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ORIGINAL ARTICLE

Chlamydia pneumoniae and *Helicobacter pylori* IgG seropositivities are not predictors of osteoporosis-associated bone loss: a prospective cohort study

Mohammad Reza Kalantarhormozi[1] · Majid Assadi[2] · Katayoun Vahdat[3] · Kamyar Asadipooya[4] · Afshin Ostovar[3] · Katayoun Raissi[1] · Hossein Darabi[3] · Shokrollah Farrokhi[3] · Sina Dobaradaran[3] · Maryam Farrokhnia[5] · Iraj Nabipour[5,6]

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Abstract The potential link between infection with *Chlamydia pneumoniae* or *Helicobacter pylori* and osteoporosis has not been investigated in population-based longitudinal studies. A total of 250 healthy postmenopausal women who participated in a prospective cohort study were evaluated for IgG antibodies directed against *C. pneumoniae* and *H. pylori*, osteoprotegerin (OPG), the receptor activator of nuclear factor kappa B ligand (RANKL), CrossLaps, and osteocalcin. Bone mineral density (BMD) was measured at the femoral neck and lumbar spine at baseline and at follow-up 5.8 years later. There were no significant differences in age-adjusted bone turnover markers, OPG, RANKL, the RANKL/OPG ratio, and BMD between the *C. pneumoniae* and *H. pylori* IgG seropositive and seronegative subjects ($P > 0.05$). Neither *C. pneumoniae* nor *H. pylori* IgG seropositivity was associated with age-and body mass index-adjusted BMD at the femoral neck and lumbar spine or bone loss at the 5.8-year follow-up. In logistic regression analysis, neither *C. pneumoniae* nor *H. pylori* IgG seropositivities predicted incident lumbar or spine osteoporosis 5.8 years later. In conclusion, neither *C. pneumoniae* nor *H. pylori* IgG seropositivity was associated with bone turnover markers, the RANKL/OPG ratio, BMD, or bone loss in postmenopausal women. In addition, chronic infection with *C. pneumoniae* or *H. pylori* did not predict incident osteoporosis among this group of women.

Keywords *Chlamydia pneumoniae* · *Helicobacter pylori* · Bone mineral density · Osteoporosis

Introduction

Chlamydia pneumoniae is an obligate intracellular human respiratory pathogen that contributes to a wide spectrum of clinical presentations, including atherosclerosis [1]. This bacterium is able to survive in host cells and can affect chronic processes, such as atherosclerosis, through augmentation of the inflammatory system, signaling pathways, and oxidative stress [2]. Thus, it is plausible to consider a contributory role for *C. pneumoniae* infection in accelerated bone loss. Indeed, in vitro and in vivo studies have shown that infection with *C. pneumoniae* produces potentially inflammatory and bone resorptive cytokines and chemokines [3, 4]. Very recently, it has been reported that chlamydial DNA was found in osteoporotic bone tissue [5].

The association of *Helicobacter pylori* seropositivity with a variety of extradigestive manifestations, such as cardiovascular, immunological, and various other pathologies, has been suggested [6]. However, the link between *H. pylori* and these extradigestive manifestations is

✉ Iraj Nabipour
inabipour@gmail.com

1 Department of Endocrine and Metabolic Diseases, The Persian Gulf Tropical Medicine Research Centre, Bushehr University of Medical Sciences, Bushehr, Iran

2 The Persian Gulf Nuclear Medicine Research Centre, Bushehr University of Medical Sciences, Bushehr, Iran

3 Department of Infectious Diseases, The Persian Gulf Tropical Medicine Research Centre, Bushehr University of Medical Sciences, Bushehr, Iran

4 Division of Endocrinology and Metabolism, University of Kansas Medical Center, Kansas City, KS, USA

5 Department of Biochemistry, The Persian Gulf Marine Biotechnology Research Centre, Bushehr University of Medical Sciences, Bushehr, Iran

6 The Persian Gulf Tropical Medicine Research Center, Boostan 19 Alley, Imam Khomeini St, 7514763448 Bushehr, Iran

controversial [7]. There are also limited contradictory studies regarding the relationship between osteoporosis and infection with *H. pylori* [8–11]. Infection with *H. pylori* in children was not associated with significant changes in bone metabolism [8]. However, infection by CagA-positive *H. pylori* strains was more prevalent in osteoporotic men [9]. Reduced levels of estradiol and increased bone turnover markers were also found in infected CagA-positive men with osteoporosis [9].

Given the contradictory data on the association between *H. pylori* infection and bone metabolism [8–11] and the very limited studies on *C. pneumoniae* infection and osteoporosis in humans [10], more studies are required to clarify the involvement of chronic infection with these organisms in bone health. The aim of this prospective, population-based study was to evaluate the potential link between infections with *H. pylori* and *C. pneumoniae* and bone mineral density (BMD) in a sample of postmenopausal women.

Materials and methods

Community sampling

In an extension arm of the Iranian Multicentral Osteoporosis Study, a community-based longitudinal study, an age-stratified random sample of postmenopausal women was randomly selected from 13 clusters in Bushehr Port (the center of Bushehr province, which has the longest border with the Persian Gulf). All were community-dwelling and ambulatory. The following exclusion criteria were used: (1) the known presence of generalized bone diseases, including hyperparathyroidism, hypoparathyroidism, thyroid disorders, rheumatoid arthritis, Cushing's disease, and steroid-induced osteoporosis, renal osteodystrophy, or other metabolic diseases; (2) a history of malignant diseases and liver diseases; (3) drug addiction; and (4) restriction to bed rest within the last 2 weeks after an illness or complete bed rest for 3 months. The baseline examination took place 4 April to 22 September 2006, and the follow-up examination occurred at a median of 5.8 years. The study design was described previously in detail [12].

The participants in the current study were a subset of 250 postmenopausal women who participated in the extension arm of the Iranian Multicentral Osteoporosis Study.

Physical examinations

A stadiometer was used to measure the subjects' height and weight. Heavy outer garments and shoes were removed before the participants' height and weight were measured. Their body mass index (BMI) was calculated. Waist circumference was defined at the midway level between the costal margins and the iliac crests. Hip circumference was measured at the level of the greater trochanters.

Bone mineral density (BMD) was determined for the lumbar spine (L2–L4) and proximal femur (neck) using dual-energy X-ray absorptiometry on an Osteocore II bone densitometer (Osteocore II Osteodensitometer; Medilink, France). To eliminate operator discrepancies, the same operator tested all the women during the study. Duplicate measurements were obtained from 30 women who agreed to undergo a repeat assessment on the same day, and the precision errors were calculated using the root mean square method. The coefficients of variation (CVs; precision) of the measurements of the lumbar spine and the femoral neck were 0.8 and 1.6 %, respectively.

Laboratory measurements

A fasting blood sample was taken. All the samples were promptly centrifuged and separated, and analyses were carried out at the Persian Gulf Health Research Center on the day of blood collection using a Selectra 2 autoanalyzer (Vital Scientific, Spankeren, The Netherlands).

IgG antibodies against *C. pneumoniae* were measured by a commercial test kit (DRG Instruments GmbH, Germany). The kit is based on an indirect solid-phase enzyme immunoassay with horseradish peroxidase as a marker enzyme; the positivity threshold was enzyme immunounits >45. Sera were screened for IgG antibodies against *H. pylori* with an ELISA kit (Radim SpA, Italy), and the samples were considered positive when IgG values were higher than 30 RU/mL for *H. pylori*.

Serum osteoprotegerin (OPG) levels were measured using an ELISA commercial kit (Biomedica Gruppe, Vienna, Austria). The detection limit of the assay was 0.14 pmol/L. The mean intra-assay and interassay coefficients of variation of the OPG assay were 4–10 % and 7–8 %, respectively.

The receptor activator of nuclear factor-κB ligand (RANKL) levels was measured using an ELISA with an additional enhancement system (ampli-sRANKL; Biomedica Gruppe). The detection limit of the assay was 0.4 pg/mL. The mean intra-assay and interassay CVs of the RANKL assay were 8–9 % and 6–3 %, respectively.

The N-MID osteocalcin ELISA (Nordic Bioscience Diagnostics A/S) was used for the quantitative measurement of osteocalcin in sera. The intra-assay CVs for the low (7.0 ng/mL), medium (21.8 ng/mL), and high (43.2 ng/mL) values were 3.4 %, 2.0 %, and 2.4 %, respectively.

The serum CrossLaps enzyme-linked immunosorbent assay (Nordic Bioscience Diagnostics A/S, Herlev, Denmark) was used for the quantification of degradation products of C-terminal telopeptides of type I collagen in sera. The intra-assay CVs for low (0.242 ng/mL), medium

(0.375 ng/mL), and high (0.476 ng/mL) values were 5.4, 5.0, and 5.1 %, respectively.

Serum alkaline phosphatase was determined by spectrophotometry using p-nitrophenylphosphate as substrate (ParsAzemon, Tehran, Iran). The intra-assay and interassay CVs were 1.5 % and 2.6 %, respectively.

Statistical methods

Normal distribution of the data was controlled with the Kolmogorov–Smirnov test. The significance of the difference in the results of any two groups was determined by Chi square analysis using 2 × 2 contingency tables for categorical variables. A two-tailed *t* test was used to compare the mean values across groups. We found that log transformation of osteocalcin, CrossLaps, sRANKL, and OPG gave a better fit to a Gaussian distribution. The geometric mean for those biochemical variables was defined as the arithmetic mean of the log-transformed data ±SD, raised to the power of 10.

Annual bone loss was defined as the change in BMD between the initial and second BMD measures, divided by the time interval (year) between the baseline and follow-up measurements.

Multiple linear regression models were used to assess the association between BMD at a number of skeletal sites (or bone loss) and IgG seropositivities against *C. pneumoniae* or *H. pylori*. The models were adjusted for age and BMI as covariates.

Binary logistic regression was used to compute the relative risks of incident osteoporosis at any skeletal sites for individuals with *C. pneumoniae* or *H. pylori* infection, controlled for age and BMI.

Probability values <5 % were considered statistically significant. All statistical analyses were performed using the PASW Statistics GradPack 18 (SPSS Inc., Chicago, IL).

Results

Table 1 shows the baseline characteristics of the studied postmenopausal women, stratified by IgG seropositivities against *C. pneumoniae* and *H. pylori*. The mean age (mean ± SD) of the women was 58.87 ± 8.02 years. The prevalence of IgG antibodies against *C. pneumoniae* and *H. pylori* among the studied population was 20.4 % (51 women) and 57.2 % (143 women), respectively.

There were no significant differences in age, anthropometric measures, bone turnover markers, markers of bone metabolism, and BMD between *C. pneumoniae* IgG seropositive and *C. pneumoniae* IgG seronegative subjects (Table 1, $P > 0.05$). Similar results were found

Table 1 The baseline characteristics of the studied postmenopausal women, stratified by IgG seropositivities against *C. pneumoniae* and *H. pylori*

	C. pneumoniae IgG			*H. pylori IgG*		
	Positive	Negative	*P* value	Positive	Negative	*P* value
Age, years	59.61 ± 8.78	58.68 ± 7.82	0.464	58.24 ± 7.55	59.88 ± 8.61	0.114
Waist circumference, cm	99.25 ± 12.34	99.19 ± 10.19	0.970	99.39 ± 9.90	98.95 ± 11.54	0.745
Body mass index, kg/m^2	28.07 ± 5.23	28.32 ± 4.60	0.738	28.52 ± 4.57	27.93 ± 4.92	0.338
Waist-to-hip ratio	0.92 ± 0.08	0.92 ± 0.06	0.752	0.92 ± 0.06	0.92 ± 0.07	0.588
Osteoprotegerin, pmol/L[b]	3.31 ± 1.82	3.66 ± 1.51	0.159	3.41 ± 1.58	3.84 ± 1.55	0.046
RANKL, pmol/L[ab]	1.62 ± 2.60	1.39 ± 3.27	0.409	1.41 ± 2.89	1.44 ± 3.51	0.916
RANKL/OPG ratio	0.41 ± 1.06	0.88 ± 1.04	0.330	0.75 ± 1.12	0.73 ± 1.05	0.899
Osteocalcin, ng/mL[b]	12.99 ± 2.23	11.62 ± 1.95	0.309	11.39 ± 1.76	12.62 ± 2.33	0.260
CrossLaps, ng/mL[b]	1.54 ± 1.65	1.72 ± 1.77	0.204	1.75 ± 1.69	1.59 ± 1.83	0.195
Alkaline phosphatase, U/L	242.41 ± 101.23	243.54 ± 94.76	0.940	237.86 ± 87.36	250.57 ± 107.53	0.307
Calcium, mg/dL	9.88 ± 0.46	9.82 ± 0.59	0.495	9.86 ± 0.54	9.79 ± 0.58	0.339
Phosphorus, mg/dL	3.90 ± 0.50	3.93 ± 0.50	0.680	3.92 ± 0.47	3.92 ± 0.53	0.979
Femoral neck BMD, g/cm^2	0.837 ± 0.182	0.856 ± 0.201	0.534	0.874 ± 0.206	0.823 ± 0.183	0.046
Lumbar BMD, g/cm^2	0.962 ± 0.230	0.951 ± 0.181	0.728	0.968 ± 0.184	0.931 ± 0.202	0.139

Data are given as means ± SD

BMD bone mineral density

[a] Receptor activator of nuclear factor-κB ligand

[b] Geometric mean ± SD

when *H. pylori* IgG positive postmenopausal women were compared with *H. pylori* negative subjects, except for marginal significant differences in serum OPG levels and BMD a the femoral neck (Table 1, $p > 0.05$). However, these significant differences were lost by adjusting for age.

BMD at the lumbar spine and femoral neck decreased progressively with increases in age ($P < 0.0001$). Annual bone loss was 0.82 % at the femoral neck and 3.55 % at the lumbar spine among the women.

Cross-sectional analysis

Neither *C. pneumoniae* nor *H. pylori* IgG seropositivities was associated with baseline age-adjusted and BMI-adjusted BMD at the femoral neck and lumbar spine (Table 2). At baseline, according to the World Health Organization (WHO) criteria [13], 12 (4.8 %) and 16 (6.4 %) women (9.2 %) were considered osteoporotic at the femoral neck and lumbar spine, respectively. *C. pneumoniae* and *H. pylori* infections were not associated with prevalent osteoporosis at the lumbar spine or the femoral neck (Table 3).

Prospective analysis

Baseline *C. pneumoniae* and *H. pylori* IgG seropositivities were not associated with age-adjusted and BMI-adjusted BMD at the femoral neck and lumbar spine during the 5.8-year period (Table 2). During the 5.8-year follow-up period, 19 (4.8 %) and 16 (6.4 %) women who were not osteoporotic at baseline developed osteoporosis at the femoral neck and lumbar spine, respectively. In logistic regression analysis, neither *C. pneumoniae* nor *H. pylori* IgG seropositivities predicted incident lumbar or spine osteoporosis after 5.8 years (Table 3).

Table 2 Multiple linear regression analysis for the association between bone mineral density (BMD) and IgG seropositivities against *C. pneumoniae* or *H. pylori* in the cross-sectional and prospective phases of the study

	Femoral neck BMD		Lumbar BMD	
	β	*P*	*β*	*P*
Cross-sectional analysis				
Chlamydia pneumoniae				
Unadjusted	−0.03	0.534	0.02	0.728
Age-, and BMI-adjusted	−0.01	0.747	0.04	0.338
Helicobacter pylori				
Unadjusted	0.12	0.046	0.09	0.139
Age-adjusted, and BMI-adjusted	0.07	0.135	0.04	0.385
Prospective analysis				
Chlamydia pneumoniae				
Unadjusted	−0.01	0.82	0.06	0.403
Age-adjusted, and BMI-adjusted	0.02	0.699	0.06	0.399
Helicobacter pylori				
Unadjusted	0.19	0.03	0.06	0.419
Age-adjusted, and BMI-adjusted	0.13	0.055	0.05	0.514

BMD bone mineral density, *BMI* body mass index

Discussion

We found that IgG seropositivities against *C. pneumoniae* and *H. pylori* were not associated with baseline bone metabolism and BMD at any skeletal sites among postmenopausal women. Chronic infection with these organisms was not involved in bone loss at 5.8-year follow-up. *C. pneumoniae* and *H. pylori* IgG seropositivities were not predictors of incident osteoporosis in postmenopausal women.

Table 3 Prediction of osteoporosis by IgG seropositivities against *C. pneumoniae* or *H. pylori* using multivariable odds ratios (OR), relative risk (RR) and their 95 % confidence intervals (CI)

	OR	95 % CI	*P* value
Cross-sectional analysis			
Chlamydia pneumoniae	1.16	0.37–3.50	0.796
Helicobacter pylori	1.02	0.39–2.55	0.997
	RR	95 % CI	*P* value
Prospective analysis			
Chlamydia pneumoniae	1.12	0.52–2.38	0.766
Helicobacter pylori	0.965	0.51–1.80	0.910

For the first time, Bailey et al. [3] demonstrated generalized bone loss associated with increased IL-6, IL-1, and T-cells that expressed RANKL due to *C. pneumoniae* infection in mice. They suggested a causal linkage between *C. pneumoniae* and bone loss following their in vitro study, which showed similar bone resorptive cytokine profiles as those in vivo in a *C. pneumoniae*-infected osteoblast cell line [3].

Rizzo et al. [4] showed that *C. pneumoniae* was able to invade and survive within an SaOS-2 osteoblastic cell line. They also suggested a pathological role of *C. pneumoniae* in generalized bone loss following their observation that infected osteoblasts produced increased levels of proinflammatory cytokines and chemokines [4].

Very recently, Di Pietro et al. [5] evaluated 32 women with osteoporosis and 27 with osteoarthritis who underwent hip joint replacement surgery for femoral neck fracture to determine whether *C. pneumoniae* infection is a risk factor for osteoporosis-associated bone loss. They found chlamydial DNA in osteoporotic bone tissue but not in non-osteoporotic bone tissue [5]. In addition, they detected a significant association between the presence of *C. pneumoniae* DNA, both in bone tissue and in peripheral blood mononuclear cells of osteoporotic women, and an increase in the sRANKL/OPG ratio and circulating resorptive cytokines [5]. Hence, Di Pietro et al. [5] considered *C. pneumoniae* infection as a new risk factor for osteoporosis-associated bone loss.

In contrast to the aforementioned in vitro and in vivo studies, which illustrated the contribution of *C. pneumoniae* infection to bone loss, we found no significant association between seropositivity to *C. pneumoniae* and BMD in postmenopausal women. In the prospective phase of our study, we did not detect a significant association between seropositivity for *C. pneumoniae* at baseline and changes in BMD and incident osteoporosis in postmenopausal women. Undoubtedly, the current study does not rule out a possible causal link between *C. pneumoniae* and osteoporosis-associated bone loss. However, it suggests that serological markers of *C. pneumoniae* infection may be ineffective in detecting any relationship between *C. pneumoniae* infection and BMD, if such a relationship exists.

Likewise, the correlation of serology and the polymerase chain reaction for detection of vascular *C. pneumoniae* infection in coronary artery disease patients was poor [14]. In a prospective study and meta-analysis, IgA and IgG *C. pneumoniae* titers were not strongly predictive of coronary heart disease in the general population [15]. However, accumulating evidence suggests that *C. pneumoniae* is an important contributory factor in the pathogenesis of atherosclerosis, from the initial stage of the inflammatory lesions to plaque rupture [1].

According to the results of the current study and discrepancies between seroepidemiological studies and polymerase chain reaction (PCR)-based investigations for detection of vascular *C. pneumoniae* infection in coronary artery disease patients [14, 15], serological markers appear not to be a predictive tool for individual vascular *C. pneumoniae* infection or osteoporosis-associated bone loss.

Gastric inflammation in patients infected with *H. pylori* has been characterized by increased activity of Th1 and Th17 that mediate a proinflammatory response toward clearance of *H. pylori* infection and increased production of IL-1, IL-6, IL-12, IL-18, IL-23, TNF-alpha, and IFN-gamma [16, 17]. This proinflammatory response and the release of inflammatory cytokines in *H. pylori* infection may indicate a plausible role for this organism in bone resorption. However, only 4 studies in the medical literature investigated H. pylori infection in relation to bone health, all of which reported contradictory findings [8–11]. In contrast to a case–control study that reported a higher prevalence of CagA-positive strains in osteoporotic men, two studies with small samples of women showed that *H. pylori* infection with or without chronic atrophic gastritis in postmenopausal women [11] and *H. pylori* associated gastritis in women [10] were not associated with decreased BMD. Consistent with the results of these studies in women [10, 11], we also found no association between IgG seropositivity with *H. pylori* and BMD/BMD loss in postmenopausal women. Therefore, *H. pylori* infection does not seem to be an important risk factor for bone loss in women.

Similar to the findings of a study in children that found that *H. pylori* infection was not accompanied by significant changes in circulating estradiol, parathyroid hormone, cross-linked collagen I carboxy terminal telopeptide, bone-specific alkaline phosphatase, N-terminal cross-links of human pro-collagen type I, N-mid-osteocalcin, calcium, and phosphate [8], we did not find significant differences in bone turnover markers between IgG *H. pylori* positive and IgG *H. pylori* negative postmenopausal women.

A balance in RNKL and OPG is essential for osteoclast function and bone remodeling [18]. In the current study, we found a significant difference in OPG between IgG *H. pylori* positive and IgG *H. pylori* negative postmenopausal women, but this significant difference was lost by further adjustment for age. Circulating levels of RANKL and OPG also did not differ significantly between IgG *C. pneumoniae* positive and IgG *C. pneumoniae* negative postmenopausal women. Previously, we showed that circulating levels of RANKL/OPG in the osteoimmunity system are associated with BMD in postmenopausal women [19]. The observed lack of a relationship between *H. pylori* or *C. pneumoniae* IgG seropositivities and the RANKL/OPG osteoimmunity system may be a clue to explain the findings of the current

study, which showed that neither *H. pylori* nor *C. pneumoniae* is involved in postmenopausal bone loss.

We acknowledge several limitations. Although this study is the first population-based study to investigate a link between *C. pneumoniae* seropositivity and BMD, its findings should be confirmed in further human studies with larger samples. We did not measure IgA or IgM titers for *C. pneumoniae* infection. However, currently, there is no valid marker to show the presence of chronic *C. pneumoniae* infection in seroepidemiological studies [1]. In addition, it has been reported that there is a significant relationship between organism-specific DNA or antigens in coronary arteries obtained at autopsy and levels of pre-existing *C. pneumoniae*-specific IgG antibody titers but not IgA or IgM titers [20]. Furthermore, the measurement of additional inflammatory markers, pro-inflammatory cytokines, and chemokines that are indicators of bone resorption merits consideration to elucidate the complex system that regulates chronic infection, the immune system, and bone resorption. We used seropositivity as a marker of infection. Although it has the advantage of clinical applicability, the assessment of infection status based on serology without further clinical or laboratory characterization is subject to diagnostic inaccuracies, especially if seropositivity is common because of the widespread distribution of the incriminated microorganism. Since we did not measure IgG seropositivity for the studied microorganisms at the follow-up period, new seroconversions indicating recent infections with *C. pneumoniae* or *H. pylori* could not be reflected in the current study. This limitation may be a pitfall for interpretation of the findings.

In conclusion, neither *C. pneumoniae* nor *H. pylori* IgG seropositivity was associated with baseline bone turnover markers, OPG, sRANKL, the sRANKL/OPG ratio, and BMD at any skeletal sites and bone loss at 5.8-year follow-up in postmenopausal women. In addition, chronic infection with *C. pneumoniae* or *H. pylori* did not predict incident osteoporosis among the women. However, this lack of predictive power of IgG seropositivities against *C. pneumoniae* and *H. pylori* for osteoporosis-associated bone loss should not be interpreted as these organisms have no contributory role in postmenopausal osteoporosis. Further investigations in human studies are warranted to illustrate any possible causal role of chronic infection with *C. pneumoniae* and *H. pylori* in postmenopausal osteoporosis.

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