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Vitamin D Status and its Correlation with Heart Rate Variability among Healthy Female Medical Students at King Abdulaziz University: A Cross Sectional Study

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Abstract: Background: Vitamin D deficiency is a global health problem and is highly prevalent among the Saudi population. A growing body of evidence indicates an association between vitamin D deficiency and risk of cardiovascular disease (CVD). Heart rate variability (HRV) is a non-invasive technique that evaluates cardiac autonomic function, which is a major determinant of cardiovascular health. Limiteddata is available on the relationship between vitamin D deficiency and HRV and none exists among the Saudi population. Therefore, this study was conducted to examine the association between vitamin D status and HRV in female medical students at King Abdulaziz University (KAU) in Jeddah, Saudi Arabia. **Methods:** This cross-sectional study was conducted from March to May, 2015 at the Faculty of Medicine, KAU. A total of 124 female students participated in the study. After completing a self- administered questionnaire, anthropometric measurements were taken for all subjects. HRV was recorded after five minutes rest in the supine position using ML870 power lab machine. Blood was obtained for the determination of serum levels of 25-hydroxyvitamin D_3 (25[OH]D), parathyroid hormone (PTH), calcium, phosphate and magnesium. Informed consent was obtained from all participants and the study was approved by the ethics committee at KAU hospital. **Results:** The mean age of the study subjects was 21.7± SD 1.1 and the mean 25 (OH) D levels was 31.2 nmol/L ± SD 16.9. Almost 86 % of the participants were vitamin D deficient. Seventy-two (72%) of the participants with 25(OH) D< 30 nmol/L had RMSSD values (an index of parasympathetic activity) below the 25thpercentile compared to 28% with 25(OH) D ≥30 nmol/L (chi-square, P=0.049). Regression analysis showed that 25(OH) D level was independently and negatively associated with LFnu (an index of sympathetic activity) (B=-0.175, P<0.028). **Conclusion:** Our findings suggest that vitamin D deficiency is associated with low HRV, a predictor of CVD risk. Larger community based studies are needed to confirm these findings. Early identification of vitamin D deficiency coupled with appropriate treatment measures can prevent the risk of CVD in otherwise healthy subjects.
[Lojine Ayoub, Hanan Al Kadi, AzraKirmani, HossamAwad. **Vitamin D Status and its Correlation with Heart Rate Variability among Healthy Female Medical Students at King Abdulaziz University: A Cross Sectional Study.** *J Am Sci* 2023;19(5):57-71]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). http://www.jofamericanscience.org 05.doi:10.7537/marsjas190523.05.

Keywords: Vitamin D status, Heart Rate Variability, (HRT Cardiovascular Disease. (CVD).

1. Introduction

Vitamin D deficiency is a global health issue (1). It is one of the most widespread endocrine disorders, and affects more than 1 billion people world-wide (2). Despite the abundance of sunshine year round, it is highly prevalent among the Saudi population, ranging from 28% to 100 %.Table (1)shows the prevalence of vitamin D deficiency as reported by some of the largest studies in Saudi Arabia, Gulf and Middle East. Another contribution factor in the prevalence recorded is the cutoff point used to define vitamin D deficiency (3–5).

There is controversy regarding vitamin D deficiency definition. The "Endocrine Society" Guidelines set the cutoff levels at 75 nmol/L(30 ng/ml) whereas the "Institute of Medicine" set the cutoff level as 50nmol/L(20ng/ml)(4).Despite the prevailing disagreements about the exact vitamin D levels needed to be healthy, many researchers adopt the following classification (34,35):

- Desirable vitamin D level = 100-250 nmol/L
- Sufficient vitamin D level = 75-99.9 nmol/L
- Insufficient vitamin D level = 50-74.9 nmol/L
- Mild vitamin D deficiency = 25-49.9 nmol/L
- Moderate vitamin D deficiency = 12.5-24.9 nmol/L
- Severe vitamin D deficiency < 12.5 nmol/L

Conversions for vitamin D_3: [serum] 2.5 nmol/L= 1 ng/mL (21).

Factors affecting the normal physiological pathway for vitamin D production are the main cause for vitamin D deficiency; these are summarized in Table 2. The pathophysiology of the vitamin D deficiency consequences, result in low serum calcium.

This in turn, stimulates the parathyroid gland to increase its production of parathyroid hormone (PTH), which results in secondary hyperparathyroidism. This gives the typical clinical picture of elevated PTH levels (>7 ng/L) but normal serum calcium levels and vitamin D level < 50 nmol/L (57,58).

The PTH increases the serum calcium level to normal physiologic range by mobilization of calcium from the skeleton, through increasingtheosteoclastic activity which results in local foci of bone weakness, as well as, an overall decrease in the bone mineral density (BMD). This decrease in BMD results in osteopenia and if severe to osteoporosis.

Table (1): The prevalence of vitamin D deficiency in the Saudi Arabia, Gulf, and Middle East.

Year	**Location**	**Author**	**Sample Size**	**Mean Age or**	**Mean Vitamin D**	**Definition**	**Prevalence**
2015	Saudi Arabia	Albuhairan et al. (42)	12,575	15.8	N/A	<50 nmol/L	95.6%
2015	Saudi Arabia	Al-Saleh et al (43)	2,226	15.1	Male = 39.0 Female= 29.5	<25 nmol/l	Males = 19.4% Females = 47.0%
2015	Abu Dhabi, United Arab Emirates	Haq et al (44)	60,979	Range: 1-114	Female = 47.41 Male = 50.37	Mild: 25-49.99 Extreme: <24.99	82.5%
2014	Saudi Arabia	Hussain et al(45)	10,709	Range: 0-60+	44.58	25 (severe), 50 (moderate) and 75 (mild) nmol/l	83.6% (31.9% severe, 32.0% moderate and 19.7% mild)
2013	Saudi Arabia	Tuffaha et al.(37)	5,590	15+	N/A	<28 ng/mL	40.6% males and 62.7% of females
2012	Saudi Arabia	Kanan et al.(46)	1,556	44.4 and 46.1 (two groups)	4 Groups: 1= 33.3 2= 44.4 3= 28.5 4= 36.3	<50nmol/l	Pre-menopausal:80%. 85% Post-menopausal: 68%, 78%
2012	Saudi Arabia	Ardawi et al (47)	834 males	42.1	29.01 nmol/l	<50 nmol/l	87.8%
2011	Jordan	Batieha et al(48)	5,640	41.9	Male = 183.25 Female = 99.5	<75nmol/l	37.3% in females, 5.1% in males
2011	Iran	Hovsepian et al (49)	1,111	41.4	Male = 52.5 Female = 45	Mild = 50-75 Moderate = 25-50 Severe= <25	Mild = 19.6% Moderate = 23.9% Severe = 26.9% Deficiency was more prevalent among women.
2011	Zahedan Southeast, Iran	Kaykhaei et al (50)	993	36.71	34.37 nmol/l	<50nmol/l	82.5%

PTH also increases excretion of phosphorus by the kidneys. The resultant phosphaturia leads to low normal or low serum phosphorus level. Phosphaturia causes an insufficient calcium-phosphorus product, which leads to defective mineralization in the skeleton. Phosphaturia is especially harmful for children with little skeletal mineral deposits, as this defect can cause various skeletal deformities, typically termed as rickets. In adults, such mineral defects can go undetected. This is because, in adults, the epiphyseal plates are closed with enough skeletal minerals. Though, this prevents formation of skeletal deformities, it can cause a mineralization defect, known as an osteomalacia. This is manifested as local or generalized pains in the bones and muscles (25,59).

Table (2). Summary of factors affecting normal physiological pathway for vitamin D production with examples(22).

Factors affecting normal physiologic pathway for vitamin D production	Example
Reduced synthesis of active vitamin D ($1,25(OH)_2D_3$)	• Decline in skin synthesis: o Applying local sun block creams. o Seasonal variation. o Different sun exposure time of the day o Skin color o Aging • Liver diseases (cirrhosis) or hepatic failure. • Kidney disease: chronic kidney disease or renal failure.
Reduce absorption	• Systemic diseases: cystic fibrosis, celiac disease, crohn's disease. • Surgeries: gastric by pass. • Medications decrease cholesterol absorption
Increase sequestration	• Obesity
Increase catabolism	• Medications: anti-convulsions, corticosteroids
Increase loss of $25(OH)_2D_3$	• Kidney disease: nephrotic proteinuria

Vitamin D deficiencyhas been associated with several acute and chronic disorders, including cardiovascular disease (CVD) (6,7). It has also been linked to all-cause mortality in the general population (8). In 2013, the "World Health Organization" (WHO) reported death due to CVD as the number one cause of death globally, which is also the number one cause of preventable death (9).Vitamin D has been found to exerts some direct effects on the cardiovascular system through the vitamin D receptor (VDR), which is present in the cardiomyocytes, endothelial cells, and vascular smooth muscle cells (80). This results in vitamin D regulating cardiomyocytes contractility, extracellular matrix turnover and anti-hypertrophic actions (14).

Evidence from a growing body of literature suggests an association between low vitamin D status and risk of CVD (10). However, limited studies examined this association using the "Heart Rate Variability" (HRV) in healthy humans (11–14). HRV is beat-to-beat interval fluctuation of heart rate (HR) that is a direct reflection of the cardiac function regulated by the autonomic nervous system (ANS)(15). It is a non-invasive and extremely sensitive investigation, which is an index of cardiovascular well-being and disease (16,17). High HRV is a healthy sign of normally functioning ANS. On the other hand, low HRV is not only indicative of CVD, but also a predictor of impending one (18). It identifies the risk of a first time cardiovascular event in apparently healthy individuals (17). Few studies have reported lower HRV in subjects with lower levels of vitamin D (14) .

The "Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology", introduced different methods for the assessment of HRV in 1996 (70,71). The most widely used methods by which variations in HR may be evaluated include (16,17,71,73):

- Frequency domain analysis: In this method, the cyclic fluctuations of R-R intervals are quantified by the frequency of the fluctuation using Fast Fourier transformation or auto regression spectral analysis(17). From a short term recording of 2-5 minutes, three main spectral components can be distinguished, namely- Very Low Frequency (VLF), Low Frequency (LF), and High Frequency (HF). The unit of measurement f is usually milliseconds squared (ms^2).

VLF is affected by temperature variations and humoral systems (frequency strength of VLF is 0.04–0.15 Hz)(14,68,70,74).It has stronger associations with all-cause mortality (60). The major contributors to the VLF band are thermoregulation associated with ANS activity, long-term regulation mechanisms, the renin-angiotensin-aldosterone system (RAAS), and hormonal factors (60,71). The VLF band is also an indicator of sympathetic function (14). This is because several experimental studies have suggested that the VLF rhythm is intrinsically produced by the heart with the amplitude and frequency modulated by efferent SNS activity (60).

LF is sensitive to variations in cardiac sympathetic and presumably PNS activity (frequency strength of LF is 0–0.04 Hz)(14,68,70,74).The LF component of the HRV spectrum represents the complex interactions between the SNS and PNS activities of the HR and the baroreceptor activity (14,71). Some authors have also stated that LF indicates SNS activity of the SAN (6,68,69).During rest, the LF region mainly reflects the baroreceptor activity. Due to this reason, this region was known previously as "baroreceptor range" or "mid-frequency band". Efferent vagally-mediated respiratory influences can also be seen in this band(60)

HF is synchronized to the respiratory rhythm

and is mainly modulated by cardiac parasympathetic innervations (frequency strength of HF is 0.15–0.4 Hz) (14,68,70,74).This band is also known as the respiratory band as it corresponds to the HR variations seen in the respiratory cycle (17,60,71). A modulated vagal tone helps maintain a stable autonomic regulation which is essential for cardiovascular health(60). The influence of vagal mediators is more rapid on the heart, principally the high frequency power (HFP)(68). A number of studies have shown that total vagal blockade can eliminate HF oscillations and reduce the power in LF (60).Decreased HF activity, due to decreased parasympathetic activity, has been associated with aging, several cardiac pathologies, and in patients suffering from panic, anxiety, or those who are under stress (60).

Of the three components, LF and HF can also be measured in normalized units (nu). The use of normalized units represents the relative value of each power component in proportion to the total power without the VLF component. When represented using normalized units, LF and HF signify controlled and balanced state of the ANS(68).

Low frequency to high frequency ratio (LF:HF ratio) represents the ratio of LF power to HF power (60). This ratio represents an index of the relative balance of sympathovagal (SNS and PNS) influences on the heart(60,68,71). At the same time, this ratio reflects the overall state of autonomic activity(14,69). It is known, that depending on the context, the LF power can be influenced by vagal, sympathetic, and baroreflex mechanisms. On the other hand, HF power is a product of efferent vagal activity resulting from respiratory activity.

It is often considered that a low LF:HF ratio represents a greater parasympathetic activity compared to sympathetic activity, and a high LF:HF ratio represents increased sympathetic activity compared to parasympathetic activity (60,71). The LF/HR ratio should be interpreted only after knowing the mean values of the HF and LF powers. Total power (TP) represents principally the overall level of modulation of cardiac ANS activities(14,69). The TP is high with high HRV and low with low HRV(14).

Time-domain analysis

Time domain method is the simplest method for HRV measurement and calculates HRV via a continuous electrocardiogram (ECG) (60,68). In this method, each QRS complex is detected and N-N intervals (which denotes all intervals between adjacent QRS complexes) or the instantaneous HR is calculated (68,71). Therefore, in time domain method, detection of heartbeat is necessary for measurement of HRV first. In time domain method, due to modulation of AV nodal conduction, fluctuations in the PR interval are neglected (68). HRV measurement using time-domain can be performed using both statistical and geometrical methods(71). Only statistical methods will be discussed, as this was the method used in the current study.

Statistical time domain measurements are taken from a series of instantaneous HR or cycle intervals(68). The variables used in the calculation can be either derived from total ECG recording or smaller segments of the ECG recording(68). These variables include themean N-N intervals of the entire ECG recording (MNN), the standard deviation of all N–N intervals (SDNN), the root mean square of the successive R-R interval difference (RMSSD), the percentage of successive R-R intervals >50 ms(pNN50) and the Mean Heart Rate (14,17,60,68,71). HR and variability in R-R interval are reciprocals of each other or to be exact value is HR =60,000/RR where HR unit is beats per minute (bpm) and RR unit is ms(68).

The SDNNis a useful way to measure HRV. It is measured in milliseconds(60,68). It represents the overall cyclic components of HRV (14).A higher SDNN is associated with a higher probability of survival(60), while, a lower SDNN is associated with reduced functioning of the left ventricle(14).In general, SDNN and LF are both affected by adrenergic and cholinergic activities along with other physiological activities (14).

In a RMSSD, first, the successive time difference between normal heartbeats is calculated in milliseconds.After that, each of the values is squared and the average result is obtained before getting the square root of the total value (60). The RMSSD is the primary method to calculate the vagally mediated changes in the HRV spectrum(14,60). Though, the RMSSD is correlated with the HF power, the effect of respiration rate on RMSSD is uncertain. It is considered that lower RMSSD values are associated with higher scores on a risk inventory of sudden unexplained death inepilepsy(60).As the RMSSD and pNN50 are completely based on comparisons between successive heart beats, they measure only short term variations in the N-N interval (68). An indicator of parasympathetic involvement in the circulatory system are the RMSDD, SDNN, and pNN50 which represent fast breathing associated beat-to-beat variations (18).

As per our knowledge, no studies determining the association between HRV and vitamin D level in healthy individuals in Saudi Arabia have been done. Therefore, this study aims to examine the correlation between vitamin D status and HRV among healthy female medical students at “King Abdulaziz University” (KAU) in Jeddah, Saudi Arabia. In so doing, we are testing the hypothesis, which states that vitamin D deficiency is associated with a decrease in

HRV.

2. Methods:

Ethical approval (number 44-15) was obtained from "The Unit of Biomedical Ethics Research Committee" at KAU, Faculty of Medicine in February 2015. This cross-sectional study was conducted at the research laboratory in the Physiology Department, Faculty of Medicine, KAU, Jeddah, Saudi Arabia. The study lasted for 3 months, from March to May 2015.

Sample size estimation was done using Epi-Info (version 6). Recruitment of the subjects was done following an announcement in the Faculty of Medicine, KAU. One hundred thirty-three young, Saudi (or born to a Saudi mother), apparently healthy adult females, medical or paramedical students at KAU, volunteered to participate in the study. The vitamin D levels for all the subjects were taken into consideration for the determination of vitamin D status per se.

A summary of the total number of subjects recruited, included and excluded is found in Figure 1.HR within the range of 60 to 100 beats per minute was considered for inclusion. The exclusion criteria were the presence of cardiac, renal, neurological and liver diseases or endocrine and malabsorption disorders as well as being on any type of regular medications except vitamin D, calcium and multivitamins. Males were not involved in the present study to avoid the impact of gender on the cardiovascular function. Further exclusion was done depending on the HRV recordings. Twenty-two of the subjects' HRV were excluded for technical errors in the HRV recordings. Moreover, nine of the subjects that were suffering from hypertension and mitral regurgitation or on regular medications like beta-blockers, ventoline, corticosteroids and anti-hypertensive medications were excluded.

All measurements were done in the physiology research laboratory. After a brief explanation about the aim and the method of the study, each volunteer signed a consent form(Appendix no. 1)and answered a questionnaire (Appendix no. 2). This was followed by anthropometric measurements. Each participant was weighed in kilograms using an electronic weighing machine (Cardinal DETECTO, No. 758C, No. E16005-0141 12VDC/300mA, U.S.A) while wearing light clothes and bare footed to the nearest 0.1 kg.The height was measured in centimeters using a stadiometer(Cardinal DETECTO, No. 758C, No. E16005-0141 12VDC/300mA, U.S.A). Each participant was barefooted and stood on the scale facing forward and the height was recorded to the nearest 0.1 cm. BMI was calculated by dividing the weight in kilograms by the height in meters squared. The waist of the subject was measured to the nearest 0.1 centimeter in the standing position using a stretch-resistant measuring tape at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest as stated by WHO recommendations (82).

HRV recording was done in the supine position and blood was withdrawn for the different biochemical determination. The difference in duration between the blood work and HRV was not more than 48 hours.

HRV was recorded using A ML870 power lab 8/30 (8 channels) and ML 408 Dual Bio Amp / Stimulator machine(AD Instruments, PowerLab 4/25T, Model number: ML865, Serial: T425-509, Australia). Power lab is a hardware device with a Chart 5 (v5.5 18) software, which controls the bio-amplifier that is connected to a computer. It can acquire, store, analyze and present the data.

HRV measurements were recorded in the following manner.

1. Each subject was asked to keep all electromagnetic appliances and metallic accessories away and then to lie down in a supine position.
2. The subject was connected to the power lab via 3 electrodes:

a) Positive electrode was attached to the left wrist.
b) Negative electrode was attached to the right wrist.
c) Ground electrode was attached to the right leg.

3. The subject was instructed to relax for five minutes.
4. The recoding took place in a complete quiet room with the following instructions given to each subject:

a) No movement or talking or sleeping is allowed.
b) No thinking of stressful topics.
c) Not to close the eyes. Blinking was allowed.

The computer software analyzed the HRV recordings under the frequency (LF, VLF, HF and TP) and time domain (MNN, SDNN, RMSSD, NN50) parameters. The report displays personal information, date of recording, average heart rate, time and frequency domain parameters of HRV.

Three test tubes with two ml of blood in each were obtained by phlebotomy. Each was centrifuged at 3000 rpm for 10 minutes and the serum obtained was separated and stored until the time of analysis. Serum levels of 25(OH)D were determined using an automated direct competitive chemiluminescent immunoassay that detects 25-OH vitamins D_2 and D_3 in serum or plasma (Siemens Advia Centaur Immunoassay System, Siemens Healthcare Diagnostics). Blood level of PTH was detected using immunoassay through direct chemiluminometric technology (Siemens Advia Centaur Immunoassay System, Siemens Healthcare Diagnostics). Blood levels of phosphate (Ph_i), creatinine, calcium, magnesium (Mg), albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), Alanine Aminotransferase (ALT) and Gamma-Glutamyl Transpeptidase (GGT) were determined using an

autoanalyzer (Siemens Dimension Vista System, Siemens Healthcare Diagnostics) at KAU Hospital Laboratory.

The Statistical Package for the Social Sciences (SPSS program version 16, Chicago, IL, USA) was used for data analysis. Data were screened for any entry mistakes using frequencies. Continuous variables were checked for normality using Shapiro-Wilk test. Log transformation was done to normalize those variables that were not normally distributed. Continuous data are presented as mean (Standard Deviation) if normally distributed and median (Inter Quartile Range) if not normally distributed. Categorical variables are presented as frequency (percentage). Student T-test was used to compare the means of different HRV measures and other variables between groups of vitamin D status and BMI groups. Spearman's correlation was used to assess the correlation between 25(OH) D and different HRV parameters. Chi-square test was used to examine the correlation between categorical variables. Multiple linear regression analysis was conducted to study the independent association between 25(OH)D and different HRV parameters adjusting for confounders. Log transformed variables were used in the model for those that were not normally distributed. For all comparisons a p-value <0.5 was considered as statistically significant.

3. Results:

A total of 124 apparently healthy female students (aged between 19-25 years old with a mean age of 21.7± Standard Deviation (SD) 1.1) participated in this study. Mean 25(OH)D for the study group was 31.2 nmol/L ± SD 16.9. HRV recordings were available for 102 subjects only. Table 3 presents anthropometric, biochemical and HRV parameters for the whole study group.

Table (3). Anthropometric characteristics, biochemical and HRV parameters of the study group.

	Mean(SD)	**Median(IQR)**
Anthropometric Measures	**n=124**	
Age (years)	21.7 ± 1.1	-
Height (cm)	157.2 ± 6.4	-
Weight (kg)	59.8 ± 14.7	-
BMI (kg/m^2)	24.1 ± 5.4	-
WC (cm)	76.9 ± 13.0	-
Biochemical measures		
PTH (pmol/L)	6.4 ± 3.1	6.2 (4.2-7.9)
25(OH)D (nmol/L)	31.2 ± 17.0	28.5 (16.0-41.8)
Ca (mmol/L)	2.28 ± 0.09	-
Ph_i (mmol/L)	1.06 ± 0.15	-
Mg (mmol/L)	0.834 ± 0.06	-
HRV parameters	**n= 102**	
HR (bpm)	77.5± 9.0	-
MeanNN (ms)	785±91	782 (726-845)
SDNN (ms)	48.6 ± 18.4	43.0 (34.6-60.5)
RMSSD (ms)	47.3 ± 25.1	41.2 (29.6- 59.9)
NN50	72.7 ± 58.0	56.0 (25.0-105.0)
TP (ms^2)	2472±1864	1795(1145-3472)
LF (ms^2)	580 ± 425	494 (224-740)
LF nu	35.4 ± 15.5	33.2 (24.3-44.1)
HF (ms^2)	1125± 1286	616 (348 -1692)
HF nu	53.1 ± 17.2	55.4 (40.7-65.7)
VLF (ms^2)	577±399	449 (321-662)
LF:HF	0.845 ± 0.67	0.639 (0.375-1.02)

Abbreviation: BMI= body mass index, WC= waist circumference, PTH= parathyroid hormone, 25(OH)D= 25-hydroxyvitamin D, Ca= corrected total calcium, Ph_i= inorganic phosphate, Mg= serum magnesium, HRV= heart rate variability, HR= average heart rate, Mean NN=mean N-N intervals of the entire ECG recording, SDNN= standard deviation N-N interval, RMSSD= root mean square of successive R-R interval difference, NN50= successive R-R interval differences measured in an entire ECG recording larger than50ms, TP= total power, LF ms^2= low frequency power, LFnu= low frequency power normalized, HF ms^2= high frequency power, HFnu= high frequency power normalized, VLF= very low frequency, LF:HF= low frequency power to high frequency power ratio. Values are expressed as mean ± standard deviation (SD).Median and interquartile range (IQR) are also presented for variables that were not normally distributed.

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The majority of the participants (76%) were medical students, 117 (94%) were Saudi, 56 (45%) were exercising regularly (14% of them often long enough to work up a sweat) and almost all were non-smokers (99%). Twenty subjects (16%) were taking vitamin D supplements, 26 (21%) were on multivitamins.

Vitamin D deficiency was highly prevalent among the study subjects. Almost 86% of them (n=106) had a 25(OH) D level that was below 50 nmol/L. Severe deficiency was evident in 12% of the subjects (n=15) with 25(OH)D levels below 12.5 nmol/L. Only 3 subjects (2.4%) had 25(OH)D levels above 75 nmol/L. Table 4 presents the prevalence of vitamin D deficiency among the study group using different cutoff points that are reported in the literature.

Table (4): Prevalence of vitamin D deficiency among the study group.

25(OH)D level	**N (%)**
<12.5 nmol/L	15 (12%)
<25nmol/L	51 (41%)
<37.5nmol/L	85 (68.5%)
<50nmol/L	106 (85.5%)
<75nmol/L	121 (98%)

Participants were split into 2 sub-groups according to the 25(OH) D level: those with 25(OH)D below 30 nmol/L and those above or equal to 30 nmol/L. This cut-off point was used as it approximated the median 25(OH)D level of the study group (28.5 nmol/L) and previous studies have shown that 25(OH)D level of 30 nmol/L was associated with increased incidence of cardiovascular events (83,84).

As expected PTH was significantly higher in the group with lower 25(OH)D level ($P<0.05$)(Table5).This negative correlation between 25(OH)D and PTH was statistically significant. There were no differences between corrected total calcium (Ca) and inorganic phosphate (ph_i) among the 2 vitamin D status groups, however, serum Mg was higher in the low vitamin D group and almost approached statistical significance (p=0.05).

Although not statistically significant, SDNN, RMSSD, and HF, which are indicators of the parasympathetic tone, were lower in subjects with 25(OH)D level below 30 nmol/L.

On the other hand, the mean LFnu (Low Frequency Power Normalised), a sympathetic activity indicator, was higher in subjects with 25(OH)D level less than 30 nmol/L(Figure 4.8).The overall sympathovagal balance represented by the LF:HF ratio was higher in the group where 25(OH)D level is less than 30 nmol/L. Nevertheless, none of these differences reached statistical significance.

None of the HRV parameters was significantly associated with any of the anthropometric or biochemical measures. Although there was a positive correlation between 25(OH)D and all HRV parameters that are regarded as indices of parasympathetic activity (SDNN, RMSSD, NN50 and HF), and negative correlation with indices of sympathetic activity (LF both absolute and normalized), none of these correlations reached statistical significance. Correlation between 25(OH) D and different HRV parameters was examined in the two 25(OH) D sub-groups (Figures 4.10-4.14). None of the correlations in any of the 2 groups was significant.

Table (5): Hormonal and biochemical characteristics of the study group according to the vitamin D status.

Variable	**25(OH)D ≥ 30 nmol/L n= 58**	**25(OH)D < 30nmol/L n= 66**	***p*-value***
PTH (pmol/L)	5.66 ± 2.79	7.07 ± 3.26	0.014
25(OH)D (nmol/L)	45.7 ± 13.3	18.5 ± 6.3	<0.0001
Ca (mmol/L)	2.28 ± 0.08	2.28 ± 0.09	0.545
Ph_i (mmol/L)	1.06 ± 0.13	1.06 ± 0.17	0.872
Mg (mmol/L)	0.822 ± 0.05	0.844 ± 0.07	0.050

Abbreviation: PTH= parathyroid hormone, 25(OH)D= 25-hydroxyvitamin D, Ca= corrected total calcium, Ph_i= inorganic phosphate, Mg= serum. Values are mean ± SD, * calculated by student T-test.

Since RMSSD is a definite index of parasympathetic activity, quartiles of RMSSD were calculated and a dichotomous variable was created with: 0= RMSSD values below the 25th percentile and 1 = RMSSD values above the 25th percentile. A total of 18 subjects (72%) with 25(OH)D < 30 nmol/L had RMSSD values below the 25thpercentile compared to only 7 subjects (28%) with 25(OH)D ≥30 nmol/L who had RMSSD values below the 25thpercentile(chi-square, P=0.049) (Table 6).

Multiple regression analysis (stepwise method) was performed to examine the independent association between 25(OH)D and the different HRV parameters. In addition to 25(OH)D, age, BMI, WC, PTH, total serum calcium (corrected for serum albumin), and exercise were entered as independent variables into the regression model. 25(OH)D was independently and negatively associated with LFnu, an index of sympathetic activity (Table 7).

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Table (6): Correlation between the vitamin D status and RMSSD in the study group.

Vitamin D Status	RMSSD	
	< 25th Percentile n (%)	≥ 25th Percentile n (%)
25(OH)D ≥ 30 nmol/L	7 (28%)	38 (51%)
25(OH)D< 30nmol/L	18 (72%)	37 (49%)

Abbreviation: 25(OH)D= 25-hydroxyvitamin D, RMSSD= root mean square of successive R-R interval difference (P=0.048)

Table (7): Associations of HRV parameters with 25(OH)D using multiple linear regression analyses in the study group

Dependant Variables	Predictor	B	SE	*p*-Value	R^2
LFnu	25(OH)D	-0.175	0.078	0.028	0.050

Abbreviation: B= unstandardized regression coefficient, SE= standard error, LFnu= low frequency power normalized, 25(OH)D= 25-hydroxyvitamin D.

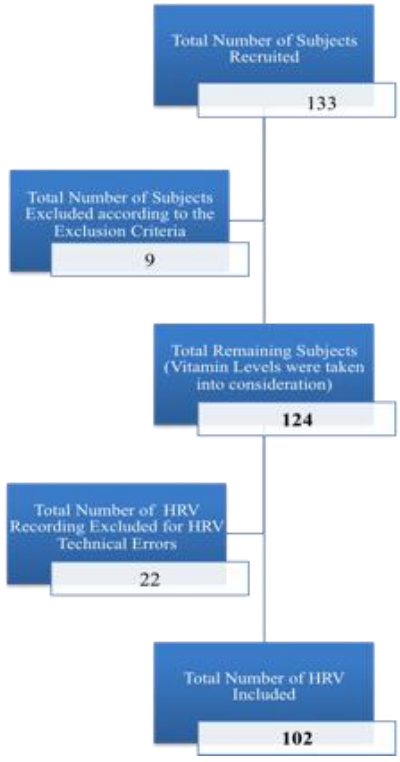

Figure (1): Summary of the total number of subjects recruited, included and excluded.

4. Discussion:

There is a paucity of studies addressing the association between vitamin D levels and HRV in healthy subjects. This study is one of the few to examine the relationship between vitamin D levels and cardiac autonomic function through HRV recordings. It is the first study to our knowledge to assess this relationship in apparently healthy Saudi Arabian women and the first in the Middle East.

Vitamin D [25(OH)D] deficiency and low HRV have been correlated to an increased risk for CVD. HRV is an adaptive physiological change in HR, modulated by both arms of the ANS. A low HRV is a marker for inadequate ANS changes in response to changing demands of the homeostatic mechanisms. A low HRV also increases the probability of cardiovascular complications in subjects with CVD. In healthy individuals without known CVD, low HRV is associated with an up to 45% increase in the risk of a cardiovascular event (17,86).

The main findings in the current study were: (i) vitamin D deficiency (defined as 25[OH]D level <30 nmol/L) was significantly associated with decreased RMSSD (an index of parasympathetic activity), (ii) 25(OH)D level was independently and negatively associated with LFnu (an index of sympathetic activity). These trends are consistent with current available evidence on the detrimental impact of low vitamin D status on cardiac autonomic function.

Vitamin D deficiency is a major health problem in the Saudi population that affects both sexes and different age groups. This has been associated with

different chronic diseases including cardiometabolic disorders. In the present study, vitamin D deficiency was highly prevalent among the study group (86% with 25(OH)D level <50 nmol/L), in accordance with the previously published literature.

Risk factors for vitamin D deficiency among Saudi females include reduced sun exposure. This may be due to traditional clothing, limited outdoor activities, high temperatures during the summer or deliberate avoidance for cosmetic reasons (87,88). Darker skin tone due to the protective effects of melanin; and obesity are also common risk factors. Although time spent outdoors and skin tone were not noted in our study population, 34% of the study subjects were either overweight or obese. Thus it is probably that obesity along with other factors may contribute to this high prevalence of vitamin D deficiency in our study.

Vitamin D deficiency has been associated with cardiac autonomic dysfunction, which can be assessed by HRV. In our analysis an association between vitamin D deficiency and reduced HRV was seen with reduced parasympathetic limb activity and increased sympathetic limb activity.

$1,25(OH)_2D_3$, easily crosses the blood brain barrier. The areas of the brain which control the ANS such as the brainstem have high percentage of VDRs. The activity of these VDRs seems to regulate the vagal and sympathetic tones. Due to the physiological role of vitamin D in the autonomic regulation of cardiac function, vitamin D deficiency has been demonstrated to be associated with increased pressor responses increasing the risk of CVD-related sudden arrhythmic cardiac death (81).

It is significant to note that apparently healthy individuals without any overt symptoms of CVD exhibit impaired cardiac autonomic function characterized by sympatho-vagal imbalance (12–14,81). In addition, normal levels of vitamin D_3 are required for the normal functioning of the aortic baroreceptors(89).

Moreover, Vitamin D has a non-genomic action on cardiac muscle calcium influx involving the regulation of the β-adrenergic –sensitive receptors. Thus, Vitamin D facilitates autonomic regulation from within the CNS, as well as, the heart. Administration of $1,25(OH)_2D_3$ might prevent or decrease the rate of progression of CVD(36).

The link between vitamin D deficiency and CVD is well documented (90). Studies in animals and humans have shown that vitamin D suppresses the RAAS (91). Vitamin D suppresses renin gene expression by binding to the gene promoter (92). Renin is a hormone that stimulates the production of angiotensin II - a strong vasoconstrictor the over stimulation of which can lead to vascular hypertrophy and hypertension (93). Angiotensin II also stimulates aldosterone, and facilitates sympathetic transmission (94). Therefore, in situations of vitamin D deficiency, renin, angiotensin II and aldosterone blood concentrations will be elevated, contributing to CVD. Low vitamin D status in persons with CVD have been shown to exacerbate the disease, leading to two-fold increased risk for a cardiovascular event (93). Recent evidence from clinical trials on diabetic patients points to enhanced parasympathetic function after 6 weeks of renin inhibition (95). This is in agreement with previous studies that found that high plasma renin activity was an independent determinant of reduced vagal tone (96). Taken all together, these findings can explain the low HRV seen in subjects with vitamin D deficiency.

The elevated levels of PTH in our vitamin D deficient group is also consistent with previous studies (13). However, the mean PTH among the study subjects was within the normal range and did not reach a pathological level. In addition to the role of PTH in regulating blood calcium and vitamin D concentrations, PTH acts on cardiomyocyte nuclear receptors causing cardiomyocyte hypertrophy, therefore, being a CVD risk factor (2,7,93).

The present study is the first among women living in Jeddah, Saudi Arabia and up to our knowledge, the first in the Middle East. Therefore, our findings were compared to similar international studies as no local studies were found in the published literature for comparison.

There are few studies addressing the relationship between HRV and vitamin D status among healthy individuals. Mann and co-workers (13) were the first to conduct a cross-sectional study, published in 2013, examining the association between 25(OH)D status and HRV among healthy subjects (n=34). Only frequency domain HRV parameters were reported. In contrast to our results, they found a significant suppression of sympathovagal balance (lower LF:HF ratio) in the vitamin D deficient group (n=7; defined as 25(OH)D≤ 50nmol/L). The small sample size (n=7) and the higher mean 25(OH)D level among their deficient group (38 nmol/L *vs.* 18.5 nmol/l in our study group), could explain the discrepancy. They also included subjects from both genders (with 5 females only in the deficient group *vs.* 58 female subjects in our study group), and older age (mean age 38 *vs.* 22 years old). Finally, the HRV recording was performed for a longer duration (90 minutes) than the 5 minutes HRV recording in our study. Nevertheless, in the same study, they also evaluated $1,25(OH)_2D_3$, and correlated it to HRV parameters at baseline and after a pressor challenge (graded angiotensin II infusion). They found that subjects in the lowest $1,25(OH)_2D_3$quartile had a lower parasympathetic activity during the angiotensin

II pressor test compared to those in the higher quartiles. They concluded that this withdrawal of the cardioprotective vagal tone may explain the increased cardiovascular risk in subjects with low vitamin D status.

Tak et al.(14), in a cross-sectional study on healthy Korean population (mean age 55 years, both sexes, with 73 females) showed that 25(OH)D level was positively and significantly associated with SDNN and RMSSD (parasympathetic indices), which is in general agreement with our findings of reduced parasympathetic tone in vitamin D deficient subjects. Surprisingly, they also reported a positive and significant correlation between 25(OH)D level the LF (sympathetic component). However, normalized LF was not reported to allow for proper comparison with our results. It is worth mentioning that the mean 25(OH)D of the study group was 53 nmol/L and that vitamin D deficiency was defined as a level below 37.5 nmol/L. Further, their groups were not equally weighted with the deficient group having less than half (n=50) the number of participants as the non-deficient (n=126) group. Their sample size was also larger (n=176), containing more males (n=103) than females (n=73). Tak*et al* also recruited older subjects with a mean age of 55.3 ± 11.7 years; another factor that can further affects HRV. While the HRV recoding time was the same, their subjects rested for longer time (30 minutes) before recording and didn't consume any caffeine; giving them more control over physiological processes that could affect HRV.

Findings reported by another cross-sectional study conducted in Turkey by Canpolat and coworkers (2014) were in agreement with our results(12). They recruited healthy subjects (n=74; both sexes with 34 females, mean age 37.5 years). Importantly, their control group was age, gender and BMI matched to the vitamin D deficient group (defined as 25(OH)D≤ 50 nmol/L). Consistent with our findings, Canpolat et al, found that serum 25(OH)D was positively and significantly correlated with parasympathetic parameters (RMSSD, PNN50 and HF nu) and negatively correlated with sympathetic parameter, namely LF nu and over all LF:HF ratio.

Jung et al.(97) have examined HRV status and cardiac autonomic neuropathy in patients with type 2 diabetes and vitamin D deficiency. In their study, (n=163, 84 males and 79 females, mean age: 56.8 years), vitamin D deficiency (25(OH)D<25 nmol/L) was significantly correlated with HRV parameters. Consistent with our findings, the vitamin D deficient group had significantly lower SDNN and RMSSD values and greater LF:HF compared to the vitamin D sufficient group (25(OH) D≥50 nmol/L). These findings confirm the association between vitamin D deficiency and low HRV in type 2 diabetes patients. They concluded that low vitamin D levels lead to an overall ANS imbalance (97).

Another cross-sectional study(11), examined the association between vitamin D levels and impaired cardiac autonomic function in chronic heart failure patients (n= 71) and healthy subjects (n= 25). Their control group included males (n=12) and females (n=13) but had more non-deficient participants (>50 nmol/L, n=21) than deficient (<50 nmol/L, n=4) participants. Their results showed that deficient subjects had higher SDNN and RMSSD values, though statistically insignificant. No frequency domain HRV indices were reported. The difference between these results and ours could be due to their smaller sample size, older study participants (mean age 56 ± 9 years old), and a higher 25(OH)D mean (84 ± 14.3).

Therefore, our findings support the hypothesis that decreased vitamin D levels affects the cardiovascular system and it is represented in cardiac autonomic inflexibility, which may increase the incidence of the CVD in otherwise healthy population. Table (8). represents a summary of the similar published studies on the association between vitamin D status and HRV in healthy and diseased subjects, including the current study results for comparison.

Table (8):Summary of the similar international studies correlating Vitamin D levels and HRV.

Year	Author	Sample Size	Mean Age	Mean 25(OH)D	Definition of Deficiency	Duration of HRV Recording	Main Significant Finding In Vitamin D Deficient Group
2016	Ayoub, et al.	124	21±1	21.7	<30 nmol/L	5 minutes	RMSSD 25(OH)D -ve correlation with LFnu
2015	Canpola et al (12)	74	N/A	N/A	≤ 50 nmol/L	24 hours	SDNN RMSSD PNN50 HF LFnu LF:HF 25(OH)D +ve correlation with RMSSD, PNN50 and HF nu -ve correlation with LF nu and LF:HF ratio
2015	Jung et al.(97)	163	56±11	37 ±7.5	<25 nmol/L	N/A	SDNN RMSSD LF:HF
2014	Cetin et al (11)	25 control	56 ±9	84 ± 14.3	≤ 50 nmol/L	24 hours	25(OH)D +ve correlation with SDNN.
2014	Tak et al (14)	176	55±11	53 ± 9.9	≤ 37.5 nmol/L	5 minutes	25(OH)D +ve correlation with SDNN, LF
2013	Mann et al (13)	34	38±2	71 ± 4	≤ 50 nmol/L	90 minutes	LF:HF

Abbreviations: N/A: not applicable, 25(OH)D= 25-hydroxyvitamin D, HRV= heart rate variability, SDNN= standard deviation N-N interval, RMSSD= root mean square of successive R-R interval difference, PNN50= proportion of successive R-R interval differences measured in an entire ECG recording larger than50ms, LF= low frequency power in ms^2, LFnu= low frequency power normalized, HF = high frequency power in ms^2, HFnu= high frequency power normalized, LF:HF= low frequency power to high frequency power ratio. Values are expressed as mean ± standard deviation (SD).

Limitations:

Although the present study was conducted on young and healthy females, to avoid the variation in HRV due to age, sex and comorbidities, our study has several limitations. The cross-sectional nature of the study leads to unsuccessful determination of the causal associations between vitamin D and HRV measures. Moreover, the small sample size and the volunteering nature of the sampling technique limit the generalizability to the whole population. Given the effect of hormonal changes during the menstrual cycle which may influence HRV (98), we did not account for the menstrual phase during our recruitment and HRV recordings. Furthermore, the questionnaire didn't include questions about autonomic function assessment such as postural hypotension or excessive sweating that would suggest autonomic imbalance (12) and we also didn't examine for cardiac autonomic dysfunction using the classic tests (99).Although our recording took place in a quiet room to avoid spikes of epinephrine due to sounds(101) we didn't determine the current psychological status of each student such as having an exam in an hour or just had the exam results. In that case, there would be higher sympathetic limb values affecting the actual effect of vitamin D level on the HRV.

Conclusion and future research:

This study is the first to address the association between vitamin D status and cardiac autonomic dysfunction as assessed by HRV, among a Saudi healthy population. Our findings are consistent with the hypothesis that vitamin D deficiency is associated with low HRV, a risk factor for CVD and all-cause mortality.

Future studies can be directed towards correction of vitamin D status with supplementation in the subjects of this study, and recording an improved HRV. This will consolidate the status of vitamin D as an independent risk factor for CVD. Further research is also required to know the exact molecular mechanisms through which vitamin D may affect the cardiovascular ANS in healthy asymptomatic subjects.Given the fact that both vitamin D deficiency and cardiovascular disorders are common health problems among the Saudi population, larger community based studies on both sexes and different age groups are needed to confirm our findings in healthy as well as diseased

individuals. Randomized clinical trials to assess the effects of vitamin D supplementation on cardiac autonomic function are also warranted. If the outcome of these trials is positive, correcting vitamin D status is a simple and inexpensive method to improve cardiovascular health and reduce mortality from cardiovascular events.

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