for 48 hours [25].

An assay of the *Candida* mannan antigen was conducted at SRL Inc. (SRL. Inc., Tokyo, Japan). Briefly, the Mannan antigen was measured using a commercial sandwich ELISA, Unimedi *Candida* (Kyokuto Pharmaceutical Industry Co., Ltd., Tokyo, Japan). The test was performed according to the manufacturer's instructions. Briefly, oral rinse solution (150 μL) was mixed with 150 μL of treatment solution (including 20 nmol/L of sodium dihydrogen phosphate), boiled for 4 minutes, and then centrifuged at 10,000×*g* for 10 minutes. The supernatant (100 μL) was added to the wells of antibody-coated microtiter plates. After an incubation at room temperature for 2 hours, the plates were washed thoroughly, and 100 μL of alkaline phosphatase-conjugated antibodies was added to the wells. After a further incubation at room temperature for 1 hour, the plates were washed. The reaction was revealed by an incubation with 100 μL of substrate solution at room temperature for 20 minutes. After the addition of 100 μL stopping solution to the wells, optical density was read at 490-500 nm on a microplate reader. Each experiment included positive and negative control samples as well as a calibration curve, which was made with a pool of normal human serum supplemented with known concentrations of mannan. The relationship between mannan concentrations in the immunoassay (U/ml) and the number of *Candida* colonies in cultures (CFU/culture) was analyzed.

Table 1. The characteristics of individuals analyzed and the concentration of oral *Candida* mannan antigen.

		Amount of Candida (CFU)		
Characteristics	**(n)**	**median (IQR)**		
Sex	Women (19)	6 (1.5 – 160)	p = 0.81*	
	Men (13)	10 (4 – 15)		
Age	20-59 (19)	4 (1 – 6)	p < 0.01*	p < 0.001**
	60 < (13)	285 (20 – 2160)		(r = 0.64)
Oral symptoms/signs	Absence (19)	4 (1.25 – 6)		
	BMS (6)	10 (1 – 162)	p = 0.19* (vs. absence)	
	Oral candidiasis (7)	2320 (160 – 2900)	p < 0.001* (vs. absence)	
Denture wearing	(–) (19)	4 (1 – 6.5)	p < 0.001*	
	(+) (13)	285 (15 – 2610)		
Systemic disease	Absence (14)	4 (1.3 – 5.8)	p < 0.01*	
	Presence (18)	24.5 (10.3 – 1813.8)		

* Mann-Whitney's U test

** Spearman's rank correction test

BMS: burning mouth syndrome

2. Specific health check-up and dental check-up

The present study performed specific health check-ups [24] at Azumino and Shiojiri City, Nagano Prefecture, Japan. In 2017, 1,935 persons insured by the national health insurance system and older than 30 years (including self-employed workers, farmers, and the elderly) underwent specific health and dental check-ups. Of these, 563 individuals (261 men and 302 women) were randomly selected and included in the present study. Written informed consent was obtained from all individuals.

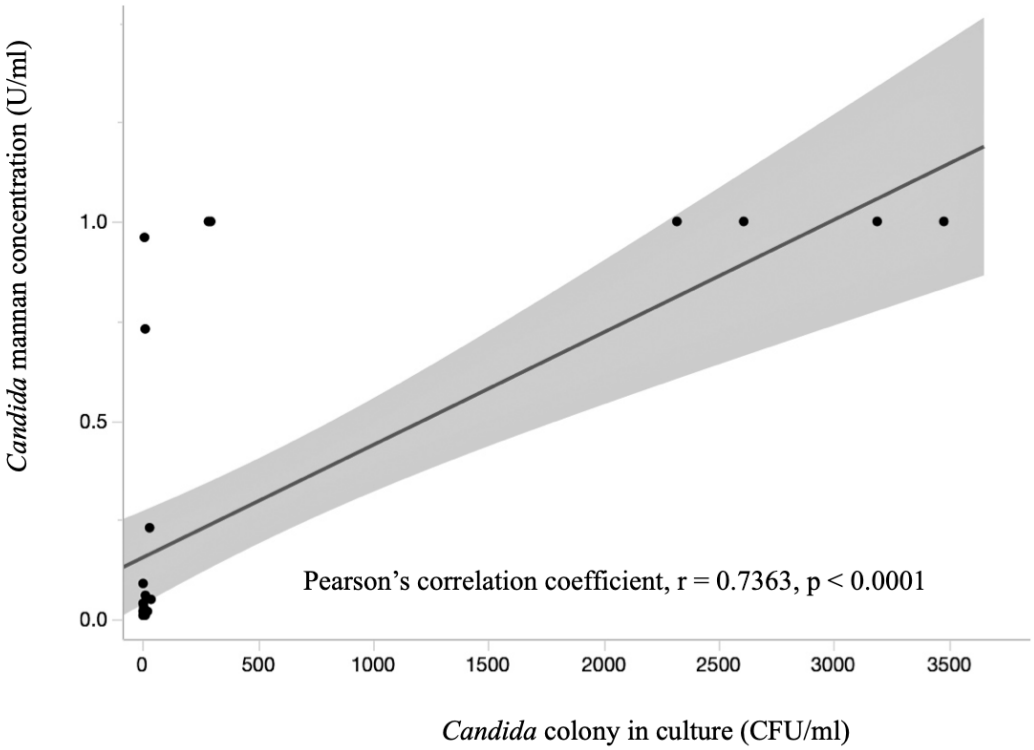
Specific health check-ups were conducted following the standard program supplied by the Ministry of Health, Labour and Welfare of Japan (2013) [24], and included a medical interview, measurements of body height, weight, abdominal circumference, and blood pressure, and a blood examination. The blood examination measured the levels of triglycerides, low/high-density lipoprotein cholesterol, blood sugar, creatinine, and HbA1c as well as the red blood cell count. Each individual also underwent a dental check-up by trained seven dentists. The dental check-up included the inspection of dental and periodontal tissues and an assessment of the dryness of the mouth, according to the method of the Japanese National Survey of Dental Diseases. All examinations were conducted using a plane dental mirror and explorer using sunlight or a flashlight. However, the explorer was only used to clean the tooth surface as necessary and not to probe teeth or tooth surfaces. In order to evaluate the prevalence of dental diseases, DMFT indices were recorded. Since 1938, DMF indices became a relevant tool for monitoring the distribution of dental caries; it was applied by the World Health Organization (WHO) in their assessment of oral health, reflecting the intensity or frequency of dental caries [26]. Additionally, the use of dentures was verified. Regarding the dentist's calibration of measuring of pocket depth, and evaluating of dental caries and oral hygiene, all dentists were trained with models. The dryness of the mouth was judged by the dentist and classified into four categories according to the clinical classification reported by Kakinoki (normal: non-dry, slight: saliva shows viscosity, moderate: saliva shows tiny bubbles on the tongue, and severe: dry tongue with little or no saliva present) [27]. Prior to the dental examination, oral rinse solution was collected by rinsing the mouth for 10 seconds with 3 ml of distilled water. Salivary pH was measured using the salivary multi-test system (Salivary Multi Test, Lion Co., Tokyo, Japan). Approximately 1 ml of the oral rinse solution was submitted to the measurement of the *Candida* mannan antigen. The assay of the *Candida* mannan antigen was described above.

The relationships between mannan concentrations and factors influencing the

prosperity (quantity) of oral *Candida*, including sex, age, dental caries, prosthetic teeth, the dryness of the mouth, salivary pH, obesity (BMI: body mass index), DM (HbA1c), renal function (estimate glomerular filtration rate: eGFR), and anemia (red blood cell count), were then analyzed.

Statistical analyses were performed using JMP ver.13 (SAS Institute Inc., North Carolina, USA). The relationship between mannan concentrations in the immunoassay (U/ml) and the number of *Candida* colonies in cultures (CFU/culture) was examined with Pearson's correlation coefficient. A univariate analysis was performed with the Wilcoxon rank-sum test and Spearman's rank correlation. A multivariate analysis was conducted with a stepwise multiple regression analysis. P-values <0.05 were considered to be significant.

Fig. 1 Relationship between the *Candida* mannan antigen and *Candida* colonies in cultures. A strong correlation was observed (Pearson's correlation coefficient, r = 0.74, p < 0.01).



Results

1. Relationship between the Candida *mannan antigen and* Candida *colonies in cultures*

Oral *Candida* was detected in the cultures of samples from all subjects. The median number of *Candida* colonies was 6.5 CFU/ml (interquartile range (IQR): 28.5 CFU/ml, range: 1 – 3,480 CFU/ml). *Candida* mannan was also detected in all samples. The median concentration was 0.025 U/ml (IQR: 0.89 U/ml) with a range between 0.01 and 1.00. The relationship between the *Candida* culture and mannan antigen concentrations is shown in Figure 1, with a strong correlation being observed (Pearson's correlation coefficient, $r = 0.74$, $p < 0.01$).

The results of univariate analyses on the relationship between the quantity of oral *Candida* colonies and oral and systemic health conditions were also shown in Table 1. Oral *Candida* was more prevalent in elderly subjects (older than 60 years), denture wearers, and those with systemic diseases ($p < 0.05$).

2. Relationship between the quantity of oral Candida *and oral and systemic health conditions*

A total of 563 out of 1,935 individuals were randomly selected and included in the present study. Their characteristics and the results of dental and health check-ups were summarized in Table 2.

The results of univariate analyses on the relationship between the quantity of oral *Candida* and oral and systemic health conditions were also shown in Table 2. *Candida* mannan concentrations correlated with age ($p < 0.01$), the number of untreated decayed teeth ($p < 0.01$), the number of prosthetic teeth ($p < 0.01$), salivary pH ($p < 0.01$), HbA1c ($p < 0.05$), and the red blood cell count ($p < 0.01$). Mannan concentrations increased with advancing age, and were high in those older than 60 years. The greater the number of decayed or prosthetic teeth, the higher the mannan concentration. The lower the salivary pH, the higher the *Candida* mannan concentration. Mannan concentrations were higher in subjects with elevated HbA1c. They were also higher in subjects with advancing anemia. Furthermore, mannan concentrations were slightly high in subjects with moderate to severe renal dysfunction (eGFR less than 45, $p = 0.073$). Mannan concentrations were also slightly higher in subjects with moderate dry mouth than in those with no or slightly dry mouth.

The results of the multivariate analysis were shown in Table 3. The multivariate analysis was included the significant variables in the univariate analysis and variable, which was detected the association with *Candida* concentration. The age decade,

number of decayed teeth, number of prosthetic teeth, dryness of the mouth, salivary pH, HbA1C, and the red blood cell count were included in the model. eGFR was excluded from the model because of its correlation with other independent variables (a correlation was observed between eGFR and salivary pH, $r = 0.025$, $p < 0.001$).

Candida mannan concentrations correlated with age ($p < 0.01$), the number of untreated decayed teeth ($p < 0.01$), number of prosthetic teeth ($p < 0.01$), salivary pH ($p < 0.01$), and the red blood cell count ($p < 0.01$). Mannan concentrations were higher in subjects older than 80 years. *Candida* mannan concentrations increased in subjects with a higher number of either untreated or prosthetic teeth. They were also higher in subjects with lower salivary pH and red blood cell counts. HbA1c was associated with the quantity of oral *Candida* ($p = 0.078$).

Table 2. Relationship between oral *Candida* mannan concentrations and results of dental and health examinations (univariate analysis).

Variables		n	Average	SE	Median	IQR	
Sex	Woman	302	0.22	0.02	0.02	0.02-0.26	*NS* †
	Man	261	0.19	0.02	0.02	0.02-0.19	
Age decade (years)	30-39	19	0.08	0.07	0.03	0.02-0.1	*p < 0.01* *
	40-49	55	0.09	0.04	0.02	0.02-0.03	
	50-59	55	0.11	0.04	0.02	0.02-0.05	
	60-69	183	0.17	0.02	0.02	0.02-0.19	
	70-79	195	0.23	0.02	0.04	0.02-0.33	
	80 ≤	56	0.46	0.04	0.31	0.02-1	
Number of untreated decayed teeth	0	402	0.18	0.01	0.02	0.02-0.2	*p < 0.01* *
	1	100	0.19	0.03	0.02	0.02-0.14	
	2	27	0.27	0.07	0.05	0.02-0.47	
	3	14	0.51	0.11	0.34	0.12-1	
	4	11	0.50	0.14	0.27	0.04-1	
	5 ≤	9	0.30	0.11	0.11	0.03-0.54	
Number of prosthetic teeth	0	273	0.11	0.02	0.02	0.02-0.08	*p < 0.01* *
	1	86	0.16	0.03	0.02	0.02-0.175	
	2	56	0.26	0.04	0.04	0.02-0.41	
	3	23	0.28	0.06	0.13	0.02-0.39	
	4 ≤	125	0.40	0.03	0.19	0.02-0.93	
Dryness of mouth	Normal	525	0.20	0.01	0.02	0.02-0.22	*NS* *
	Slight	26	0.25	0.07	0.02	0.02-0.38	
	Moderate	5	0.58	0.20	0.64	0.12-1	
pH of saliva	7.04 <	78	0.13	0.03	0.02	0.02-0.07	*p < 0.01* *
	6.7-7.04	115	0.17	0.03	0.02	0.02-0.19	
	< 6.7	365	0.23	0.02	0.04	0.02-0.32	
BMI	< 18.5	50	0.20	0.04	0.02	0.02-0.27	*p < NS* *
	18.5 - 24.9	381	0.21	0.02	0.02	0.02-0.21	
	25.0 ≤	131	0.21	0.03	0.03	0.02-0.31	
HbA1c (NGSP)	≤ 5.5	197	0.17	0.02	0.02	0.02-0.15	*p < 0.05* *
	5.6-6.4	314	0.22	0.02	0.02	0.02-0.3	
	5.6 ≤	51	0.26	0.05	0.07	0.02-0.35	
e-GFR	≥ 60	471	0.19	0.01	0.02	0.02-0.21	*p = 0.073* *
	45-59	74	0.29	0.05	0.025	0.02-0.55	
	< 45	16	0.31	0.10	0.06	0.02-0.52	
Red blood cell count	M: ≥ 400, F: ≥ 360	544	0.20	0.01	0.02	0.02-0.22	*p < 0.01* *
	M: 360-399 , F: 330-359	15	0.36	0.09	0.23	0.02-0.55	
	M: < 360, F: < 330	3	0.53	0.22	0.73	0.09-0.78	

†: Wilcoxon rank-sum test

*: Spearman's rank correlation

Table 3. Relationship between oral *Candida* mannan concentrations and results of dental and health examinations (multivariate analysis).

	Partial regression coefficient	Standard error	standard partial regression coefficient	t-value	p-value	Variance Inflation Factor
Intercept	0.362	0.163	0.000	2.23	0.0263*	.
Decade of age (Over 80 vs. 30s&40s&50s&60s&70s)	0.087	0.021	0.166	4.26	<.0001*	1.106
Number of untreated decade teeth	0.027	0.009	0.116	3.12	0.0019*	1.009
Number of prosthetic teeth	0.017	0.002	0.304	7.88	<.0001*	1.083
Dryness of mouth	0.056	0.042	0.050	1.34	0.1821	1.015
pH of saliva	-0.003	0.001	-0.181	-4.85	<.0001*	1.017
HbA1c (NGSP)	0.031	0.018	0.066	1.77	0.0775	1.008
Red Blood Cell count	-0.001	0.000	-0.152	-4.00	<.0001*	1.047

Discussion

Candida species are frequently isolated from the oral cavity of healthy individuals of all ages, and have been reported at a prevalence of 15-75% [28-30]. The amount of oral *Candida* has been shown to increase under specific oral conditions, including denture wearing, hyposalivation, low salivary pH, and the presence of dental carious lesions [1-6]. Although other systemic conditions or diseases impair immunity, such as aging, malnutrition, obesity, malignancy, DM, and anemia, the impact of these conditions/diseases on the prosperity of oral *Candida* colonies remains unclear. Therefore, the aim of the present study was to investigate the relationship between the quantity of oral *Candida* and the systemic condition/diseases of the host.

The Unimedi Candida kit is was reported to have high sensitivity for mannanemia with *C. albicans* [21,23]. Furthermore, we previously demonstrated that *Candida* mannan antigen detection has potential as a sensitive technique for the detection of oral *Candida*, and also that the conditions of the ELISA-based assay need to be optimized for oral rinse solutions [31]. *Candida* mannan was detected in all samples in the present study, and a correlation was observed between *Candida* cultures and mannan antigen concentrations. Therefore, the quantity of oral *Candida* using the *Candida* mannan antigen in subjects who underwent specific health check-ups was examined in the present study.

The multivariate analysis revealed that *Candida* mannan concentrations correlated with some intra-oral conditions, including decayed teeth, prosthetic teeth, and salivary pH. These results were consistent with previous findings. A correlation was reported between the number of caries teeth and *Candida* colony-forming units in saliva [32]. The enzymes secreted by *C. albicans* degrade collagen, particularly type I, which represents more than 90% of the dentin organic matrix [33-39]. *C. albicans* colonization and proliferation is facilitated, which may contribute to the promotion of the carious process in dentin [40-42]. The elimination of dentinal caries may reduce the number of

sites for *Candida* colonization and, consequently, the risk of fungal infections [43]. Oral *Candida* colonization was previously reported to increase by six-fold in denture wearers [44,45]. A spongy denture tissue surface, the surface characteristics of denture base acrylic resins, such as hydrophobicity, and an abundance of nutritive substances are considered to be an ideal incubator for species such as *C. albicans* [46,47]. A previous study demonstrated that salivary pH was lower in complete denture wearers than in non-denture wearers [48], and low pH levels promoted the adhesion and proliferation of *Candida* yeast. Decreased salivary gland hypofunction and pH changes have been noted in the elderly, the main wearer of dentures [48]. Furthermore, a reduction in the salivary flow rate with aging induced an increase in the concentration of microbes in saliva [48]. Since these factors support the present results, a strong correlation may exist between the number of untreated decayed teeth, number of prosthetic teeth, salivary pH, and the prevalence of oral *Candida*. However, in recent preliminary genetic study of the oral mycobiome of children with and without dental caries , *C. albicans* was reported to be frequently isolated from both caries-affected and caries-free dentition, and not to be more abundant in children with dental caries [49]. The further investigation will be needed to clarify the association between dental caries and *C. albicans*.

On the other hand, the results of the multivariate analysis revealed that some systemic factors also had a significant impact on the *Candida* load in the oral cavity. Besides the local factors described above, age and the red blood cell count independently influenced oral *Candida* mannan concentrations. Furthermore, HbA1c influenced the quantity of oral *Candida*. Aging is one of the important factors for oral *Candida* infection. The elderly have been reported to have several metabolic disorders including decreased hepatic and renal functions [50], the use of multiple drugs, and poor nutrition [51], which are considered to contribute to colonization and infection by *Candida* [52]. A previous study investigated the relationship between *Candida* carriage and drugs, and a multivariate analysis revealed that the variables of an older age (80 years and older) had a significant impact on the quantity of oral *Candida* besides the presence of dry mouth [53]. These findings are consistent with the present results. The immune system becomes weaker with aging, and this decline with age is reflected in increased susceptibility to infectious diseases, poorer responses to vaccinations, and the increased prevalence of cancer and autoimmune and other chronic diseases. Innate and adaptive immune responses are affected by the aging process [54]. Aging of the immune system may contribute to the increased load of oral *Candida*.

A high prevalence of oral Candida infection has been reported in patients with iron deficiency anemia [55]. A high incidence of *Candida* infection of 85% was found

in iron-deficient patients. Iron deficiency is the most common nutritional deficiency and causes half of all anemia cases worldwide [55]. The possibility of *Candida* infection in iron deficiency was reportedly due to impaired cellular immunity [56,57]. Iron is essential for the growth of all cells. Hassan et al. reported that humoral immunity, non-specific immunity (phagocytic activity and oxidative burst), and IL-6 levels were affected in patients with iron deficiency anemia [58]. Ekiz C et al. also showed that humoral, cell-mediated, and non-specific immunities and the activity of cytokines, which play an important role in various steps of immunogenic mechanisms, were influenced by iron deficiency anemia [59]. Other types of anemia, such as aplastic anemia and myelodysplastic syndromes, are conditions in which impaired immunity is induced. These results suggest that subjects with anemia have impaired immunity and, consequently, an increased load of oral *Candida*.

In the present study, HbA1C affected the quantity of oral *Candida*. Patients with type 2 DM are known to be at an increased risk of opportunistic infections, including oral candidiasis [60]. Due to elevated serum glucose levels and a weakened cellular immune system, patients with DM are susceptible to opportunistic infections. A previous study also reported that the oral carriage of *Candida* species was significantly higher in patients with DM than in controls [61]. The present results appear to support the effects of DM on the prevalence of oral *Candida*.

The strength of the present study was that it was an investigation based on a relatively large number of individuals who participated in specific health check-ups. Its limitation was that since this was a cross-sectional study, other confounding effects, such as medications, were unclear. Another limitation of this study was that dentist's calibrations of DMFT indea and oral hygine measurements might affect the outcomes of this study. For the evaluation and standardization of examiner's skill, all dentists participated in this study were trained with dental models for dentist's calibration.

In conclusion, the cross-sectional relationship between *Candida* mannan concentrations and health check-up results was analyzed and local conditions that influence the prevalence of oral *Candida* were considered. A strong correlation was observed between *Candida* cultures and mannan antigen concentrations. *Candida* mannan concentrations correlated with age, the number of untreated decayed teeth, number of prosthetic teeth, salivary pH, HbA1C, and the red blood cell count in the univariate analysis. In the multivariate analysis, *Candida* mannan concentrations correlated with age, the number of untreated decayed teeth, number of prosthetic teeth, salivary pH, and the red blood cell count. Mannan concentrations were higher in subjects older than 80 years, with a higher number of either untreated or prosthetic teeth,

lower salivary pH, and a low red blood cell count. The relationship between the quantity of oral *Candida* and systemic condition/diseases of the host was demonstrated in the present study.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest. The authors alone are responsible for the content and the writing of the paper.

Ethical Approval: This study protocol was approved by the Committees on Medical Research of Shinshu University (#2795 and #3683) and Aizawa Hospital (#2012-091).

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