Original Article

Association of Human Whole-blood NAD$^+$ Levels with Nabothian Cyst*

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Abstract

Objective Little is known about the association between whole-blood nicotinamide adenine dinucleotide (NAD$^+$) levels and nabothian cysts. This study aimed to assess the association between NAD$^+$ levels and nabothian cysts in healthy Chinese women.

Methods Multivariate logistic regression analysis was performed to analyze the association between NAD$^+$ levels and nabothian cysts.

Results The mean age was 43.0 ± 11.5 years, and the mean level of NAD$^+$ was 31.3 ± 5.3 μmol/L. Nabothian cysts occurred in 184 (27.7%) participants, with single and multiple cysts in 100 (15.0%) and 84 (12.6%) participants, respectively. The total nabothian cyst prevalence gradually decreased from 37.4% to 21.6% from Q1 to Q4 of NAD$^+$ and the prevalence of single and multiple nabothian cysts also decreased across the NAD$^+$ quartiles. As compared with the highest NAD$^+$ quartile (≥ 34.4 μmol/L), the

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adjusted odds ratios with 95% confidence interval of the NAD^+ Q1 was 1.89 (1.14–3.14) for total nabothian cysts. The risk of total and single nabothian cysts linearly decreased with increasing NAD^+ levels, while the risk of multiple nabothian cysts decreased more rapidly at NAD^+ levels of 28.0 to 35.0 μmol/L.

Conclusion: Low NAD^+ levels were associated with an increased risk of total and multiple nabothian cysts.

Key words: Nicotinamide adenine dinucleotide; Nabothian cyst; Female; Risk factor

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INTRODUCTION

Nabothian cysts are commonly observed in post-pubertal females and arise from the obstruction of squamous epithelium-covered columnar epithelium secretions in the uterine cervix[1]. Nabothian cysts can be caused by multiple conditions including inflammation, cervical trauma, pregnancy, and hormonal imbalance. The size of the cyst ranges from 2 to 10 mm and can occur as single or multiple cysts. Nabothian cysts are usually asymptomatic unless they become very large or present as numerous cysts on the cervix, which can cause hematometra and pelvic pain due to compression of the urethra or bladder neck[2-4]. Hysterectomy is often considered the best treatment option, particularly in participants with large nabothian cysts and unusual symptoms[2-4].

Nicotinamide adenine dinucleotide (NAD^+), a co-substrate for Poly (ADP-ribose) polymerases, sirtuins, and cyclic ADP-ribose synthases, is a coenzyme of many dehydrogenases in the body that are involved in the tricarboxylic acid cycle and the respiratory chain. It regulates cellular signal transduction by affecting the activity of NAD^+-consuming enzymes. Additionally, it plays an important role in a wide range of processes, including metabolism, oxidative stress, inflammation, and aging[5-8]. Uterine trauma significantly affects NAD kinase activity and NAD metabolism[9]. Sirtuin 1 encoded by the *SIRT1* gene is the most conserved mammalian NAD^+ dependent histone deacetylase[10,11]. Activation of the β4/SNAI1/SIRT3 signaling pathway could promote epithelial cell migration in cervical cancer[12]. SIRT1 and SIRT7 show low levels of positive in the basal layer of the non-neoplastic squamous epithelium of the cervix[13,14]. In addition, some studies have demonstrated that SIRT1 is overexpressed in squamous intraepithelial neoplasia and squamous cell carcinoma[14]. Our previous study found that whole-blood NAD^+ was associated with aging and its levels decreased in women aged 18–49 years[15]. However, little is known about the association between whole-blood NAD^+ levels and nabothian cysts. Therefore, we aimed to investigate the association between NAD^+ and total nabothian cysts as well as single or multiple cysts in a relatively large-scale community-based population.

METHODS

Study Design and Participants

Data used in this study were obtained from the Jidong community in Tangshan, northern China. A total of 1,532 participants aged 18–99 years were recruited for this study between August 2019 and January 2020. We analyzed 665 women after excluding 804 men and 63 women with missing data on nabothian cyst diagnosis (Figure 1). The study was

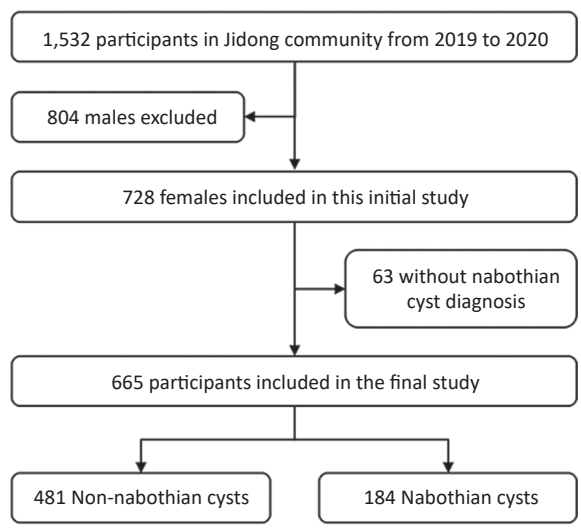


Figure 1. Flowchart of the inclusion and exclusion criteria of this study.

conducted according to the guidelines of the Helsinki Declaration, and the study protocol was approved by the Ethics Committee of the Staff Hospital of the Jidong Oil-Field of Chinese National Petroleum (approval number 2018 YILUNZI 1). All participants provided written informed consent before participating in the study.

Data Collection

Baseline data were collected by well-trained research coordinators using standardized questionnaires. The baseline data included participant demographics, education, physical activity, medical conditions, and other relevant factors. Height and weight were measured using standard anthropometric techniques, and the body mass index (BMI) was calculated by dividing the measured weight (kg) by the square of the measured height (m^2). Systolic and diastolic blood pressures (SBP and DBP, respectively) were measured using an automatic digital blood pressure monitor. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes, and laboratory measurements were performed in the central laboratory of the Staff Hospital of the Jidong Oil-Field, as previously described[15,16]. Alanine transaminase, aspartate transaminase, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride (TG), total cholesterol, and red blood cells (RBC) were performed with an auto-analyzer (Hitachi 747; Hitachi, Tokyo, Japan)[15,16]. Medical conditions included hypertension and dyslipidemia according to documented or self-reported history, medication for corresponding diseases, or clinical or laboratory examinations.

Measurement of NAD^+ Levels

After overnight fasting, blood samples were collected from the large antecubital vein and placed in vacuum tubes containing EDTA. Cycling assays and liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses were used to measure NAD^+ levels in the laboratory using methods described previously[15,16].

Outcome Measure

Nabothian cysts are diagnosed based on gynecological ultrasound examination and are classified into single and multiple cysts based on the number of cysts in the cervix. Multiple nabothian cysts were defined as the presence of more than two cysts. All reports and ultrasound images were obtained by an experienced gynecologist.

Statistical Analysis

The participants were separated into four groups according to NAD^+ level quartiles. The quartiles were as follows: Q1, < 27.6 μmol/L (n = 166); Q2, 27.6–30.9 μmol/L (n = 165); Q3, 30.9–34.4 μmol/L (n = 167); and Q4, ≥ 34.4 μmol/L (n = 167). Values for categorical variables were presented by the frequency and percentage and tested using the chi-square (χ^2) test. Values for continuous variables were expressed as mean ± standard deviation and tested using t-test or one-way analysis of variance (ANOVA).

Logistic regression analyses were used to assess the association of NAD^+ levels with total nabothian cysts and single or multiple cysts. The multivariate model was adjusted for age, BMI, history of dyslipidemia, SBP, TG level, and number of RBCs. Associations were measured using odds ratios (*OR*) and the corresponding 95% confidence intervals (*CI*). In addition, restricted cubic splines with four knots (5th, 35th, 65th, and 95th percentiles) were used to assess the association between continuous whole-blood NAD^+ levels and nabothian cysts after adjusting for confounders. $P < 0.05$ was considered statistically significant. All statistical data were analyzed using SAS 9.4.4 (SAS Institute Inc., Cary, NC, USA), R 4.1.0, and GraphPad Prism (version 8).

RESULTS

Characteristics of Participants at Baseline

Table 1 shows the baseline characteristics of the participants according to NAD^+ quartile. The mean age of participants was 43.0 ± 11.5 years, and the mean level of NAD^+ in the whole population was 31.3 ± 5.3 μmol/L. The mean levels of NAD^+ were 25.0 ± 2.6, 29.3 ± 0.9, 32.5 ± 1.0, and 38.3 ± 3.5 μmol/L for the Q1, Q2, Q3, and Q4 groups, respectively. Participants with high NAD^+ levels were more likely to have many more RBC, higher SBP, higher TG levels, and a history of dyslipidemia than those with low NAD^+ levels. There were no significant differences in the other variables of interest, such as age and BMI, across the NAD^+ quartile groups.

Nabothian Cyst Prevalence among NAD^+ Quartiles

Among the 665 eligible participants, nabothian cysts occurred in 184 (27.7%) participants with, single cysts in 100 (15.0%), and multiple cysts in 84 (12.6%) (Supplementary Table S1, available in

www.besjournal.com). The prevalence of nabothian cysts showed a decreasing trend across NAD^+ quartiles (P for trend = 0.09). Figure 2A does not show a significant correlation between age and whole-blood NAD^+ levels in participants with nabothian cysts (r = 0.04) and in those without nabothian cysts (r = 0.07). Figure 2B shows the prevalence of nabothian cysts according to NAD^+ quartiles. The prevalence of total nabothian cysts among participants in the NAD^+ quartiles Q1, Q2, Q3, and Q4 were 37.4%, 24.9%, 27.0%, and 21.6%, respectively. The lowest NAD^+ level group (Q1) had the highest percentage of total nabothian cysts as well as single and multiple nabothian cysts (P < 0.05).

Association of Whole-blood NAD^+ Levels with Nabothian Cysts

Analysis of the association between NAD^+ and nabothian cysts is shown in Table 2. In the unadjusted model, as compared with the NAD^+ Q4, ORs with 95% *CI* of NAD^+ Q1 was 2.17 (1.34–3.52) for total nabothian cysts, 1.89 (1.04–3.44) for the single

Table 1. Baseline characteristics of the study population within quartiles of NAD^+ level

Characteristics	Overall (n = 665)	NAD^+ groups				P-value
		Q1 (< 27.6) (n = 166)	Q2 (27.6–30.9) (n = 165)	Q3 (30.9–34.4) (n = 167)	Q4 (≥ 34.4) (n = 167)	
NAD^+ (μmol/L)	31.3 ± 5.3	25.0 ± 2.6	29.3 ± 0.9	32.5 ± 1.0	38.3 ± 3.5	< 0.01
Age (y)	43.0 ± 11.5	41.0 ± 9.4	43.9 ± 12.8	43.9 ± 11.8	43.4 ± 11.6	0.06
Age at menarche (y)	13.9 ± 1.5	13.8 ± 1.3	13.9 ± 1.6	14.1 ± 1.7	13.8 ± 1.2	0.11
Age at menopausal (y)	50.0 ± 3.7	50.2 ± 3.7	50.6 ± 3.3	49.9 ± 3.3	49.5 ± 4.5	0.54
Body mass index (N, %)						0.39
< 24 kg/m^2	414 (62.3)	109 (65.7)	104 (63.0)	106 (63.5)	95 (56.9)	
≥ 24 kg/m^2	251 (37.7)	57 (34.3)	61 (37.0)	61 (36.5)	73 (43.1)	
Education level (N, %)						0.39
Middle school or below	206 (31.0)	46 (27.7)	59 (35.8)	48 (28.7)	53 (31.7)	
College or above	459 (69.0)	120 (72.3)	106 (64.2)	119 (71.3)	114 (68.3)	
Physical activity (N, %)						0.17
Inactive	188 (30.1)	52 (33.1)	38 (24.5)	41 (26.5)	57 (36.7)	
Moderate active	116 (18.6)	32 (20.4)	31 (19.6)	27 (17.4)	26 (16.8)	
Active	321 (51.4)	73 (46.5)	89 (56.3)	87 (56.1)	72 (46.5)	
Hypertension (N, %)	106 (17.2)	18 (11.6)	28 (17.7)	28 (18.1)	33 (21.4)	0.14
Dyslipidemia (N, %)	254 (38.2)	47 (28.3)	64 (38.8)	66 (39.5)	77 (46.1)	0.01
SBP (mmHg)	121.1 ± 17.3	117.5 ± 14.8	121.9 ± 19.4	122.5 ± 16.2	122.3 ± 18.3	0.04
DBP (mmHg)	75.3 ± 12.0	74.0 ± 11.1	75.2 ± 11.7	75.7 ± 11.2	76.6 ± 13.6	0.26
RBC (10^{12}/L)	4.4 ± 0.3	4.3 ± 0.4	4.4 ± 0.3	4.4 ± 0.3	4.5 ± 0.3	< 0.01
LDL-C (mmol/L)	2.2 ± 0.7	2.1 ± 0.7	2.2 ± 0.6	2.3 ± 0.8	2.2 ± 0.8	0.22
HDL-C (mmol/L)	1.3 ± 0.3	1.4 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	0.48
TG (mmol/L)	1.4 ± 0.9	1.3 ± 0.8	1.5 ± 1.0	1.3 ± 0.7	1.6 ± 1.0	< 0.01
TC (mmol/L)	5.1 ± 1.0	4.9 ± 0.9	5.1 ± 0.8	5.2 ± 1.0	5.2 ± 1.0	0.08
ALT (U/L)	26.4 ± 21.8	24.2 ± 17.6	27.0 ± 27.2	28.2 ± 24.8	26.2 ± 16.0	0.38
AST (U/L)	24.3 ± 14.1	22.8 ± 8.6	24.6 ± 15.2	25.9 ± 20.9	23.7 ± 7.8	0.24

Note. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NAD^+, nicotinamide adenine dinucleotide; RBC, red blood cell; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides. Data are shown as frequency and percentage for categorical variables and mean ± standard deviation for continuous variables.

cyst, and 2.61 (1.31–5.19) for multiple cysts. In the multivariate model, as compared with the NAD+ Q4, ORs with 95% *CI* of NAD+ Q1 was 2.02 (1.21–3.40) for total nabothian cysts, 1.56 (0.82–2.97) for the single cyst, and 2.98 (1.44–6.18) for multiple cysts.

Subgroup analysis showed associations of NAD+ levels with nabothian cyst in participants stratified by age (< 40 *vs.* ≥ 40 years), BMI (< 24 *vs.* ≥ 24 kg/m^2), SBP (< 120 *vs.* ≥ 120 mmHg), and dyslipidemia as see Supplementary Table S2 (available in www.besjournal.com). Age, BMI, SBP, and history of dyslipidemia had no impact on the

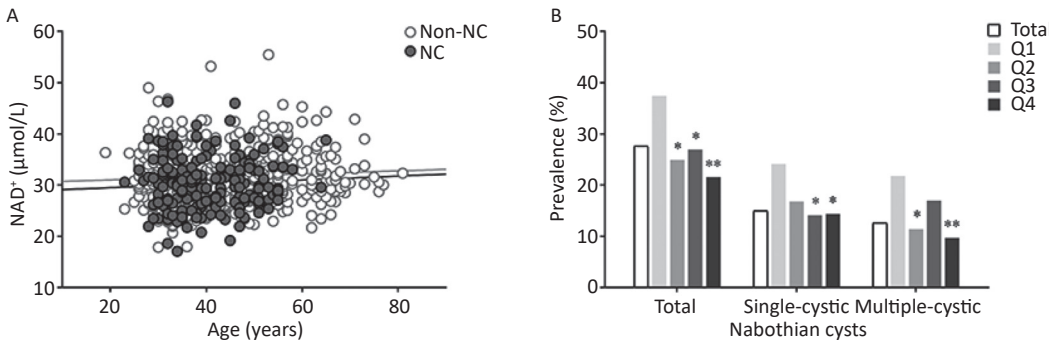
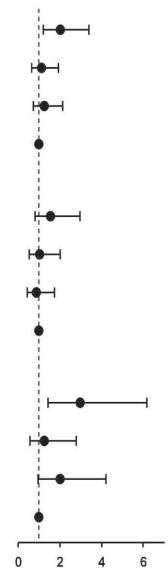


Figure 2. Association between whole-blood NAD+ levels and nabothian cysts in the study population. (A) Correlation between age and NAD+ levels in non-nabothian or nabothian cysts. (B) Comparison of the prevalence of nabothian cysts among the NAD+ quartile groups. NAD+, nicotinamide adenine dinucleotide; Non-NC, non-nabothian cyst; NC, nabothian cyst; Q1, first quartile; Q2, second quartile; Q3, third quartile; Q4, fourth quartile. *indicates statistical difference between the quartile of interest and the first quartile of NAD+ levels. *$P < 0.05$, **$P < 0.01$.

Table 2. Association of whole-blood NAD+ levels with nabothian cysts in all participants

Nabothian cysts	Events, *N* (%)	Odds ratios (95% *CI*)	
		Unadjusted	Adjusted
Total	184 (27.7)		
Q1	62 (37.4)	2.17 (1.34, 3.52)	2.02 (1.21, 3.40)
Q2	41 (24.9)	1.20 (0.72, 2.00)	1.13 (0.66, 1.95)
Q3	45 (27.0)	1.34 (0.81, 2.22)	1.26 (0.74, 2.14)
Q4	36 (21.6)	Ref	Ref
Single-cystic	100 (17.2)		
Q1	33 (24.1)	1.89 (1.04, 3.44)	1.56 (0.82, 2.97)
Q2	25 (16.8)	1.20 (0.64, 2.24)	1.04 (0.54, 2.02)
Q3	20 (14.1)	0.98 (0.51, 1.88)	0.88 (0.44, 1.75)
Q4	22 (14.4)	Ref	Ref
Multiple-cystic	84 (14.9)		
Q1	29 (21.8)	2.61 (1.31, 5.19)	2.98 (1.44, 6.18)
Q2	16 (11.4)	1.21 (0.57, 2.58)	1.26 (0.57, 2.80)
Q3	25 (17.0)	1.92 (0.95, 3.86)	2.02 (0.97, 4.22)
Q4	14 (9.7)	Ref	Ref



Note. *CI*, confidence interval; Ref, reference; Q1, first quartile; Q2, second quartile; Q3, third quartile; Q4, fourth quartile. Adjustments were made for age, body mass index, history of dyslipidemia, systolic blood pressure, triglyceride level, and number of red blood cells.

association between whole-blood NAD^+ levels and nabothian cysts ($P > 0.05$ for interaction).

Restricted Cubic Spline Curves for the Associations of NAD^+ with Nabothian Cyst

Figure 3 shows the adjusted dose-response association of whole-blood NAD^+ levels with nabothian cysts. The risk of total and single nabothian cysts decreased linearly with increasing NAD^+ levels (Figure 3A and B). The occurrence of multiple cysts showed non-linear decline along with the NAD^+ level (Figure 3C). The risk of multiple nabothian cysts was stable before NAD^+ levels of < 28 μmol/L, after which it linearly declined at NAD^+ levels of 28 to 35 μmol/L, and then became stable after NAD^+ levels of > 35 μmol/L.

DISCUSSION

In the current study, we found that approximately 28.0% of the participants had nabothian cysts among the entire study population. Low levels of whole-blood NAD^+ are associated with an increased risk of total and multiple nabothian cysts. The risk of total and single nabothian cysts linearly decreased with increasing NAD^+ levels. The risk of multiple nabothian cysts decreased more rapidly at NAD^+ levels of 28.0 to 35.0 μmol/L. The effect of NAD^+ levels on nabothian cysts was not altered by age, BMI, SBP, or history of dyslipidemia.

Our study showed that the prevalence of nabothian cysts among the participants was as high as 28%. Yilmaz et al. reported that the percentage of nabothian cysts in patients without adenomyosis was approximately 28%[17], which is consistent with our findings. Bajo et al. reported that 20% of all specimens had nabothian cysts, which is similar to our results[18]. However, Zidan et al. reported that the prevalence of nabothian cysts in Sudan is 2%[19]. The very low prevalence in this study was mainly due to discrepancies in the study population. The previous study recruited patients complaining of recurrent back or thoracic pain with a period of symptoms, whereas our study population comprised community-based women. Therefore, the prevalence of nabothian cysts should be evaluated in future large-scale cohort studies.

In the present study, low NAD^+ levels were associated with an increased risk of total and multiple nabothian cysts. Chronic uterine inflammation is likely to be the main reason for the development of nabothian cysts. These cysts are filled with mucus and may contain proteinaceous material, neutrophils, or neutrophil debris[20]. Furthermore, uterine trauma affects NAD kinase activity and NAD metabolism[21]. Mounting evidence has shown that NAD^+ and SIRT play essential roles in inflammation[8,22,23]. Previous studies showed that cervical non-neoplastic squamous epithelium showed weak positivity of SIRT1, SIRT2, and SIRT7[13,14]. SIRT1 has shown potential therapeutic effects in patients with polycystic ovary syndrome by regulating oxidative stress, mitochondrial function, and glucose and lipid metabolism[24]. Another study showed that inhibiting SIRT3 promoter activity promotes the transition between epithelial and mesenchymal cervical cancer cells[12]. Further research has suggested that SIRT1 inhibits the phosphorylation of p66Shc, regulates the activation of fibrogenic factors, enhances ovarian morphology, and diminishes ovarian oxidative stress[25]. These results emphasize the possibility of targeting SIRT1

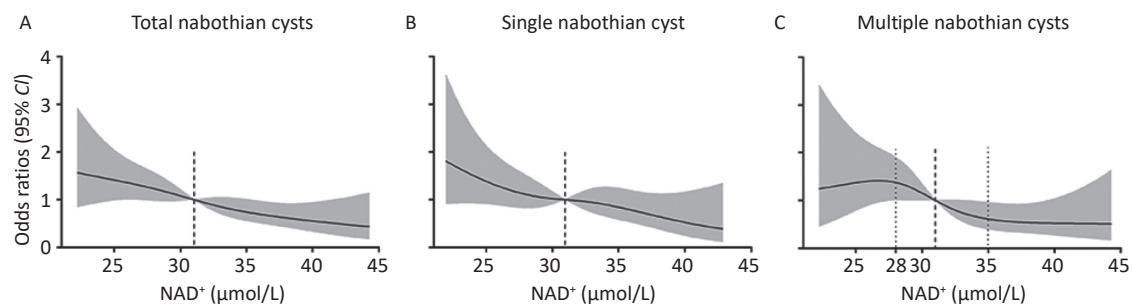


Figure 3. Dose-response association of whole-blood NAD^+ levels with nabothian cysts. (A) Restricted cubic spline curves for the association of NAD+ levels with total nabothian cysts; (B) single nabothian cyst; and (C) multiple nabothian cysts. The model was adjusted for age, body mass index, history of dyslipidemia, systolic blood pressure, triglyceride level, and number of red blood cells. The black line represents the odds ratios and the gray shaded area represents the 95% *CI*. NAD^+, nicotinamide adenine dinucleotide; *CI*, confidence interval.

as a therapeutic strategy for many gynecological diseases. Our data suggest that NAD^+ insufficiency is a common characteristic of nabothian cysts. We did not find any association between whole-blood NAD^+ levels and nabothian cysts, which were altered by age, BMI, SBP, and a history of dyslipidemia. Epidemiological studies have reported that nabothian cyst lesions enlarge in approximately 30% of patients during a long follow-up period[26,27]. Generally, the prevalence of cystic lesions increases with age; intracellular NAD^+ and NAD-dependent dehydrogenase (15-PGDH) levels decrease with aging at a cellular, tissue, and organismal level[28-30]. Supplementation with NAD^+ precursors delays aging[7]. SIRT1 mRNA expression decreased in uteri of aged mice, and uterine-specific deletion of SIRT1 results in premature uterine aging[29,31]. We did not find any correlation between age and nabothian cystic lesions in the current study. It is necessary to conduct further studies with a larger cohort of participants to confirm the association between aging and nabothian cysts.

Dose–response analyses showed that NAD^+ levels were inversely associated with total and single nabothian cysts in all participants. We found that the risk of multiple nabothian cyst declined non-linearly with whole-blood NAD^+ levels, while it declined linearly at NAD^+ levels of 28 to 35 μmol/L. This phenomenon may be attributable to multigravid pregnancy. Tunnel clusters, a specific type of multiple nabothian cysts, are commonly observed in multigravid women[32]. In addition, systemic changes, such as metabolic dysfunction and dramatic changes in hormone levels during and after pregnancy, might also be major factors associated with NAD^+ levels and nabothian cysts. A recent study found that patients with tunnel clusters and nabothian cysts also had inflammation[33]. It is necessary to further elucidate the detailed biochemical and molecular mechanisms of NAD^+ in nabothian cysts in future experimental studies.

Our study had several limitations. First, there was a lack of nabothian cyst size records in our study, although clinical studies have shown that the large size of nabothian cysts can cause many complications. Second, whole-blood NAD^+ may not adequately reflect the NAD^+ level in nabothian cysts, because it is distributed in all tissues. Finally, we analyzed the association between NAD^+ levels and nabothian cyst using cross-sectional data. Therefore, there was no causal effect of NAD^+ on nabothian cysts in our study.

In conclusion, we found a negative association between whole-blood NAD^+ levels and nabothian cysts, particularly multiple cysts. NAD^+ would be considered as a biological marker for nabothian cysts development. Our findings provide new evidence for the association of blood NAD^+ levels and nabothian cysts.

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AUTHOR CONTRIBUTIONS

XU Ling and WANG Yue Xuan contributed equally to this study. ZHOU Yong and WANG Deng Liang designed the study. XU Ling and WANG Yue Xuan conducted statistical analyses and drafted the manuscript. FAN Xue, CHEN Xue Yu, and LIU Yu He contributed to the material preparation and data collection. WANG Wei, YANG Fan, and JU Zhen Yu performed detection tests on blood samples. ZHOU Tian Yun and YU Ye supplied the administrative, technical, and material support. All authors listed have made substantial, direct, and intellectual contributions to the work and approved it for publication.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY

The datasets used and analyzed in the current study are available from the corresponding author upon reasonable request.

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