**Introduction**

More than 50% of the world’s population may have been infected with *Helicobacter pylori* (*HPY*), a micro-aerophilic and Gram-negative bacterium inhabiting the gastric mucosa. [1] *HPY* infection is associated with a higher risk of gastrointestinal disorders, such as dyspepsia, gastric and duodenal ulcer, and gastric cancer. [2,3] The disease may be present without symptoms for many years after primary infection. [1,4] Furthermore, *HPY* infection is mentioned to be associated with 25-OH-Vitamin-D3 (Vit.D3) absorption. We supposed that *HPY* disrupts the Vit.D3 absorption. We evaluated the association between Vit.D3 and anti-*HPY* immunoglobulins (Igs) and the Vit.D3 potency as a predictive biomarker for *HPY* infection.

**Abstract**

**Background:** *Helicobacter pylori* (*HPY*) provokes gastrointestinal disorders and gastric cancer. We supposed that *HPY* disrupts the 25-OH-Vitamin-D3 (Vit.D3) absorption. We evaluated the association between Vit.D3 and anti-*HPY* immunoglobulins (Igs) and the Vit.D3 potency as a predictive biomarker for *HPY* infection.

**Materials and Methods:** 603 patients’ raw data were gathered from a private clinical laboratory. Anti-*HPY* Igs including serum IgG, IgA, and IgM, in addition to *HPY*-stool antigen, were assessed by the immunoassay methods. Vit.D3 was determined by high-pressure liquid chromatography. Correlations, ordinal comparisons, cutoff points (COP), and odds ratio (OR) were estimated.

**Results:** The age mean ± standard deviation was 39.83 ± 18.426 for female and 38.82 ± 16.937 for male participants (*P* = 0.521). Significant correlations existed after age and gender adjustment between Vit.D3 serum levels and the *HPY* IgG (*R* = 0.298) and IgA (*R* = 0.271) but not for IgM (*R* = −0.103). Approximately, 48% of males and 36% of females had insufficient/deficient Vit.D3 serum levels (male/female OR: 1.65: 1.16–2.33; *P* = 0.0051). After age and gender adjustment, the best COP of Vit.D3 to predict an *HPY* IgG-positive patient was Vit.D3 >32.80 ng/mL with 66.23% diagnostic accuracy (DAAC), 30.43% specificity (SPC), and 90.41% sensitivity (SEN). For the *HPY* IgA, the values were Vit.D3 >37.83 ng/mL, DAAC = 60.45%, SPC = 58.82%, SEN = 64.20%. For *HPY* IgM, the values were Vit.D3 >37.32 ng/mL, DAAC = 58.97%, SPC = 57.33%, and SEN = 100%.

**Conclusions:** Vit.D3 had a good association with anti-*HPY* Igs and may be a good biomarker for immunity competence against *HPY* infection if the patient’s age and gender are considered when interpreting the laboratory results.

**Keywords:** Biomarker, cholecalciferol, chromatography, *Helicobacter pylori*, immunoglobulins
with special neurological disorders,[5–7] cardiac problems and dysfunctions,[8,9] hepatitis or immunodeficiency virus infections,[10–13] and so forth. As it is obvious, different disorders and involvements may be present with HPY infection, concurrently. Therefore, it is notable that HPY infection has a multifaceted feature that is affected by various causes and affects various disease processes.

1,25-dihydroxy-vitamin-D3 (Vit.D3), the active form of cholecalciferol or 25-OH-Vit.D3, has an immunomodulatory impact.[14,15] In addition to HPY-stool antigen (STHP), at least three types of antibodies are evaluated for diagnosis of HPY infection including immunoglobulin A (IgA), G (IgG), and M (IgM).[4,16] Therefore, in the present study, we are supposed to survey the association between HPY antibodies and Vit.D3 serum levels, considering the immunomodulatory impact of Vit.D3 and immunologic responses of the human body to HPY. If there is an association between HPY antibodies and Vit.D3 serum levels, theoretically, Vit.D3 status could be an accurate predictor of its infection. On the other hand, Vit.D3 is a fat-soluble vitamin that is absorbed from the gastrointestinal tract during the lipid emulsification and digestion process. In addition, the cholesterol that is the precursor of Vit.D3 is absorbed via the gastrointestinal tract and transported by lipoprotein particles throughout the reticuloendothelial system.[16,17] Hence, we assumed that HPY-infected persons may suffer Vit.D3 deficiency/insufficiency. In other words, if a patient has higher HPY antibodies or is positive for STHP, he/she may suffer low levels of Vit.D3. A published study (2019) has proposed that the relationship between Vitamin D and HPY remains to be fully elucidated.[18] Therefore, more evidence is necessary for confirmation of Vit.D3 role in controlling HPY infection or the effect of HPY on Vit.D3 serum levels. Results of this study will help clarify the probable association between Vit.D3 and HPY infection and prescribing proper therapeutics or considering protective strategies.

**Materials and Methods**

We have reviewed 1-year results of a private clinical laboratory in Tehran city, the capital of Iran. Stored data were surveyed from February to November 2017 in a blinded manner. The patient’s personal information was not added to the data repository. In total, 11,892 records were explored for data extraction from the laboratory database. All gathered data became blinded by removing the patient’s name, and only the test values were transferred into the statistical software. Figure 1 depicts the study steps and sample size in each step.

**Ethical aspects**

This study was done by data gathering and purification from patients’ records registered from a private clinical laboratory electronic reservoir, and there was not an approved proposal from a university. In addition, the researchers, in special, the data managers, had not any access to the patient’s characterization data, including the name and family name, inhabituation address, and any others, except the values of laboratory tests. The data source was provided by the quality manager of the laboratory with the permission of the laboratory technical manager. Therefore, only the laboratory results of each patient, without any informative data about the patient characterization, were delivered to data managers. We did not do an extra measurement on the patient’s samples, except at the physicians’ request. Only recorded data were included in the statistical analysis 3 years later.

**Vitamin D3 measurement using high-performance liquid chromatography**

To separate and determine Vit.D3, we used an HPLC system (KNAUER Co, Germany) and the previously described method by Lipkie et al.[19] in this method, the concentration of serum Vit.D3 was determined based on a standard curve plotted for the Vit.D3 to internatinal standard ratio; the internal standard was Vit.D3–D6 (Vitamin D3-26,26,26,27,27,27-d6). The area under the curve (AUC) was obtained from Vit.D3 measurement using high-performance liquid chromatography.

**Figure 1:** The flowchart of study steps and sample size from patient’s admission to test results. In total, 603 patients from a database containing 11,892 records of different tests were explored. We did not do any test except based on his/her physician request. Only data sets were considered that include the Vitamin D3 request. All 603 patients had at least serum Vitamin D3 and one *Helicobacter pylori* antibody or *Helicobacter pylori* antigen-requested tests.

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**Patient’s reception in clinical laboratory and taking samples**

- **Performing requested tests and saving the data**
  - 11,892 records (603 patients) were explored for data extraction from lab database.
  - VitD3 was assessed for all patients
  - 161 patients; stool antigen
  - 455 patients; Hpy-IgG
  - 268 patients; Hpy-IgA
  - 78 patients; Hpy-IgM

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and internal standard; these areas were used for the calculation of the ratio which was necessary for plotting the calibration curve and calculating each sample concentration. However, the presented data were based on an in-house setup. Briefly, to determine Vit.D3 levels, a whole blood sample was gathered from each patient, and serum separation was done using centrifugation. For Vit.D3 extraction, 100 µL of each patient’s serum was deproteinized by the addition of 50 µL methanol: 2-propanol (80:20 ratio) followed by 200 µL n-hexane mixed with internal standard (800 ng/mL final concentration). The mixture was mixed for 2 min and centrifuged for 15 min with 4500 G-force. Ten microliters of supernatant were injected into the HPLC system for the assessment of Vit.D3. Internal standard existed in extraction solution with a concentration equal to 800 ng/mL. Both Vit.D3 and internal standard were separated from the injected solution by an isocratic method; a reverse-phase C18 column was used and two different mobile phases were applied. Mobile phases composed of the acetonitrile: methanol and methanol: water (both 95%:5% v/v) with 0.7 mL/min flow rate and in a 50°C incubator. Vit.D3 separation was performed for 6 min while being monitored by a photometric system equipped with a Deuterium lamp at 265 nm to plot the separation curve. The limit of detection was 5 ng/mL, and the linear range was up to 500 ng/mL, with $R^2 = 0.999$. Coefficient of variation was 0.55% for the low control sample (mean target value = 22.30 ng/mL; standard deviation [SD] = 0.1229 ng/mL) and was 0.06% for the high control sample (mean target value = 186.59 ng/mL; SD = 0.1226 ng/mL). Figure 2 shows the sample chromatogram of the described method in our work showing separation time, AUC, and so on.

**Statistical analysis**

IBM SPSS Statistics for Windows, version 20 (IBM Corp, the Armonk, N.Y. area; USA) or MedCalc Statistical Software version 15.8 (MedCalc Software bvba, Ostend, Belgium statistical software) was used for obtaining descriptive statistics, mean ± SD, quartiles, comparisons, and correlations. Independent $t$-test or Mann–Whitney test were used for comparison between groups. The relationship between variables was examined using Spearman’s ρ correlation. Receiver-operating curve (ROC) analysis was used for evaluating the proper cutoff point (COP) and accuracy indices for each studied variable. Based on the defined reference ranges for HPY IgG, IgA, and IgM, the patients were divided into serum-positive or -negative individuals, and Vit.D3 serum levels were compared between categorized groups. For the estimation of the gender effect on the Vit.D3 serum levels, the odds ratio (OR) was calculated. At least a 95% confidence interval was considered for the determination of significant results. In addition to routine statistical methods, we have done age and gender adjustments to reduce the effect of confounding bias.

**Results**

In total, from 11,892 records, 603 patients from both genders, 413 females and 190 males, were included in the study. The age mean ± SD was equal to 39.83 ± 18.426 years in females and 38.82 ± 16.937 years in males ($P = 0.521$). STHP was assessed for 161 patients, 78 patients had IgM, 268 had IgA, and 455 had IgG test requests, of course, after primary clinical evaluation by physicians [Figure 1]. There were 51/104 (49%) females and 29/57 (50.9%) males with STHP-positive results ($P = 0.9535$), showing an insignificant frequency difference of HPY between the two genders. Vit.D3 serum levels were measured for all participants ($n = 603$) from which 80/161 patients (49.7%) were STHP positive. The STHP was assessed for 104 females (49% positive result) and 57 males (50.9% positive result). The patient’s gender was not a risk factor for the presence of HPY antigen in stool ($P = 0.823$; OR = 1.076; 0.564–2.054). Age- and gender-adjusted Vit.D3 mean ± SD was equal to 34.2 ± 7.69 ng/mL in STHP-negative patients and 37.51 ± 6.61 ng/mL in STHP-positive patients ($P = 0.004$).

**Vitamin-D3 status between male and female patients**

In our laboratory, for the HPLC method, Vit.D3 values lower than 30 ng/mL are considered insufficient or deficient, and the higher values are considered sufficient up to 150 ng/mL. Vit.D3 levels exceeding 150 ng/mL are defined as toxic in our laboratory reference range. Based on the 30 ng/mL COP, 47.9% of male and 35.8% of female individuals had insufficient/deficient Vit.D3 serum levels. Table 1 shows descriptive statistics of age and serum HPY IgG, IgA, and IgM levels among patients with Vit.D3 insufficiency/deficiency (upper part of the table) and sufficiency (down of the table). Figure 3 is a box plot for comparing male and female participants, which shows that females had significantly higher Vit.D3 mean values than males ($P = 0.005$).

**Anti-Helicobacter pylori immunoglobulins among Vitamin-D3-sufficient and -insufficient/deficient patients**

Detailed serum levels of anti-HPY Igs and Vit.D3 are reported in Table 1 (unadjusted values). The comparative box plot of age- and gender-adjusted HPY IgG, IgA, and IgM among Vit.D3-sufficient and -insufficient/deficient patients is shown in Figure 4. There was a significant difference between Vit.D3-sufficient and -insufficient/deficient participants for HPY IgG (adjusted mean ± SD in the Vit.D3-sufficient group = 90.17 ± 11.24 AU/mL and in the Vit.D3-insufficient/deficient group = 84.46 ± 10.19 AU/mL; $P < 0.001$) and HPY IgA (adjusted mean ± SD in the adjusted Vit.D3-sufficient group = 34.59 ± 10.7 AU/mL and in the Vit.D3-insufficient/deficient group = 30.26 ± 9.83 AU/mL; $P = 0.002$). Anyway, there was not a meaningful difference of IgM levels between Vit.D3-sufficient and -insufficient/deficient participants (adjusted mean ± SD in the sufficient group = 4.54 ± 0.72 AU/mL and in the insufficient/deficient group = 4.57 ± 0.82 U/mL; $P = 0.740$).

**Vitamin-D3 association with Helicobacter pylori antibodies and patient’s age**

To determine Vit.D3 association with HPY antibodies titer, we have assayed IgA, IgG, and IgM. Mean ± SD of HPY IgG, IgA,
and IgM was 87.89 ± 78.14 AU/mL, 32.92 ± 36.91 AU/mL, and 4.54 ± 4.18 U/mL, respectively. The mean ± SD Vit.D3 serum level was 37.85 ± 21.829 ng/mL. Based on the Spearman’s rho correlation [Table 2], there was not a significant association between Vit.D3 and HPY IgG before adjustment \((R = 0.017; P = 0.723)\). Anyway, a positive and significant correlation existed after adjustment \((R = 0.298; P < 0.001)\). Vit.D3 was positively and meaningfully correlated with HPY IgA both before \((R = 0.122; P = 0.0.046)\) and after \((R = 0.271; P < 0.001)\) adjustment. Vit.D3 serum levels were negatively associated with HPY IgM before \((R = −0.324; P = 0.004)\) but not after \((R = −0.103; P = 0.37)\) adjustment. The HPY-IgG and HPY-IgA were positively correlated before and after adjustment. Further, the HPY-IgM and HPY-IgA pairs were negatively correlated only after adjustment. Patient’s age, without any adjustment, was positively and significantly correlated with Vit.

D3 \((R = 0.283; P < 0.001)\), HPY IgG \((R = 0.233; P < 0.001)\), and IgA \((R = 0.439; P < 0.001)\) but not associated meaningfully with HPY IgM \((R = −0.155; P = 0.177)\).

**Vitamin D3 cutoff values that are representative of immunity status against Helicobacter pylori**

Vit.D3 serum levels were not statistically different between HPY IgG serum-positive and -negative individuals before age and gender adjustment \((P = 0.733)\) but were different after \((P < 0.001)\) adjustment [Figure 5a, dot plot]. Similar results were obtained for IgA before \((P = 0.121)\) and after \((P < 0.001)\) adjustment. However, the Vit.D3 serum levels were not considerably different between HPY IgM-positive and -negative patients both before (exact \(P = 0.981)\) and after (exact \(P = 0.215)\) adjustment.

To determine a cutoff value and diagnostic accuracy (DAAC) of Vit.D3 serum levels for anti-HPY Igs serum positive...
and negative, the ROC analysis was done. In the current study, the ROC analysis determines which Vit.D3 serum levels are usable for a physician to expect a sufficient immunologic response, i.e., IgG, IgA, or IgM production, against HYP infection. Note that to calculate the reported percentages and to do the ROC analysis, we have used the age- and gender-adjusted values. Unadjusted values showed insignificant results (data are not presented). As is shown in Figure 5, after age and gender adjustment, most patients with serum positive HYP IgG [Figure 5a], IgA [Figure 5b], and IgM [Figure 5c] had Vit.D3 levels ≥30 ng/mL (sufficient value). Only 7/271 (2.58%) HYP IgG-positive patients had Vit.D3 <30 ng/mL and the others (264/271; 97.42%) had Vit.D3 ≥30 ng/mL. In addition, 27/184 (14.7%) of HYP IgG-negative patients had Vit.D3 <30 ng/mL and the others (157/184; 85.3%) had Vit.D3 ≥30 ng/mL. There were 22/81 (27.16%) HYP IgA serum-negative patients with Vit.D3 <30 ng/mL, and the others (59/81; 72.84%) had Vit.D3 ≥30 ng/mL. There were not any HYP IgM serum-positive patients with Vit.D3 <30 ng/mL, and only three patients with HYP IgM serum-positive status had Vit.D3 ≥30 ng/mL (100%). Therefore, it seems that the sufficient immunologic response against HYP infection may be dependent on the Vit.D3 normal levels. Hence, using ROC analysis, we seek the best cutoff values of Vit.D3 in which the anti-HYP Igs are expected to be produced competently. Table 3 shows the results of ROC analysis when the HYP IgG, IgA, and IgM were considered positive or negative, and the Vit.D3 levels were evaluated for a proper cutoff value. Figure 5, right hand curves, shows the ROC curves for age- and gender-adjusted Vit.D3 levels.

Figure 4: Comparative box plot of age- and gender-adjusted Helicobacter pylori immunoglobulin G, immunoglobulin A, and immunoglobulin M among Vitamin D3-sufficient and -insufficient/deficient patients. There was a significant difference between Vitamin D3-sufficient and -insufficient/deficient participants for Helicobacter pylori immunoglobulin G (mean ± SD in the sufficient group = 90.17 ± 11.24 AU/mL and in the insufficient/deficient group = 84.46 ± 10.19 AU/mL; P < 0.001) and immunoglobulin A (mean ± SD in the sufficient group = 34.59 ± 10.7 AU/mL and in the insufficient/deficient group = 30.26 ± 9.83 AU/mL; P = 0.002) but not for immunoglobulin M (mean ± SD in the sufficient group = 4.54 ± 0.72 AU/mL and in the insufficient/deficient group = 4.57 ± 0.82 U/mL; P = 0.740). Note that the reported mean ± SDs are adjusted values and the comparisons were done using Mann–Whitney U statistical method. Unadjusted values are reported in Table 1. SD: Standard deviation.

Table 1: Descriptive statistics of Vitamin D3, Helicobacter pylori immunoglobulin G, immunoglobulin A, and immunoglobulin M serum levels among Vitamin D3-insufficient/deficient (upper part) and sufficient (lower part) patients; these data were not adjusted for age and gender.

| Statistics | Vitamin D3 (ng/mL) | HYP-IgG (AU/mL) | HYP-IgA (AU/mL) | HYP-IgM (U/mL) |
|------------|--------------------|----------------|----------------|----------------|
| n          | 239                | 180            | 103            | 35             |
| Mean±SD    | 19.58±6.86         | 88.08±77.54    | 29.31±31.31    | 5.02±3.12      |
| Median     | 20.00              | 59.25          | 17.90          | 4.70           |
| Minimum    | 5                  | 2.50           | 1.30           | 1.00           |
| Maximum    | 30                 | 200 and higher | 154.20         | 15.20          |
| Percentiles|                    |                |                |                |
| 25         | 14.70              | 16.05          | 9.90           | 2.80           |
| 50         | 20.00              | 59.25          | 17.90          | 4.70           |
| 75         | 25.90              | 187.50         | 36.00          | 6.60           |
| n          | 364                | 275            | 165            | 43             |
| Mean±SD    | 49.84±19.88        | 87.76±78.67    | 35.16±39.94    | 4.14±4.87      |
| Median     | 45.30              | 48.70          | 18.00          | 3.20           |
| Minimum    | 30                 | 2.20           | 2.40           | 1.10           |
| Maximum    | 206                | 200 and higher | 196.70         | 32.00          |
| Percentiles|                    |                |                |                |
| 25         | 36.95              | 17.10          | 10.15          | 1.90           |
| 50         | 45.30              | 48.70          | 18.00          | 3.20           |
| 75         | 56.35              | 194.60         | 48.25          | 4.60           |

SD: Standard deviation, IgA: Immunoglobulin A, IgG: Immunoglobulin G, IgM: Immunoglobulin M, HYP: Helicobacter pylori
The HPY IgG will be positive probably when the patient has Vit.D3 serum levels >32.80 ng/mL, and this cutoff value has 66.23% DAAC, 30.43% specificity (SPC), 90.41% sensitivity (SEN), 65.7% positive predictive values (PPV), and 68.3% negative predictive value (NPV). The HPY IgA will be positive probably when the patient has Vit.D3 serum levels >37.83 ng/mL, and this cutoff value has 60.45% DAAC, 58.82% SPC, 64.20% SEN, 40.3% PPV, and 79.1% NPV. The HPY IgM will be positive probably when the patient has Vit.D3 serum levels >37.32 ng/mL, and this cutoff value has 58.97% DAAC, 57.33% SPC, 100% SEN, 8.6% PPV, and 100% NPV. Note that in the ROC analysis, the results were significant for mentioned cutoff values after adjustment; however, before adjustment, only the result of Vit.D3 cutoff for HPY IgM were statistically meaningful.

## Discussion

In the current study, adjusted Vit.D3 serum levels significantly were higher in the STHP-positive group than in STHP-negative patients. One interpretation is that the HPY involvement may not be an interfering factor for Vit.D3 absorption via the gastrointestinal tract, although such a claim needs complementary evaluations. Anyhow, this assumption is rejected as the higher Vit.D3 levels existed in patients with higher HPY IgG, IgA, and IgM and STHP-positive patients. As the quality control manager of laboratory reports, there were a few patients with STHP-negative results whereas were positive in repeating the sampling and vice versa. Such evidence suggests that STHP may not enter into the feces continuously and during anti-HPY Iggs production. Nevertheless, this finding was only a signal in our work that necessitates the surveying and reporting the quality control criteria of STHP immunoassay methods. In the introduction, we have assumed that HPY-infected persons may suffer Vit.D3 deficiency/insufficiency. Vit.D3 serum levels were lower than normal values in approximately 48% of male and 36% of female participants. Based on the OR, the male patients have been at a higher risk of hypovitaminosis-D3 than females (1.65 folds; \( P = 0.00051 \)). Higher Vit.D3 levels among females could be due to the administration of complementary regimens prescribed by physicians for females in Iran, in previous years. Nowadays, Iranian females routinely use the Vit.D3 complementary drugs, and hence, their Vit.D3 serum levels are significantly higher than males. As our data show that 9/413 females (2.17%) and only 2/190 males (1.05%) had Vit.D3 serum levels upper than 100 ng/mL, anyhow, the maximum Vit.D3 serum level was reported for a male patient and was equal to 206 ng/mL. Furthermore, there were 107/413 (25.9%) females, but only 29/109 (15.26%) males had Vit.D3 levels upper than 50 ng/mL. Yet, evidence from the other parts of the world with different cultural and social structures showed insignificant differences between male and female for Vit.D3 levels. Han et al. have done a multicenter cohort study and declared that the most demographic factors, including age and gender, did not have a meaningful effect on the eradication of HPY infection by Vit.D3; for instance, indoor/outdoor occupation, body mass index (BMI), residential area (urban/town/rural), marital status, education level, family size, annual income, cigarette smoking, and hygiene evaluations (source of drinking water, periodontal disease, and so on). However, successful HPY infection eradication is less frequent in alcohol consumers.\[20\] In the current study, we did not consider such demographic factors, except the patient’s age and gender. Gender was a determinative factor in our study, and the females had higher levels of serum Vit.D3 levels than males. Therefore, it is expected that Iranian females benefit from Vit.D3-protective effects against HPY infection and subsequent gastrointestinal cancers compared with Iranian males. Notably, alcohol consumption is forbidden in Iran because of religious
instructions mentioned in the holy Quran and constitutional law. In addition, cigarette smoking and opium usage are highly limited among Iranian women based on cultural foundations, and most Iranian women hate behaviors that affect their pride, dignity, and beauty. Therefore, evaluating alcohol consumption or smoking among the Iranian population would be biased-prone for the study outcomes.

Considering the immunoregulatory role of Vit.D3, assessment of the association between Vit.D3 and a highly

Figure 5: Left side: Comparative dot-plot of age- and gender-adjusted Vitamin D3 serum levels between Helicobacter pylori immunoglobulin G (a), immunoglobulin A (b), and immunoglobulin M (c) positive or negative patients. The most Helicobacter pylori immunoglobulin G-, immunoglobulin A-, and all immunoglobulin M-positive patients had higher Vitamin D3 serum levels compared with immunoglobulin-negative patients. Using receiver-operating curve analysis, the best cutoff values were calculated for Vitamin D3 serum levels and receiver-operating curve immunoglobulins. Right side: The receiver-operating curve curves plotted to determine the best cutoff values and accuracy data of Vitamin D3 as a differential diagnosis/prediction biomarker for Helicobacter pylori infection. Details are presented in the body text.
prevailing infectious disease, i.e., *HPY* bacteria, is valuable medically/clinically; this was done in the presented study. Several reports suggested the association between viral or bacterial infections with Vit.D3. However, clinical associations between mentioned variables are not well known. In the current study, although there was not a significant association between Vit.D3 levels and *HPY* IgG, after age and gender adjustment, a positive and meaningful correlation was obtained. This finding is representative of the effect of age or gender or both on the Vit.D3 and IgG association. Vit.D3 serum levels were well correlated with *HPY* IgA, positively and meaningfully both before and after adjustment. Vit.D3 was associated with *HPY* IgM, negatively and significantly before adjustment, but not after adjustment. Positive and significant correlation results mean that when the higher Vit.D3 serum levels exist, the higher *HPY* IgG and IgA are expected. A negative and significant correlation means that when the higher Vit.D3 is present, the lower *HPY* IgG serum levels are expected. Therefore, we suggest the Vit.D3 serum level affects the immunologic responses to *HPY* in a way that the lower Vit.D3 levels predispose the patient to *HPY* infection which is manifested as an acute phase of disease and production of IgM and secretory IgA. Therefore, it can be suggested that immunologic responses to *HPY* are dependent on the patient’s age, gender, and immunoregulation by the Vit.D3. The immunoregulation effect of Vit.D3 and its related mechanism is well known and described in a review article by Di Rosa et al. Vit.D3 exerts many biological impacts such as innate immunity promotion, inflammation, and calcium and phosphorus metabolism, via complex signal transduction pathways.

As the age was associated with serum levels of Vit.D3, *HPY*-IgG, and *HPY*-IgA, positively and meaningfully, it can be concluded that the older patients, probably, are protected by this type of antibodies, because of previous infection and reactivation of memory T-cells and B-cells. Published works insist on the role of Vit.D3 in the regulation of the immune cells’ function in various diseases. Han et al. and Yang et al. have shown that positive *HPY* patients had lower Vit.D3 levels than negative *HPY* individuals. Using *HPY* urea breath tests, they have shown that Vit.D3 levels >10 ng/mL have a role in the eradication of this infection. Eradication rate was 71.7% in patients with Vit.D3 <10 ng/mL and 87.3% in those with Vit.D3 >10 ng/mL. These findings confirm that hypovitaminosis-D3 predisposes the patient to *HPY* infection. In the present study, we have seen that Vit.D3 levels were well correlated with *HPY*-IgG and IgA blood concentrations, after age and gender adjustment. Nevertheless, IgM blood level was negatively well correlated with Vit.D3 levels only before adjustment. Therefore, our results confirm the findings by Han et al. and Yang et al.

In the introduction, we have stated that the gastrointestinal tract is the site of the Vit.D3 absorption, and accordingly, we supposed that the patients with *HPY* infection may suffer Vit.D3 deficiency/insufficiency. Thus, the question was: whether

### Table 3: Diagnostic accuracy criteria calculated by receiver-operating curve analysis for Vitamin D3 levels in *Helicobacter pylori* immunoglobulin A and Helicobacter pylori immunoglobulin M serum-positive or -negative patients

| Serum Factor | n | Positive | Negative | AUC | Cutoff (95% CI) | P | Sensitivity% | Specificity% | NPP% | PPV% |
|--------------|---|----------|----------|-----|----------------|---|-------------|-------------|------|------|
| IgG (AU/mL)  | 455 | Positive | 271 (59.50) | 184 (40.44) | 0.668 (66.23) | <0.001* | 0.644 (60.45) | 0.573 (45.48) | 0.573 (45.48) | 0.644 (60.45) |
| IgA (AU/mL)  | 228 | Positive | 81 (35.20) | 147 (64.80) | 0.644 (60.45) | <0.001* | 0.682 (51.46) | 0.582 (51.46) | 0.682 (51.46) | 0.582 (51.46) |
| IgM (U/mL)   | 78  | Positive | 3 (3.15) | 75 (96.85) | 0.670 (58.97) | <0.001* | 0.851 (73.3) | 0.731 (65.04) | 0.851 (73.3) | 0.731 (65.04) |

* n: sample size, CI: Confidence interval, AUC: Area under curve, PPV: Positive predictive value, NPV: Negative predictive value, HPY: *Helicobacter pylori*.

* Significant result, For accuracy estimations the online tool is used: https://www.omnicalculator.com/statistics/accuracy. IgA: Immunoglobulin A, IgG: Immunoglobulin G, IgM: Immunoglobulin M.
the HPY infection affects the Vit.D3 serum levels. Present results showed that patients with sufficient values of Vit.D3 serum levels had lower IgM serum concentrations compared with insufficient/deficient Vit.D3 individuals. IgM is an acute phase-specific antibody, and therefore, the response to the question is positive; the patients suffering acute-phase HPY infection may encounter digestive problems, resulting in disturbed Vit.D3 absorption. Vit.D3 malabsorption from the gastrointestinal route results in its low serum level and reduced innate and acquired immunity responses.\textsuperscript{[33]}

Therefore, it is supposed that the Igs related to the early stage of the disease, i.e., IgM and the IgA, may not be sufficiently produced. Anyhow, the demonstration of Vit.D3’s effects on the physiology and pathology of the diseases is not simple because of the pathways’ complexities. Therefore, molecular studies are needed to obtain confirmatory results for the clinical evidence. Mut Surmel\textit{ et al.} evaluated the patients with gastric biopsy and categorized them into HPY-positive or HPY-negative. They have shown that the HPY-positive patients had Vit.D3 deficiency compared with HPY-negative individuals. Furthermore, they have reported increasing odds of HPY infection and Vit.D3 deficiency, after adjustment for age and gender.\textsuperscript{[18]} In the current study, we have reported significant HPY-IgG and IgA differences after age and gender adjustment where Vit.D3-sufficient individuals had higher levels of these antibodies than deficient/insufficient patients [Figure 4].

Still, most investigators have not considered the problem in a reverse direction, i.e., the effect of HPY infection on the Vit.D3 metabolism. We suggest this problem be evaluated in detail using molecular techniques, tissue culture models, and tracing methods. It should be noted that Mut Surmel\textit{ et al.} have evaluated patients aged 65 and over,\textsuperscript{[18]} whereas we have not eliminated patients based on age. Further, we have interpreted the results based on the three different Igs, i.e., IgG, IgA, and IgM, in addition to HPY coproantigen, although not for all studied patients.

Evidence from molecular and animal models has shown that Vit.D3 could suppress the MAPK signaling pathway and inhibit colitis or even colon cancer.\textsuperscript{[34,35]} Vit.D3 induces Paneth cell defensins that are effective for maintaining the gut microbiota in animal models.\textsuperscript{[36]} In mice models, the deficiency of Vit.D3 upregulated protein-1 promotes HPY-induced gastric cancer. Researchers have suggested when HPY tumorigenesis is occurring, the TNF-alpha, NF-kappa-B, and PTGS-2 signaling pathways could be targeted by the Vit.D3.\textsuperscript{[37]} As it is obvious from animal, cellular, and molecular studies, the inflammatory pathways are involved in the HPY infection, and Vit.D3 targets them. Totally, the results of the current and other evidence by investigations emphasize the medical and clinical impacts of the Vit.D3 through the signaling and immunomodulatory pathways.

Figure 4 is plotted for HPY-IgG, IgA, and IgM based on the adjusted data and shows a significant difference between participants with sufficient and insufficient/deficient Vit.D3 serum levels for IgG and IgA, but not for IgM. Accordingly, the assessment of Vit.D3 is suspected to be valuable for HPY infection differential diagnosis or prediction. Therefore, this question existed: Does the Vit.D3 status have differential diagnostic or predictive accuracy, as a determinative biomarker for HPY infection. In addition, what are the best COPs of Vit. D3 to predict HPY serum-positive and -negative patients? Based on the ROC analysis, after age and gender adjustment, the best cutoff values of Vit.D3 were >32.80 AU/mL, >37.83 AU/mL, and >37.32 U/mL for diagnosis of anti-HPY IgG, IgA, and IgM, respectively. Based on these cutoff values, the Vit. D3 levels lower than 32 AU/mL are expected or be reported for HPY IgG- and IgA-negative patients. In addition, Vit.D3 levels upper than 38 U/mL may be reported by the clinical laboratories for HPY IgM-negative patients, considering the negative correlation between Vit.D3 and HPY IgM, but without adjustment. We think that there are not enough studies to clarify the role of Vit.D3 in the differential diagnosis of HPY IgG-, IgA-, and IgM-positive and -negative patients. Hence, we propose that the other researchers gather more evidence by repeating the ROC analysis on the Vit.D3 level association with anti-HPY Igs.

**Conclusion**

A simple interpretation for the sum of the results is that the Vit.D3 deficiency/insufficiency predisposes patients to the HPY infection, whereas an individual may have sufficient Vit.D3 but become infected with HPY. Age and gender are important confounding factors and should be considered when interpreting the anti-HPY human serum antibodies. Vit.D3 may have a role in the prevention of and defense against HPY infection, probably via immunomodulatory and immunoregulatory functions. Therefore, Vit.D3 status could be an applicable biomarker for HPY infection predictor for clinicians, both diagnostically and clinically. In summary, Vit. D3 is well associated with anti-HPY Igs and could be a valuable biomarker for immunity competence during the disease. Proposed COPs of Vit.D3 serum levels to consider a patient as HPY positive or negative are suggested to be reassessed and reevaluated by other researchers to establish more evidence for reaching the consensus diagnostic ranges.

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**Conflicts of interest**

There are no conflicts of interest.
Radiology, Gastroenterology, Immunology, and Virology

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