Association of Vitamins D, B₉ and B₁₂ with Obesity-Related Diseases and Oral Microbiota Composition in Obese Women in Croatia

Running head: Oral microbiota and vitamin levels in obese women

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Received: 24 August 2021
Accepted: 4 April 2022

SUMMARY

Research background. Oral microbiota has become an important factor in obesity, but its association with obesity-related diseases and serum 25-hydroxy vitamin D [25(OH)D] and B complex levels is still uncertain. The main aim of the paper was to determine variation in oral microbiota composition regarding vitamin status and obesity-related diseases in obese females from Croatia. We hypothesized that the prevalence of probiotic or pathogen bacteria in the oral cavity of obese women in Croatia depends on vitamin B₉ (folic acid), B₁₂, and 25(OH)D serum levels and/or hypertension, diabetes, and prediabetes diagnosis.

Experimental approach. To test the defined research hypothesis, female individuals with body mass index (BMI) ≥30 kg/m² (N=70) were recruited to participate in this study. Obese women were divided into groups according to BMI value, diagnosis of obesity-related diseases, and

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micronutrients blood levels. For the quantitative determination of folic acid, vitamin B$_{12}$, and 25(OH)D serum levels, an electrochemiluminescence protein binding assay (ECLIA) was performed. Isolated microorganisms from the saliva of obese women were analyzed by MALDI-TOF mass spectrometer.

Results and conclusions. The present results do not support the hypothesis that the prevalence of probiotic or pathogen bacteria in the oral cavity of obese women in Croatia depends on the level of micronutrients in obese women from Croatia. On the other hand, hypertension and diabetes/prediabetes diagnosis favor the growth of oral pathogens, specifically increased levels of *Candida* sp.

Novelty and scientific contribution. To the best of our knowledge, this is the first study showing the relationship between obesity, micronutrient level, oral microbiota composition, and the incidence of obesity-related disease. We included only obese women from Croatia, so it is regionally specific. Also, we have shown that oral microbiota composition is not connected with micronutrient deficiencies but only with obesity-related diseases.

**Keywords:** obesity, obesity-related diseases, oral microbiota composition, vitamin D, vitamin B$_{12}$, folic acid

**INTRODUCTION**

Obesity is a complex disease and a great medical problem that increases the risk of acquiring comorbidities such as heart disease, prediabetes, diabetes, high blood pressure and cancer development (1). The association between diabetes and obesity is clearly visible from the fact that as many as 80 % of people with type 2 diabetes are obese and, from the opposite perspective, that 10 % of obese people have type 2 diabetes (2). However, the mechanisms underlying obesity-associated with diabetes and prediabetes or other obesity-related diseases like hypertension have not yet been defined. The most common strategies for preventing prediabetes, diabetes and hypertension include lifestyle modifications and pharmacological interventions. Still, there are indices that the development of the diseases is in tight connection to a deficit of certain micronutrients, including D and B complex vitamins. Fat-soluble 25-hydroxy vitamin D has anti-inflammatory and immunomodulatory properties, and it can reduce insulin resistance (3). More specifically, it increases the sensitivity of fat and muscle tissue to existing insulin and decreases gluconeogenesis in the liver, which is an additional contribution to reducing blood sugar concentrations (4).
Probiotic bacteria, mainly belonging to the genera *Lactobacillus* and *Bifidobacterium*, besides many health benefits, can produce vitamins (5) such as folate (6), and some other members of the gut microflora can produce cobalamin (B₁₂). Folic acid and vitamin B₁₂ are both required for normal cell division because of their involvement in DNA synthesis and amino acid metabolism. Additionally, folic acid is involved in DNA repair through *de novo* DNA synthesis and methylation, while vitamin B₁₂ deficiency leads to fatty acid metabolism dysregulation (7).

New findings suggest a causal link between microbial dysbiosis (which is responsible for the chronic systemic inflammation which affects insulin resistance) and obesity indicating the change of intestinal microbiota composition in obese subjects, with an increase in the phyla *Firmicutes* and a decrease in the phyla *Bacteroidetes*, which is associated with the high-fat diet and dysbiosis of gut microbiota (8).

Oral microbes play an important role in maintaining systemic and oral health by resistance to microbial colonization, digestion of nutrients, and immune response (9). Based on bacterial-mediated inflammatory processes, changes in the oral microbiome appear to be connected with obesity. It has been observed that with an increase in BMI, the diversity in bacterial species in saliva decreases (10-12).

In this work, the changing trend of oral microbiota composition depending on serum 25(OH)D, vitamin B₁₂ and folic acid levels or presence of diabetes/prediabetes and hypertension in obese women in Croatia was investigated. Furthermore, we defined the association between probiotic or pathogen oral microbiota, obesity, and obesity-related diseases.

**MATERIALS AND METHODS**

This study was conducted over a period of one year by the Faculty of Food Technology and Biotechnology University of Zagreb, Croatia, and University Hospital Centre Zagreb, Zagreb, Croatia. All scientific procedures were approved by the Ethics Committee of the University Hospital Centre Zagreb, Croatia ( Permit class: 8.1-18/161-2, No. 02/21 AG). In accordance with ethical principles, all of the patients signed an informed consent form before undergoing saliva and blood sampling procedures.

**Subjects and study design**

The experimental population consisted of obese women from Croatia aged 20 to 74 years (*N*=70, median (years)=45.00 (37.00, 55.00)). All participants were patients of the Reference Centre of the Ministry of Health of the Republic of Croatia for Obesity of the Division of Endocrinology, University Hospital Centre Zagreb. We focused only on obese women with
BMI ≥ 30 kg/m² which were subdivided into three classes according to BMI ranges (13). Association of each BMI class with hypertension classification, diabetes/prediabetes diagnosis and micronutrients blood levels were observed. The study design is shown in Fig. 1. In Priori power analysis, T test approach was conducted and minimum sample size using G*Power 3.1.9.7 software (Heinrich Heine University, Duesseldorf, Germany; http://www.gpower.hhu.de/) was calculated (14). Accordingly, the minimum sample size with 0.80 pre-specified effect size (d), 0.05 significance level (α) and 0.80 power level (1-β) was calculated as 21 per group. Considering that, we decided in this study include 70 voluntary participants as appropriate sample size to have at the end a possible significant difference between the two or three groups depending on the examined parameter.

In this study only females not taking any form of supplements that contained vitamin D, B₁₂, or folic acid were included. Furthermore, exclusion criteria were pituitary and/or adrenal disease, untreated thyroid disease, prior myocardial infarction, stroke, transient ischemic attack, or oncological disease. All analyses were performed during the first visit of obese patients to the Reference Centre for obesity (University Hospital Centre Zagreb) without prior education in proper and balanced nutrition and controlled diet. Therefore, the diet of the patients was without accurate data on the composition and daily intake of calories. We include only female patients for several reasons: the Reference Centre for Obesity has a much higher prevalence of obese females than male patients; gut microbiota differences between men and women, which can be influenced by the grade of obesity (15); the observed divergence could play a dominant role in defining gender differences in the incidence of metabolic and intestinal inflammatory diseases; studies in humans have produced conflicting results due to high inter-individual heterogeneity in age, diet and hormonal factors, and the largely unexplored influence of gender (16). Total body mass (kg), fat (%), fat mass (kg), muscle (%) and BMI (kg/m²) of the study participants were measured with the Tanita Body Fat Scale (Tanita, Tokyo, Japan) using Bioelectrical Impedance Analysis at the University Hospital Centre Zagreb.

**Fig. 1**

**Vitamin and obesity-related test correlations**

25(OH)D serum values below 50 nmol/L represented a deficiency, between 50.1-75 nmol/L were defined as an insufficiency, and anything above 75 nmol/L was considered as a sufficient level of serum 25(OH)D in a human adult (17). Folic acid values between 13.5-45.3 nmol/L are considered normal range and above 45.3 nmol/L as elevated folic acid levels. When folic acid levels in the blood are less than 6.8 nmol/L it is considered a folic acid deficiency.
Insufficiency stood for folic acid values between 6.8 and 13.4 nmol/L (18). It has been well-established that people with vitamin B₁₂ blood levels between 200 and 350 pg/mL (147.6–258.3 pmol/L) have clear vitamin deficiency symptoms (19). Thus, in this study a cut-off division was 250 pmol/L. The division of obese women into groups according to vitamin levels is shown in Fig. 1.

Classification of obese women according to obesity-related disease was made using criteria: only diabetes/prediabetes diagnosis, only hypertension diagnosis, and without diseases (control group; Fig. 1).

Saliva and blood sampling

For analytical procedures, saliva and blood samples of obese female patients were taken at the Division of Endocrinology, University Hospital Centre Zagreb. Venous blood sampling (5 mL) was performed in VACUETTE® tube CAT Serum Separator Clot Activator (Greiner Bio-One GmbH, Kremsmünster, Austria). 25-hydroxy vitamin D, B₁₂, and folic acid levels were determined from the serum of fresh pooled blood samples. Furthermore, after rinsing the mouth with 15 mL of sterile saline solution to prevent the effect of previously eaten food on the appearance of microorganisms in the oral cavity, 2 mL of saliva samples were collected using sterilized centrifuge tubes (TPP, Trasadingen, Switzerland) stored at −20 °C (20).

Isolation and identification of oral microorganisms

The isolation and identification procedures have been described in Huđek et al. (20). Original saliva and a 1:1 dilution of saliva (0.5 mL) in phosphate buffer (pH=7.2) were inoculated on Nutrient agar plates (de Man-Rogosa-Sharpe and Lauria Bertani). Furthermore, the incubation was performed over two days under aerobic conditions in a thermostat at 37 °C. Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) was used for the identification of colonies with different phenotypes. MALDI Biotyper 3.0 software package (Bruker Daltonik, Bremen, Germany) with standard settings was used to process the recorded mass spectra.

From the results of this work with 118 different species of isolated bacteria (5 different phyla – Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Fusobacteria) and 13 different species of fungi (2 different phyla – Ascomycota and Basidiomycota), 34 potentially pathogenic and 22 potentially probiotic bacteria were determined (Table 1). The classification of probiotic bacteria was adapted according to Fijan (21) and pathogens according to Werth (22). For the statistical analyses, “microbiota function” presents the number of different species that
fit into each of three categories – probiotic, pathogen, or none. Bacteria were identified as binary variables (present/not present).

Table 1

Laboratory blood testing

The laboratory where all analysis was performed has accreditation by HAA (Croatian Accreditation Agency). The Roche Diagnostics 25(OH)D total assay as competitive electrochemiluminescence protein binding assay (ECLIA) was used for the quantitative determination of serum 25(OH)D, vitamin B₁₂, and folic acid in human serum samples (23,24). Further, the ECLIA assay was used for the in vitro quantitative determination of vitamins on a Cobas 6000cee analyzer (Roche Diagnostics, Mannheim, Germany). The method used for serum 25(OH)D measurement was verified with an assay validated by the Vitamin D External Quality Assessment Scheme (DEQAS; http://www.deqas.org/) (25). The validation of methods used for folic acid and vitamin B₁₂ measurement was performed by DGKL (The German Society for Clinical Chemistry and Laboratory Medicine) at the Reference Institute for Bioanalytic, Bonn.

Data analysis and statistics

First, the One-Sample Kolmogorov–Smirnov normality test was used to determine if a data set is normally distributed. Correlations among variables including BMI, fat percentages, fat mass, muscle percentages, age, 25(OH)D, folic acid, vitamin B₁₂, microbiota classification (probiotic, none and pathogen), hypertension classification and prediabetes/diabetes diagnosis was determined using correlation matrix and Spearman’s test. We used factorial ANOVA to determine statistically significant interactions between independent variables of the oral microbiota composition and diabetes-related diseases or vitamin levels. Multiple comparisons were adjusted using Tukey’s HSD test. Statistically significant values were those that differed at p<0.05. Statistical analysis was carried out using IBM-SPSS v.22 software (26).

RESULTS AND DISCUSSION

In this study, only the female population was involved because obesity has a much higher prevalence in female than male patients. Also, results obtained in female and male population cannot be compared because there are significant gut microbiota and hormonal status differences, which impact metabolic and intestinal inflammatory diseases development.
Correlations between obesity factors, vitamin blood level, and obesity-related diseases

In this study, BMI (kg/m²) values positively correlated with a fat percentage (%), fat mass (kg) and age, while a negative correlation was demonstrated with serum 25(OH)D levels and muscle percentages (%) values (Table 2). The same correlations between BMI, fat (%), fat mass, and muscle (%) have been confirmed in other worldwide studies with obese individuals (28,29). A statistically significant positive correlation was found between serum 25(OH)D and folic acid values in the tested obese women (Table 2). According to a recent meta-analysis, the prevalence of vitamin D deficiency is 35 % higher in people with obesity than in people with normal body mass (30). Through a complicated chemical process, it is possible that excess body fat requires a surplus of vitamin D, which diminishes the amount available for other processes.

In Gominak (31) research, vitamin D deficiency was shown to alter the intestinal microbiome reducing B vitamins’ production in the gut. As well as for vitamin D, Kerns et al. (32) have confirmed that obese individuals have a deficiency in vitamin B₉ at a very high percentage (16-29 %). Obese women in Croatia follow the worldwide trend of a correlation between higher obesity levels and lower 25(OH)D blood levels (33).

Table 2

In this study, we showed a statistically significant positive correlation between the BMI values and incidence of hypertension and diabetes/prediabetes diagnosis (Table 2). By dividing the study group of obese women into 4 categories according to obesity-related diseases, a statistically significant difference in BMI values was determined between the obese women without any disease and the group of women with both hypertension and prediabetes/diabetes diagnosis (p<0.01; Fig. 2). As in our study, results from two national surveys have shown that the prevalence of hypertension and diabetes occurred across all ranges of BMI and increased with higher BMI (34). In vitamin D deficient patients with a diabetes diagnosis, supplementation of vitamin D increased serum 25-(OH)D, sirtuin 1, irisin, and glycosylated hemoglobin level, and these improvements may reduce insulin resistance. Furthermore, vitamin D can increase transport glucose in the gut, insulin receptor gene expression in β-cells, and intestinal calcium absorption may serve as a stimulus for insulin release. Vitamin D deficiency, obesity and oxidative DNA damage are significant predictors of genomic instability (34). Vitamin D via genomic and epigenomic mechanisms could control gene expression, and that could be a reason why it has such wide-ranging no skeletal health benefits (35). Epigenetic influence of 25(OH)D is through histone modifications, especially acetylation. However, there is the study that showed that serum 25 (OH)D could change the methylation ratio of the CYP2R1 gene, which is a potential candidate for predicting serum 25(OH)D variation (36).
**Relationship between oral microbiota composition and vitamin blood level, obesity or obesity-related diseases**

There was a positive correlation between numbers of oral Proteobacteria and Firmicutes bacteria (Spearman’s rho CC=0.284, p=0.017). Zeigler et al. (12) also reported bacteria from Proteobacteria phylum and individual Neisseria mucosa to be present in six-fold higher amounts in the saliva of obese adolescent subjects. Furthermore, Yang et al. (37) showed that Proteobacteria and Firmicutes bacteria were significantly associated with an increased obesity prevalence in obese individuals from the Southern Community Cohort Study.

What should be emphasized first is that there is a positive correlation between the incidence of hypertension or prediabetes/diabetes and pathogenic bacteria in the oral cavity in obese women from Croatia (Table 2). In obese women with diabetes/prediabetes, there is a greater variety of fungi (number of different species) of the genus Ascomycota compared to all 13 isolated fungi in this study (Mann-Whitney U=462.00, p=0.04). The identification results of the oral microbiota showed the predominance of Candida sp. in the Ascomycota phylum. The higher BMI values (class 1 – class 3 obesity level), depending on the presence of diabetes/prediabetes, positively affected the number of Ascomycota in the oral cavity (Factorial ANOVA, F=4.091, p=0.021). Further, a statistically significant difference in the number of Ascomycota between different obesity levels was seen only in the diabetes/prediabetes group (Tukey’s HSD post-hoc multiple comparison for diabetes/prediabetes group: class 1–class 2, p=0.004; class 1–class 3, p=0.023). The predominance of Candida sp. in the saliva of women with diabetes/prediabetes can be explained by the fact that diabetes is a metabolic disorder that increases the likelihood of a fungal infection, especially that associated with Candida sp. pathogens, due to an immunosuppressive effect of the disease on the patient (38). Also, it was proven that the fungi belonging to Ascomycota phylum produce biologically active secondary metabolites that impact diabetes and cardiovascular disorders (39). Because of the too general hypothesis, we had many post hoc observations like the present one, which had the potential impact on the quality of work.

In general, in the saliva of obese women with 25(OH)D blood levels ≥50 nmol/L significantly more species of Actinobacteria were found compared to those with lower values (p=0.029). In the study of Gominak (31), a three-month therapy with vitamins D and B resulted in improved sleep. They hypothesized that the combination of serum 25(OH)D and vitamin B creates an environment that favors the return of the bacteria species Actinobacteria, Firmicutes,
Bacteroidetes, and Proteobacteria (normal human microbiome). The same foursome of bacterial species is found in every human with a “healthy gut” all over the world, so they are called the "healthy foursome". The oral vitamin D supplementation in healthy volunteers has decreased the relative amount of pathogen Escherichia spp, Shigella spp., Helicobacter spp., and Pseudomonas spp. (40). Long-term vitamin D deficiency may produce changes in the microbiota composition that promoted body mass gain and permanently changed environmental conditions that no longer favor the normal human microbiota. On the other hand, lower serum 25(OH)D values change the microbiota composition reducing B vitamin production in the gut. With the decrease in Actinobacteria in the gut, the vitamin B_{12} blood level was also lower. Therefore, vitamin B_{12}, as it exists in nature, maybe produced by Actinobacteria (41).

Female individuals with 25(OH)D blood levels <50 nmol/L had a statistically significant number increase of fungi Candida sp. (p=0.022) and Lactobacillus sp. in the saliva (p=0.027). Since class 3 obesity was the most prevalent in the group with lower 25(OH)D blood levels (<50 nmol/L), it can be concluded that the presence of Candida sp. and Lactobacillus sp. in the mouth of obese women is associated with vitamin D deficiency and morbid obesity. In our previous study (20), significantly more Lactobacillus sp. were observed in the saliva of obese women compared to controls with normal body mass, which was explained by their increased consumption of snacks and food rich in sugar. Vitamin D has a signaling role in modulating the host immune system. VitaminD/vitamin D receptor (VDR) signaling is a major contributor to the gut microbiome at the genetic, environmental and immune level (42). VDR gene is the first gene identified as a factor that shapes the gut microbiome at the genetic level. VDR knock-out decreased the level of Lactobacillus and increased the level of Clostridium and Bacteroides in mice feces (43). Furthermore, one of the roles of vitamin D in the human immune response is to induce an antimicrobial peptide called cathelicidin LL-37. Cathelicidin is a critical component for the body's ability to fight infections, such as candidiasis, a fungal infection caused by Candida (44). This could be the reason for the increased oral concentration of Candida sp. in the saliva of obese women with a vitamin D deficiency. In this study, the presence of Candida sp. was also positively associated with blood glucose levels (diabetes/prediabetes). Our results are confirmed by Man et al. (45), who also showed that higher glucose concentration is directly related to Candida albicans growth, which may be associated with fungal infections that occur in non-controlled diabetic patients.

The mechanism of microbiota composition by which bacterial and fungal metabolism impact the level of vitamin D in obese women is less known. Vitamin D by VDR signaling can directly control the immune system. On the other hand, some microbes like Lactobacillus reuteri
increased 25(OH)D levels (46). Supplementation with *Lactobacillus rhamnosus* and *Lactobacillus Plantarum* enhances the expression of VDR in intestinal epithelial cells (47). Khosravi et al. (48) showed that the increase of 25(OH)D blood level had a positive effect on body mass loss, which is partly attributed to the anti-inflammatory effects.

Our study is subject to several limitations. We performed a preliminary study with only the female population involved. Defined selection reasons are given in the Methods, paragraph Subjects. Briefly, differences in diet, age and hormonal factors may affect the development of obesity. In further research we plan to do a study on the male population. Since the initial hypothesis that changing trend of oral microbiota composition depends on micronutrient levels and/or presence of various obesity-related diseases in obese women in Croatia was not precise enough, manipulating a large amount of data generated a number of post hoc observations that contributed to the overall quality of work. Therefore, this study can be defined in part as exploratory and hypothesis-generating.

**CONCLUSIONS**

There is no relationship between micronutrient deficiency and imbalanced oral microbiota. On the other hand, we showed positive correlations between the incidence of hypertension or diabetes/prediabetes, BMI values and pathogenic bacteria in the oral cavity of obese women in Croatia. This study highlights the potential influence of oral microbiota (with a predominance of probiotic bacteria) on BMI reduction and stabilization of obesity-related diseases in obese women in Croatia.

**FUNDING**

Financial support was obtained from the Ministry of Science, Education and Sport, the Republic of Croatia, as part of the project ‘Stress-induced diversity and evolution of mixed bacterial cultures’ (grant number 0583444-3466).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHORS' CONTRIBUTION**

A. Huđek Turković and M. Matovinović contributed equally. Both authors conceptualised the manuscript, participated in formal analysis and statistical analysis, and wrote the original draft. K. Žuna was involved in investigation, methodology and formal analysis. L. Škara did data
analysis and interpretation of experimental data. S. Kazazić has provided certain resources and was involved in the visualization and formal analysis. V. Bačun-Družina performed the investigation, methodology and writing review and editing. K. Durgo was in charge of visualization, supervision, project administration, writing-review and editing.

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https://doi.org/10.3390/nu12041093


Obese women from Croatia

Body mass index (BMI) ≥ 30 kg/m²; N=70; median (kg/m²) = 40.88 (36.23, 47.83)

Obesity
- class 1
  - (BMI 30-34.9 kg/m²)
  - 18.6 %
- class 2
  - (BMI 35-39.9 kg/m²)
  - 25.7 %
- class 3
  - (BMI ≥ 40 kg/m²)
  - 55.7 %

Vitamin test correlations

Vitamin D
- N=70
- group 1 (<50 nmol/L, N=46)
  - median (nmol/L) = 34.14 (25.00, 40.00)
- group 2 (≥50 nmol/L, N=24)
  - median (nmol/L) = 66.05 (60.30, 75.70)

Folic acid
- N=60
- group 1 (<13.5 nmol/L, N=36)
  - median (nmol/L) = 10.20 (9.60, 12.10)
- group 2 (≥13.5 nmol/L, N=24)
  - median (nmol/L) = 17.80 (15.85, 20.90)

Vitamin B₁₂
- N=60
- group 1 (<250 pmol/L, N=30)
  - median (pmol/L) = 106.50 (142.0, 214.0)
- group 2 (≥250 pmol/L, N=30)
  - median (pmol/L) = 315.00 (269.00, 402.00)

Obesity-related diseases test

N=70
- diabetes / prediabetes
- hypertension
- *control group

Oral microbiota test correlations

Fig. 1. Flowchart of participant selection for the analysis. The control group (*) represented a female population without diabetes/prediabetes and/or hypertension.
Table 1. Oral bacteria and fungi isolated from the saliva of obese women from Croatia. The classification of probiotic bacteria was made according to Fijan (21; marked in green) and pathogens according to Werth (22; marked in red).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Proteobacteria</th>
<th>Actinobacteria</th>
<th>Ascomycota</th>
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<tbody>
<tr>
<td><strong>Firmicutes</strong></td>
<td><strong>Proteobacteria</strong></td>
<td><strong>Actinobacteria</strong></td>
<td><strong>Ascomycota</strong></td>
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<td>Streptococcus salivarius</td>
<td>Clostridium cochlearium</td>
<td>Lactobacillus rhamnosus</td>
<td>Pseudomonas abietaniphila</td>
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<td>Granulicatella adiacens</td>
</tr>
</tbody>
</table>
Table 2. Descriptive statistic and Spearman rank correlation matrix for selected variables

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>1.</th>
<th>2.</th>
<th>3.</th>
<th>4.</th>
<th>5.</th>
<th>6.</th>
<th>7.</th>
<th>8.</th>
<th>9.</th>
<th>10.</th>
<th>11.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Age</td>
<td>46.10</td>
<td>12.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Body mass index</td>
<td>42.20</td>
<td>8.09</td>
<td>0.408**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>25(OH)D</td>
<td>44.78</td>
<td>20.02</td>
<td>-0.048</td>
<td>-0.273*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Folic acid</td>
<td>15.63</td>
<td>5.86</td>
<td>0.021</td>
<td>-0.135</td>
<td>0.293*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>270.53</td>
<td>128.31</td>
<td>0.020</td>
<td>-0.221</td>
<td>0.198</td>
<td>0.248</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>6.</td>
<td>Hypertension classification</td>
<td>0.53</td>
<td>0.50</td>
<td>0.514**</td>
<td>0.402**</td>
<td>0.127</td>
<td>-0.027</td>
<td>0.026</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Diabetes/prediabetes diagnosis</td>
<td>0.44</td>
<td>0.50</td>
<td>0.225</td>
<td>0.251*</td>
<td>-0.023</td>
<td>-0.027</td>
<td>0.019</td>
<td>0.208</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Microbiota function</td>
<td>0.84</td>
<td>0.67</td>
<td>0.159</td>
<td>0.196</td>
<td>0.085</td>
<td>0.059</td>
<td>0.025</td>
<td>0.319**</td>
<td>0.684**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Fat %</td>
<td>46.51</td>
<td>6.11</td>
<td>0.082</td>
<td>0.488*</td>
<td>0.073</td>
<td>0.106</td>
<td>0.143</td>
<td>0.196</td>
<td>0.142</td>
<td>0.095</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Fat mass</td>
<td>55.01</td>
<td>16.04</td>
<td>0.254</td>
<td>0.776**</td>
<td>0.086</td>
<td>0.097</td>
<td>0.130</td>
<td>0.290</td>
<td>0.130</td>
<td>0.011</td>
<td>0.705**</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Muscle %</td>
<td>48.64</td>
<td>7.47</td>
<td>-0.115</td>
<td>-0.685**</td>
<td>-0.175</td>
<td>-0.034</td>
<td>-0.006</td>
<td>-0.048</td>
<td>0.039</td>
<td>0.036</td>
<td>-0.689**</td>
<td>-0.573**</td>
</tr>
</tbody>
</table>

N=70 (except N=60 for folic acid and vitamin B<sub>12</sub>). Hypertension classification: 0-none; 1-hypertension; Diabetes/prediabetes diagnosis: 0-none, 1-diabetes/prediabetes; Microbiota function (the number of different species that fit into each of three defined categories; for example, probiotic is represented by the variable of how many probiotic species were detected within the swab of the oral cavity): 0-probiotic, 1-none, 2-pathogen. *p<0.05; **p<0.01.

* for the same body mass index (BMI), women usually contain ~ 10% more body fat than men (27).
Fig. 2. Scatter plot visualizing the relationship between the control group (no obesity-related disease diagnosis) and obese women from Croatia with diabetes/prediabetes, hypertension or both diagnoses based on body mass index (BMI) values. The black line represents the median value in each group while the black circles symbolise class 1 (BMI 30-34.9 kg/m²), white circles class 2 (BMI 35-39.9 kg/m²) and grey circles class 3 obesity (BMI≥40 kg/m²). *Statistically different values compared to the control group (p<0.05).