Evaluation of the Status of Vitamin D_3 and Vitamin K_2 of Bangladeshi Postmenopausal Women: A Cross-Sectional Study

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Abstract

Background: Postmenopause is the name given to the time when a woman stops periodical bleeding for an entire year. In this stage of postmenopause, women remain at increased risk of several health anomalies, such as osteoporosis, diabetes, vision problem, dental problem, and heart disease. Hence, the purpose of the study was to assess the status of vitamin D3 and vitamin K2 [menaquinone-4 (MK-4)] among Bangladeshi postmenopausal women especially those confined to Dhaka city. Methods: A cross-sectional analytical study was done in the Retired Government Employees Hospital in the year 2019 where 55 postmenopausal women below 60 years were selected. The blood sample was collected from them and the level of serum vitamin D3 [25(OH)D] and vitamin K2 [menaquinone-4 (MK-4)] were determined by the high-performance liquid chromatography (HPLC) method. Binary logistic regression was used for model fitness and its significance. Results: The mean ages of the postmenopausal women were 55.80±3.55 years. Their body mass index and waist-hip ratio were 26.03±2.66 kg/m2 and 0.93±0.05 cm, respectively. Serum levels of vitamin D3 [25(OH) D] and menaquinone-4 (MK-4) from postmenopausal women were 14.80±6.36 ng/mL and 0.38±0.21 ng/mL, respectively. The regression model used in this study was 74% fit, and diabetes, cardiovascular disease (CVD), dental problems, vision problems, and hearing problems were significant for postmenopausal women. However, serum vitamin D3 and serum vitamin K2 were non-significant at the 5% level of significance. A high incidence of insufficient serum vitamin D3 [25(OH) D] level and sufficient serum vitamin K2 (MK-4) level were found among postmenopausal women with pre-obese and high waist-hip ratio (WHR). Conclusion: The level of serum vitamin D3 and K2 and their correlation with BMI, WHR, and overall health status may provide insight to medical professionals and dietitians to take necessary action to address various health issues faced by postmenopausal women.

Keywords: Vitamin D3; Vitamin K2; Postmenopause; Women; Health anomalies.

Abbreviations: FSH, follicle-stimulating hormone; MK-4, menaquinone-4; HCC, hepatocellular carcinoma; CHD, coronary heart disease; BMI, body mass index; WHR, waist-hip ratio; HPLC, high performance liquid chromatography; SPSS, statistical package for social sciences; RT, retention times; SD, standard deviation; CVD, cardiovascular disease; APHA, American public health association; WHO, world health organization.

Introduction

It has been estimated that more than 840 million people over the age of 60 will live in developing countries by the year 2025, representing 70% of all older people worldwide, and hence their health should be a prime concern (World Health Organization, 2005). Menopause is defined as the permanent cessation of menses in women which occurs normally between the ages of 45 and 55 years worldwide (Ashrafi et al., 2010). The onset of menopause is featured in the decreasing

Significance | Status of Vit D_3 and Vit K_2 of Postmenopausal women of Dhaka

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production of estradiol and increased levels of follicle-stimulating hormone (FSH). During the postmenopausal transition period, women experience several irritating symptoms, such as hot flashes, night sweats, vaginal atrophy and dryness, dyspareunia, sleep disturbance, and mood swing (Sandhu et al., 2011).

Vitamin D is a steroid prohormone ("conditional" vitamin) synthesized in the skin during its exposure to ultraviolet (UV) light. UV light above the wavelength of 290–300 nm (UVB) is absorbed by 7-dehydrocholesterol in the skin to synthesize vitamin D_3. Vitamin D_3 often called the "sunshine vitamin", is a vital nutrient for the human body. Vitamin D_3 has a role in calcium metabolism and bone mineral metabolism and it works with the parathyroid hormone (PTH), acts on the kidneys, bone, intestine, and influences gene expressions such as Y-RNA, MINPP1 (Multiple inositol polyphosphate phosphatases 1), COPB2 (COP1 coat complex subunit beta 2), PUS3 (Pseudouridine synthase 3), and CD83 (Cluster of differentiation 83) (Adams et al., 2007). It is essential in preventing cancer, osteoporosis, rheumatoid arthritis, multiple sclerosis, hypertension, cardiovascular disease, obesity, psoriasis, and psychiatric diseases (Viljakainen et al., 2010).

Vitamin K exists naturally as vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone, MK-4 through MK-10) (Krueger et al., 2009; Beulens et al., 2013; Schurgers et al., 2008). Vitamin K_1 and vitamin K_2 are required for the γ-glutamyl carboxylation of all vitamin K-dependent proteins. Although the mammalian bacterial intestinal flora can produce vitamin K_2, the amount produced is thought to be inadequate (Booth et al., 1998). Menadione (unsubstituted 2-methyl-1,4-napthoquinone, a chemical analog of 1,4-naphthoquinone with a methyl group in the 2-position, and that are also called vitamin K_3) is a water-soluble synthetic form of vitamin K that functions as an intermediate in the metabolic conversion of phylloquinone to MK-4. Menadiol sodium phosphate (also called vitamin K_4) is a synthetic water-soluble form derived from reduced menadione. Vitamin K helps to prevent fractures due to osteopenia and osteoporosis (Cheung et al., 2008; Knapen et al., 2007; Cockayne et al., 2006), prevent liver cancer and death in patients with liver cirrhosis and hepatocellular carcinoma (HCC) (Yoshida et al., 2011; Kojima et al., 2010; Yoshiji et al., 2009; Hosho et al., 2008; Hotta et al., 2007; Kakizaki et al., 2007; Mizuta et al., 2006; Habu et al., 2004), prevent vascular calcifications (especially in patients on warfarin) (Beulens et al., 2013; Beulens et al., 2009; Geleijnse et al., 2004), reduce the risk of coronary heart disease (CHD), CHD mortality and all-cause mortality (Beulens et al., 2013) and improve insulin sensitivity (Yoshida et al., 2008).

Since Vitamin D and Vitamin K play important roles in the quality of health of postmenopausal women, the present study was undertaken to determine their levels of serum vitamin D_3 [25(OH) D] and vitamin K_2 [menaquinone-4 (MK-4)] and nutritional status to assess their influences.

Methodology

Human subjects

A total number of 55 postmenopausal women aged below 60 years were selected from Retired Government Employees Hospital, Abosar Bhaban, Satmasjid Road, Dhanmondi, Dhaka in 2019. It was a cross-sectional analytical study, and purposive sampling was used. Subjects were excluded if they were under 50 years of age and above 60 years of age and took tablet forms of calcium (Ca), vitamin D_3, and vitamin K_2 supplements.

Ethical approval

The study was approved by the Faculty of Biological Sciences, University of Dhaka, and ethical clearance reference No:88/Biol.Scs./06.10.2019. All participants were provided written informed consent.

Survey questionnaire development

A standard questionnaire was developed and filled with relevant information such as socio-demographic information, health status information, exposure to sunlight, and various diseases condition (hypertension, cardiovascular disease, diabetes, gastric, malabsorption, asthma, vision problem, hearing problem, dental problem, and osteoporosis). Anthropometric measurements such as height, weight, and waist-hip ratio (WHR) were included in the questionnaire to obtain data from the respondents. In the end, about 5 ml of blood was collected from each respondent by an expert medical technologist to assess serum vitamin D_3 [25(OH) D] and vitamin K_2 (MK-4) levels.

Reagent

Chemicals and solvents including vitamin D_3 (C-9756; 1 mg), vitamin K_2 (Menaquinone, Lot# LRAB8835), methanol, acetonitrile, 2- propanol, and hexane used in this study were purchased from Sigma-Aldrich Chemie, GmbH, Taufkirchen, Germany.

Chromatographic equipment

A liquid chromatographic system, SIL 20 series Prominence HPLC (Shimadzu, Japan) consisted of an auto-sampler (Model SIL-20 AC), dual pumps (Model 20 AD), column oven (Model CTO-20A), vacuum degasser (Model DGU-20A_3), UV-visible detector (Model SPD-20A), and LC solution software that runs on Windows was used. All solutions were prepared by using sonication in an ultrasonic bath (Ultrasons Medi-II, Spain). Analytical reversed-phase C-18, Luna 5µ, 250 x 4.6 mm, Phenomenex, Inc., Japan was used. The chromatographic condition was maintained as described with significant modification. Particle separation was done using a hypersil column with C18 selectively (Supelco USA, column dimension: 250×4.6 mm, particle size: 5 µm) and LC-solutions software was used for the analysis of the sample (Saima et al., 2012).

Sample preparation

Extraction of vitamin D_3 and vitamin K_2 (MK-4) was done in a similar process. To begin with, all storage samples (-20°C) were kept at room temperature. After that, 500 µl serum was taken in a falcon

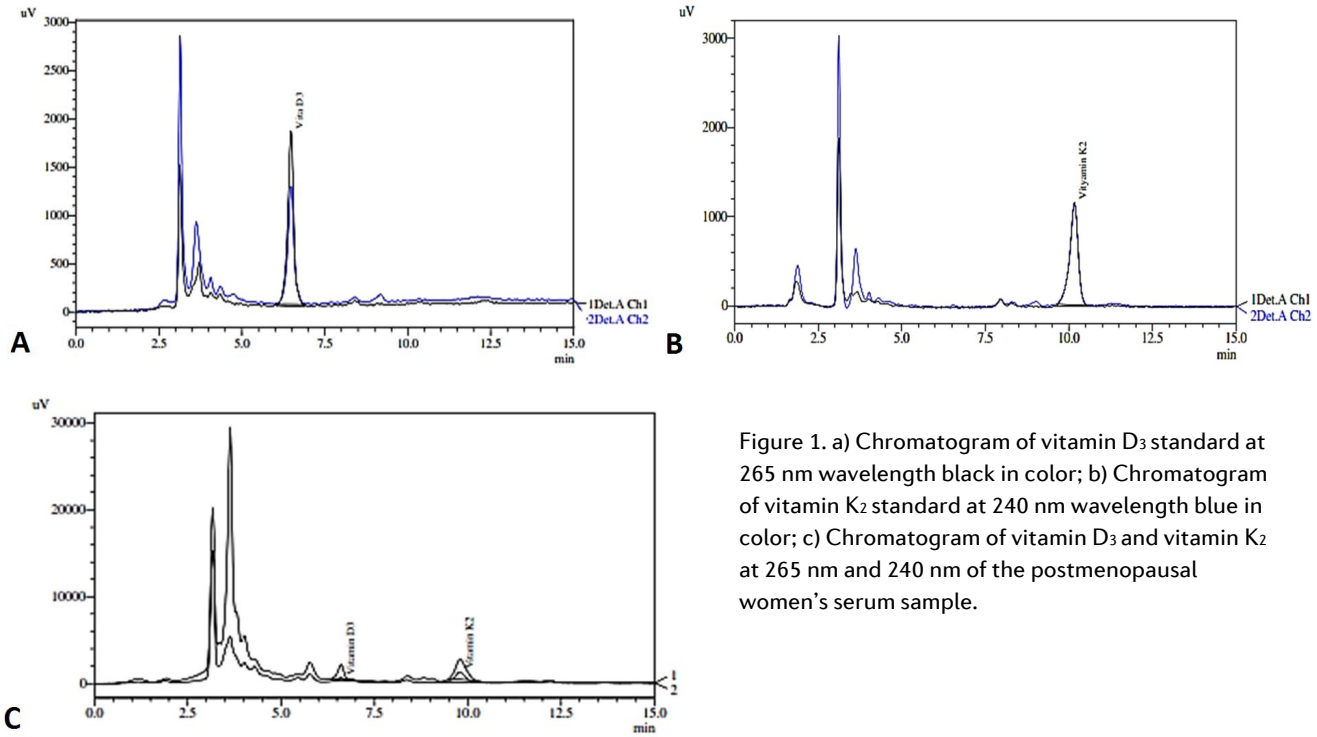

Figure 1. a) Chromatogram of vitamin D_3 standard at 265 nm wavelength black in color; b) Chromatogram of vitamin K_2 standard at 240 nm wavelength blue in color; c) Chromatogram of vitamin D_3 and vitamin K_2 at 265 nm and 240 nm of the postmenopausal women's serum sample.

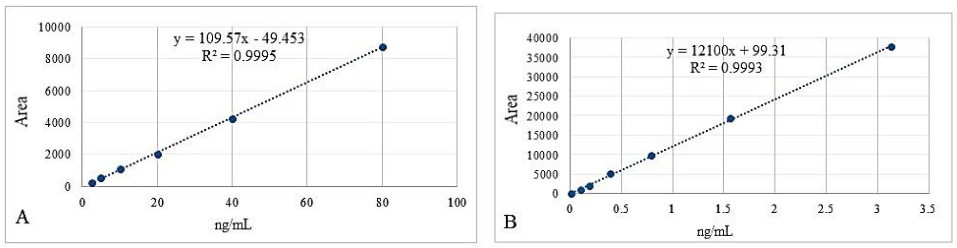

Figure 2. a) Calibration curve of vitamin D_3; b) Calibration curve of vitamin K_2 .

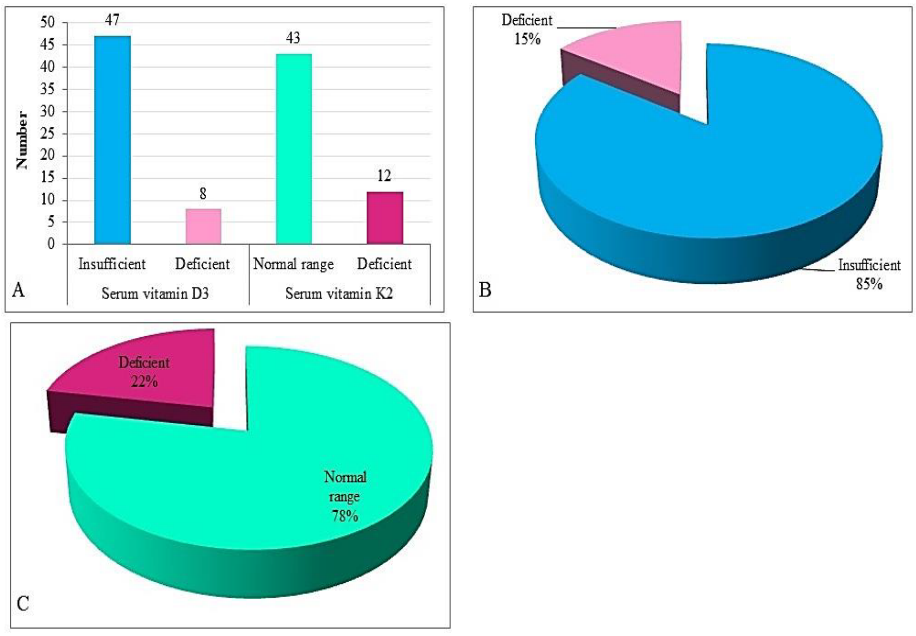

Figure 3. a) Vitamin D_3 & K_2 level[b&c] in serum of the postmenopausal women (N=55); b) Percentage of serum vitamin D_3 level[b] of the postmenopausal women (N=55); c) Percentage of serum vitamin K_2 level[c] of the postmenopausal women (N=55).
[[b] denotes reference ranges [Normal range (30 ng/mL -100 ng/mL), Insufficient ((≥ 20 ng/mL - < 30 ng/mL) & Deficient (< 20 ng/mL)] of serum vitamin D_3. Source: American public health association (APHA); [c] denotes reference ranges [Normal range (0.2 ng/mL -3.2ng/mL) & Deficient (<0.2ng/mL)] of serum vitamin K_2. Source: https://emedicine.medscape.com/article/2088738-overview]

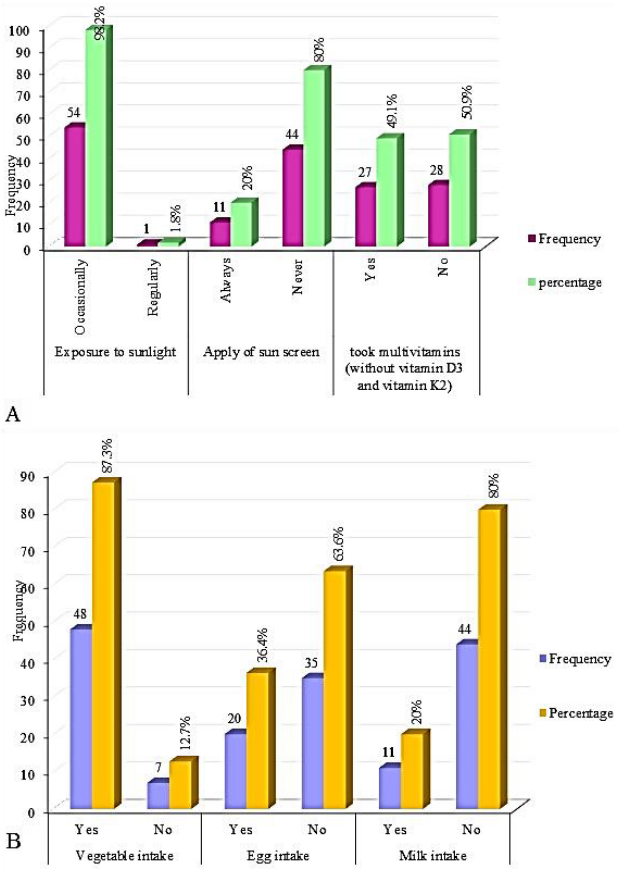

Figure 4. a) Exposure to sunlight, applying sunscreen, and taking multivitamins (without vitamin D_3 and vitamin K_2) of the postmenopausal women (N=55). b) Vegetable intake, egg intake, and milk intake of the postmenopausal women (N=55)

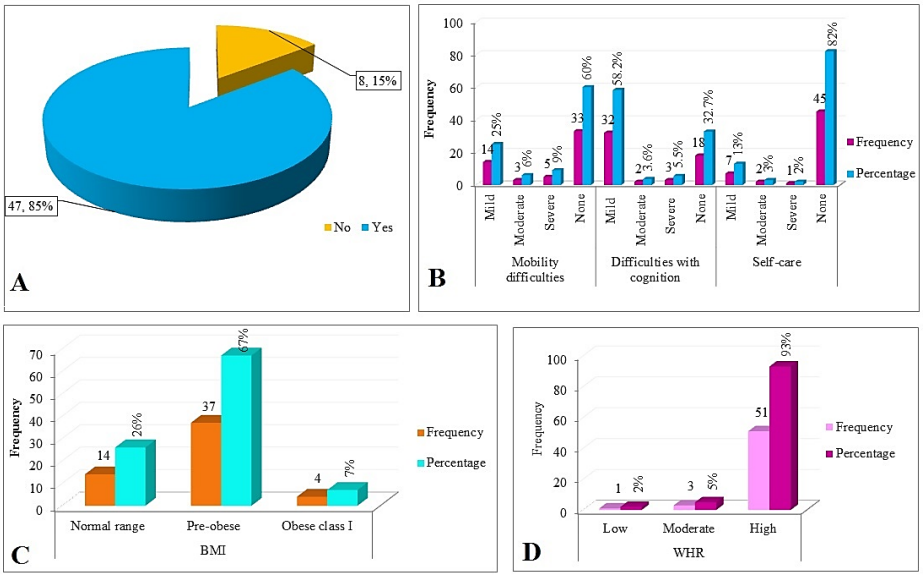

Figure 5. a) Depression of postmenopausal women (N=55); b) Mobility difficulties, cognition difficulties, and self-care of postmenopausal women (N=55); c) BMI [d] of the postmenopausal women (N=55); d) WHR[e] of the postmenopausal women (N=55)

[[d] denotes reference ranges [Normal range (18.5 kg/m^2 - 24.9 kg/m^2), Pre-obese (25.0 kg/m^2 - 29.9 kg/m^2) & Obese class I (30.0 kg/m^2- 34.9 kg/m^2)] of BMI: body mass index. Source: world health organization (WHO); [e] denotes reference ranges [Low (0.80 cm or less); Moderate (0.81 cm - 0.85 cm) & High (0.86 cm or high)] of WHR: waist-hip ratio. Source: world health organization (WHO)].

tube and added 500 µl diluent in it and vortex for 5 minutes. Then, 2 ml hexane was added three times and vortex for each time for 2 minutes (total 6 minutes). After completing the vortex, samples were centrifuged at 4000 rpm for 15 minutes for phase separation. Later, the organic phase (upper) was transferred to a falcon tube and dried under nitrogen gas. Residue was dissolved with 2 ml diluents [methanol: 2-propanol; 80:20 (v/v)]. At last, 20 µl samples were kept in a small vial and injected into the HPLC system (Saima et al., 2012) .

Standard preparation

A stock solution of standard vitamin D_3 & K_2 (MK-4) (0.5 µg/ml) was prepared for each standard in methanol and diluted to different concentrations ranging from 1.9 ng/ml to 500 ng/ml.

Mobile phase preparation

The mobile phase consisted of acetonitrile and methanol in the ratio of 95:05 (v/v) over the C-18 column (250 × 4.6 mm, 5 µm, Phenomenex, Inc.).

Chromatographic condition

Analytical reversed Phase C-18 Column was used in this study with a flow rate of the mobile phase- 1.5 ml/min. The UV detection was performed at 265 nm for vitamin D_3 and 240 nm for vitamin K_2. The experiment was performed at room temperature (~26°C). The injection volume was 20 µl for both standard and the sample solution.

Statistical analysis

Data were analyzed as mean± standard deviation, frequency distributions, percentages, binary logistic regression, and categories of the different variables. We considered dummy variables (two values of either 1 or 0 to indicate the absence or presence of some categorical effect) of the postmenopausal women who were suffering from different diseases (hypertension, cardiovascular disease, diabetes, gastric, malabsorption, asthma, vision problem, hearing problem, dental problem, and osteoporosis) were used as a dependent variable one after another and other four factors [serum vitamin D_3, serum vitamin K_2 (MK-4), BMI, & WHR] were used as an independent variable. Due to dummy variables, binary logistic regression was used to measure the fitness of the regression model and to test its level of significance. In this study, the statistical package for social sciences (SPSS) software (version 25, IBM, Chicago, United States) was used for data analysis.

Results

The standard curves were constructed by plotting the peak area ratios of vitamin D_3 [25(OH) D] and vitamin K_2 (MK-4) against the sample of serum vitamin D_3 [25(OH) D] and vitamin K_2 (MK-4) level of the postmenopausal women. The chromatogram of vitamin D_3 [25(OH) D] and the retention time for vitamin D_3 was 6.5 minutes at 265nm wavelength black in color (Fig. 1a).

In addition, the chromatogram of vitamin K_2 (MK-4) and the retention time for vitamin K_2 were 10 minutes at 240nm wavelength blue in color (Fig. 1b). The chromatogram of vitamin D_3 and vitamin K_2 in the postmenopausal women's serum sample is shown in (Fig. 1c), together with the respective retention times at 265 nm (for vitamin D_3) and 240 nm (for vitamin K_2). This study's calibration curve for vitamin D_3 was R^2 = 0.9995, while it was R^2 = 0.9993 for vitamin K_2 (MK-4) (Fig. 2a & 2b). For vitamin D_3, plotted data made a straight line with a slope of 109.57, showing a strong positive correlation between vitamin D_3 concentration and vitamin D_3 area. Additionally, for each increase of 1 ng/mL of vitamin D_3 concentration, the expected vitamin D_3 area was increased by 109.57 (Fig. 2a). Similar results were obtained for vitamin K_2, with a straight line and a slope of 12100 indicating a strong positive correlation between vitamin K_2 concentration and vitamin K_2 area. Also, the predicted vitamin K_2 area rose by 12100 for every rise in vitamin K_2 concentration of 1 ng/mL (Fig. 2b). As Bangladesh is a Muslim-majority country, most of the postmenopausal women in this study were Muslim (93%) and the rest of them were of another ethnic background (7%), housewives (50.9%), and aged 55.80±3.55 years. Additionally, 5.5% of postmenopausal women had no formal schooling, whereas 32.7% had completed their graduation (Supplementary table 1). Our study found that none of the postmenopausal women had normal serum vitamin D_3 [25(OH) D] levels, 47 of them had insufficient levels, and 8 of them had low levels. In contrast, serum vitamin K_2 (MK-4) levels were normal in 43 postmenopausal women and deficient in 12 of them (Fig. 3a). According to our work, 85% of postmenopausal women had insufficient serum vitamin D_3 [25(OH) D] levels (Fig. 3b). Because 98.2% of them were occasionally exposed to sunlight, did not take vitamin D_3 and vitamin K_2 multivitamins (Fig. 4a), and consumed less egg and milk 36.4% and 20%, respectively (Fig. 4b) whereas, 78% of postmenopausal women had normal serum vitamin K_2 (MK-4) levels (Fig. 3c) since 87.3% of them consumed a higher amount of vegetables (Fig. 4b). Overall health status of the postmenopausal women was poor because 60% had back pain, 89% had waist and joint pain, and 51% had urinary incontinence (Supplementary table 2). Moreover, 85% of women had depression (Fig. 5a) along with mobility difficulties, cognition difficulties, and self-care-related problems (Fig. 5b). We also observed that most of them were pre-obese (67%) (Fig, 5c) and high waist-hip ratio (93%) (Fig. 5d). Our study found that they were suffering from different chronic diseases e.g. hypertension for 7.56±5.55 years, a cardiovascular disease for 7.57±5.77 years, diabetes for 6.81±5.65 years, asthma for 9.75±8.01 years, dental problems for 5.30±4.83 years, gastric for 7.44±5.40 years, malabsorption for 2.83±2.50 years, vision problem 7.61±6.12 years, the hearing problem for 3.96±2.98, and osteoporosis for 3.06±2.50, respectively (Supplementary table 3). According to binary logistic regression analysis, it was shown that postmenopausal women were suffering from different diseases, and their maximum R^2 = 0.74. Where R^2 represents how well the model fits the data and it measures the strength of the relationship between the model and the dependent

variable. In this binary logistic regression diabetes, cardiovascular disease (CVD), dental problems, vision problems, and hearing problems were significant but serum vitamin D_3 and serum vitamin K_2 were non-significant at the 5% level of significance (Supplementary table 4-7). The level of the confidence interval of this analysis was 95% and hence, the p-value ≤ 0.05 (5%) indicated statistically significant findings. In binary logistic regression analysis, we could not use the other four factors [serum vitamin D_3, serum vitamin K_2 (MK-4), BMI, & WHR] as dummy variables due to their different range value.

Discussion

This study aimed to assess the relationship between postmenopausal women's serum vitamin D_3 [25 (OH) D] and vitamin K_2 (MK-4) levels and different health anomalies. As the high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection method is one of the most accurate and convenient analytical techniques compared to other chromatographic techniques, we used it in our work to determine serum vitamin D_3 and serum vitamin K_2 status. In HPLC, a liquid solvent is forced through a solid adsorbent material using a pump rather than gravity, with the different chemical components separating as they pass at different speeds. The procedure yields a high resolution and takes between 10 and 30 minutes to complete. Since no report of a correlation between vitamin D_3 and vitamin K_2 in postmenopausal women was found, the attempt was to evaluate such correlations. We also observed the combined impact of these two vitamins on their body mass index (BMI), waist-hip ratio (WHR), and different diseases such as hypertension, cardiovascular disease (CVD), diabetes, gastric, malabsorption, asthma, dental problem, vision problem, hearing problem, and osteoporosis. We used binary logistic regression to measure regression model fitness and test its significance level. According to the model, five diseases including diabetes, CVD, dental problems, hearing problem, and vision problems were significant at the 5% level of significance. In the human body, the normal range of vitamin D_3 is 30–100 ng/mL although it was found that most of the postmenopausal women had insufficient serum vitamin D_3 levels in this study, and the mean value was 14.80±6.36 ng/mL. This vitamin D_3 insufficiency was due to poor exposure to sunlight, data collection during the winter season, and inadequate intake of vitamin D_3 and calcium-rich foods. In Bangladesh, 133 postmenopausal women aged 45 years and above participated in a single-center cross-sectional study, which indicated that the mean vitamin D_3 level was higher than that of our study with 22.1±11.3 ng/mL (Ahmed et al., 2018). Our study's vitamin D_3 level was lower than another cohort study, which examined 150 ambulatory postmenopausal women (≥50 years old) in a semi-urban area of Southern India. Their level was 20.85±8.63 ng/mL, which was higher than ours (Paul et al., 2008). Determination of vitamin K_2 levels is quite difficult, because of the low concentration of circulating form of vitamin K_2 in plasma, and interfering compounds in plasma, especially triglycerides. However, this study attempted to assess vitamin K_2 levels because when calcium is broken down in the human body, vitamin K_2 activates a protein that helps the calcium bind to human bones to perform its work. According to studies, consuming more vitamin K_2 improves bone density, lowers the risk of bone fracture, and also improves blood clotting and vascular health (Knapen et al., 2015; Maresz, 2015; Park et al., 2015; Tsugawa, 2015; Flore et al., 2013; Ferland, 2012; Okano et al., 2008). We measured the serum levels of vitamin K_2 menaquinone-4 [MK-4] of 55 postmenopausal women, and the majority of them were possessing within the normal range of vitamin K_2 0.38±0.21 ng/mL. The serum levels of MK-4 in postmenopausal women with and without osteoporosis were measured by Klapkova using an HPLC method with fluorescence detection, and their findings were greater than ours. Their findings were 0.89±0.29 ng/mL and 0.82±0.27 ng/mL, respectively (Klapkova et al., 2018). Data obtained from 20 healthy subjects and 10 osteoporotic patients who took MK-4 supplements showed a large variability of vitamin K_2 levels. MK-4 levels were 0.15±0.17 ng/mL, lower in healthy subjects than in our work, whereas levels were 46.83±46.41 ng/mL, greater in osteoporotic patients taking MK-4 supplements (Kamao et al., 2005). MK-4 levels were found to be 0.02±0.04 ng/mL in a study of 344 healthy postmenopausal women in Japan, which is significantly lower than our results (Tsugawa et al., 2006). As opposed to the MK-5 to MK-10 standard, the MK-4 standard was chosen for this work because Sigma-Aldrich Chemie, GmbH, Taufkirchen, Germany, is the only supplier for it in Bangladesh.

Furthermore, the results of this study showed that postmenopausal women had a high waist-hip ratio (WHR) and pre-obesity. Therefore, it is essential to pay attention to these inadequate serum vitamin D_3 levels. The following preventive measures could be implemented, including daily exposure to direct sunlight for at least 30 minutes, consuming enough foods high in vitamin D_3 and calcium, engaging in outdoor activities, and vitamin D_3 and calcium supplementation in women of this age group.

Conclusion

The study's findings showed that postmenopausal women's serum vitamin D_3 levels were insufficient due to the data of winter season, infrequent exposure to sunlight during the outings, diet failing to provide an adequate amount of vitamin D_3, inadequate calcium-rich food intake, and no use of vitamin D_3 supplement. In addition, adequate serum vitamin K_2 (MK-4) levels were found in postmenopausal women because of their high consumption of vegetables. This study is important because it will help to determine the level of serum vitamin D_3 and K_2 and their correlation with BMI, WHR, and overall health status of that certain age group. This work may provide insight to medical professionals and dietitians to take necessary action to address various health issues faced by

postmenopausal women. However, the sample sizes used in this study were not appropriate enough. Due to the smaller sample size, further investigations are required to provide more information on the vitamin D_3 and vitamin K_2 status of postmenopausal women.

Author Contribution

FA conceptualized the study, developed the methodology and performed formal analysis, data collection, investigation, and preparation of the manuscript. AR assisted in software-related issues, validation of the methods, data collection, investigation, and revision of the manuscript. MLB contributed to the conceptualization, investigation, supervision, and preparation of the manuscript. MAH assisted in software-related issues, data collection, and statistical analysis.

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Competing financial interests

Authors have declared that no competing interest exists.

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