Prospective studies of total and ionized serum calcium in relation to incident and fatal ovarian cancer

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HIGHLIGHTS
► Two independent nationally representative population-based cohorts are examined.
► Higher ionized and total serum calcium are associated with ovarian cancer mortality.
► Confirmation that higher total serum calcium is associated with incident ovarian cancer in a second cohort

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ABSTRACT
Objective. Biological markers that could aid in the detection of ovarian cancer are urgently needed. Many ovarian cancers express parathyroid hormone-related protein, which acts to raise calcium levels in serum. Thus, we hypothesized that high serum calcium levels might predict ovarian cancer.

Methods. We examined the associations between total and ionized serum calcium and ovarian cancer mortality in the Third National Health and Nutrition Survey (NHANES III) using Cox proportional hazard models. We then examined the associations of serum calcium with incident ovarian cancer in a second prospective cohort, the NHANES Epidemiological Follow-up Study (NHEFS).

Results. There were eleven deaths from ovarian cancer over 95,556 person-years of follow-up in NHANES III. After multivariable adjustment, the risk for fatal ovarian cancer was 52% higher for each 0.1 mmol/L increase in total serum calcium (RH = 1.52, 95% CI 1.06–2.19) and 144% higher for each 0.1 mmol/L increase in ionized serum calcium (RH = 2.44, 95% CI 1.45–4.09). Associations persisted after adjusting for nulliparity and the use of oral contraceptives. Eight incident ovarian cancers occurred over 31,089 person-years of follow-up in the NHEFS. After adjusting for covariates, there was a 63% higher risk for ovarian cancer with each 0.1 mmol/L increase in total serum calcium (95% CI 1.14–2.34). Similar results were observed for albumin-adjusted serum calcium.

Conclusions. Higher serum calcium may be a biomarker of ovarian cancer. This is the first report of prospective positive associations between indices of calcium in serum and ovarian cancer. Our findings require confirmation in other cohorts.

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Introduction
Ovarian cancer is the most fatal of the gynecologic cancers. The high fatality rate results from the late stage of presentation, at which time ovarian cancers have metastasized and their curability is low. In theory, early diagnosis of ovarian cancer might be accomplished through the use of biomarkers in blood or urine. However, the most widely studied serum marker for ovarian cancer, CA-125, is elevated in only 50% of women with curable (Stage 1) disease [1]. Consequently, there is great interest in the discovery of additional biomarkers that could help to detect ovarian cancers at a curable stage.

One approach to cancer biomarker discovery is to identify a factor(s) that is differentially expressed in individuals with and without cancer and to examine that factor's ability to detect cancer in an independent sample of individuals with and without cancer [2,3]. Many ovarian cancers express increased levels of parathyroid hormone-related protein (PTHrP), an oncogenic protein that is the principal agent of hypercalcemia of malignancy [4]. PTHrP acts to increase the release of...
calcium from bone and to retard the excretion of calcium in the kidney, causing calcium levels in serum to rise [5]. Although only a small minority of ovarian cancers are characterized by hypercalcemia (i.e., serum calcium levels > upper limit of the normal reference range), the evolution of hypercalcemia in ovarian cancer may be gradual. That is, tumors likely evolve from normocalcemia to high normocalcemia before causing hypercalcemia. Thus, we hypothesized that high serum calcium levels might detect ovarian cancer at a preclinical phase.

We tested this hypothesis using data on serum calcium in two nationally representative prospective cohorts, the Third National Health and Nutrition Examination Surveys (NHANES III) and the NHANES Epidemiologic Follow-up Study (NHEFS).

Methods

Baseline data and serum samples were collected as part of NHANES III between 1988 and 1994 [6]. Total and ionized serum calcium levels were measured using ion-specific electrodes and were pH-adjusted. Because the protein binding of calcium is affected by pH, ionized calcium in blood is commonly corrected to standard pH [7]. Approximately half of total serum calcium is in the “free” or ionized state; approximately 40% is bound to serum proteins, principally albumin, and the remainder is bound to anions. Ionized serum calcium is the biologically active fraction of total serum calcium. Because the measurement of ionized calcium is technically more challenging and more expensive than the measurement of total serum calcium, ionized calcium levels often are estimated by calculating serum calcium levels adjusted for serum albumin. We computed levels of albumin-adjusted serum calcium for women with a serum albumin below 4.0 g/dL using a standard formula (0.8 times the difference between 4.0 g/dL and the observed albumin, plus the observed total serum calcium in mg/dL) [8].

The outcome in NHANES III was death attributed to ovarian cancer on the death certificate with mortality linkage through December 31, 2006. Follow-up time was computed as the number of months between the baseline exam and death from ovarian cancer (events) or any other cause (censored), or December 31, 2006 if participants were alive. We excluded women who reported that they had no ovaries at baseline but included women with a prior personal history of non-ovarian cancers because they remain at risk for ovarian cancer. No follow-up for incident cases was performed for NHANES III and information about sub-types of ovarian cancer was not available.

We conducted a second, confirmatory, population-based prospective study using the first NHANES Epidemiologic Follow-up Study (NHEFS) with incident ovarian cancer as the outcome. We included women ages 25 to 75 years at the baseline examination in 1971 to 1975. Follow-up questionnaires for incident medical conditions were administered in 1982, 1986, 1987, and 1992. Women who reported having no ovaries at baseline were excluded but women with a prior personal history of non-ovarian cancers were included, as in NHANES III. We computed follow-up time as the interval between baseline examination and date of diagnosis with ovarian cancer (events) or a report of removal of both ovaries (censored), or the end of follow-up in December 1992 [9–12]. Data on ionized calcium were not available in NHEFS.

We used Cox proportional hazard regression models accounting for survey weights and the complex sampling design to estimate relative hazards and 95% confidence intervals (CI) for ovarian cancer death by incremental (0.1 mmol/L) differences in total serum calcium, albumin-adjusted serum calcium, and ionized serum calcium. We examined potential confounding by age, height, body mass index (BMI), race/ethnicity (Black versus all other), cigarette smoking status (ever vs. never), nulliparity (no live births versus any), and use of oral contraceptives (ever vs. never). Statistical analyses were performed using R v 2.15.0 with the “survival” package [13].

Results

Table 1 shows selected characteristics of women in NHANES/NHEFS and NHANES III by categories of total serum calcium at baseline.

Eleven ovarian cancer deaths were observed over 95,556 person-years of follow-up through December 31, 2006, representing 137,404 ovarian cancer deaths in the United States. The range in total serum calcium in cases was 2.14 to 2.44 mmol/L and for ionized serum calcium was 1.17 to 1.31 mmol/L. The normal reference range for total serum calcium is approximately 2.17 to 2.52 mmol/L [8.7 to 10.1 mg/dL] and 1.12 to 1.32 mmol/L [4.5 to 5.3 mg/dL] for ionized serum calcium [14]. The range of times from calcium measurement to death was 28 to 208 months. In multivariable Cox models, the relative hazard for fatal ovarian cancer was 1.52 per 0.1 mmol/L increase in total serum calcium (95% CI 1.06–2.19) and 2.44 per 0.1 mmol/L increase in ionized serum calcium (95% CI 1.45–4.09).

Adjustment for race, cigarette smoking, height and BMI did not materially alter the estimates vs. age-adjusted estimates. Further adjustment for nulliparity and the never use of oral contraceptives yielded relative hazards (RHs) of 1.46 (1.02–2.09) and 2.11 (1.16–3.83) for total and ionized serum calcium, respectively. Due to the small number of events, we could not explore the interactions between serum calcium concentration and covariates (Table 2).

We sought to confirm these findings using a second prospective population-based cohort, the NHEFS. There were 8 incident ovarian cancer cases in the NHEFS over 31,089 person-years of follow-up. The range of total serum calcium was 1.98 to 2.93 mmol/L. The range of times from calcium measurement to diagnosis with ovarian cancer was 12 to 240 months. The multivariable adjusted relative hazard for ovarian cancer for each 0.1 mmol/L increase in total serum calcium was 1.63 (95% CI 1.14–2.34). Adjusting for BMI, height, and cigarette smoking status did not materially change the association compared to adjusting for age alone. Further adjustment for nulliparity and ever use of oral contraceptives moderately

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Selected characteristics of women in the First National Health and Nutrition Examination Survey (NHANES), NHANES Epidemiology Follow-up Study (NHEFS) and Third National Health and Nutrition Examination Survey (NHANES III) by tertile of total serum calcium concentration at baseline.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline total serum calcium tertile in NHANES/NHEFS</td>
<td>Baseline total serum calcium tertile in NHANES III</td>
</tr>
<tr>
<td>Total calcium range (mmol/L)</td>
<td>1.98–2.38</td>
</tr>
<tr>
<td>Number of participants</td>
<td>725</td>
</tr>
<tr>
<td>Weighted population</td>
<td>19,842,226</td>
</tr>
<tr>
<td>Ovarian cancer cases through 1992</td>
<td>1</td>
</tr>
<tr>
<td>Person-months of follow-up</td>
<td>158,280</td>
</tr>
<tr>
<td>Mean total calcium (mmol/L)</td>
<td>2.17 (0.08)</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>45.80 (16.18)</td>
</tr>
<tr>
<td>Mean body mass index (kg/m²)</td>
<td>25.17 (5.40)</td>
</tr>
<tr>
<td>Mean albumin (g/dL)</td>
<td>4.20 (0.39)</td>
</tr>
</tbody>
</table>

Mean and standard deviations account for the complex sampling design and survey weights in NHANES/NHEFS and NHANES III. Where means are given, standard deviations appear in parentheses.
Table 2
Multivariable adjusted relative hazards for ovarian cancer mortality by ionized, total, and albumin-adjusted serum calcium concentrations at baseline in the Third National Health and Nutrition Examination Survey (NHANES III).

<table>
<thead>
<tr>
<th>Calcium Type</th>
<th>Relative Hazard</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionized calcium (per 0.1 mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age adjusted</td>
<td>1.97</td>
<td>(1.27, 3.04)</td>
</tr>
<tr>
<td>Age and covariates adjusted</td>
<td>2.44</td>
<td>(1.45, 4.09)</td>
</tr>
<tr>
<td>Nulliparity and oral contraceptive adjusted</td>
<td>2.11</td>
<td>(1.16, 3.83)</td>
</tr>
<tr>
<td>Total calcium (per 0.1 mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age adjusted</td>
<td>1.33</td>
<td>(0.92, 1.92)</td>
</tr>
<tr>
<td>Age and covariates adjusted</td>
<td>1.52</td>
<td>(1.06, 2.19)</td>
</tr>
<tr>
<td>Nulliparity and oral contraceptive adjusted</td>
<td>1.46</td>
<td>(1.02, 2.09)</td>
</tr>
<tr>
<td>Albumin-adjusted calcium (per 0.1 mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age adjusted</td>
<td>1.30</td>
<td>(0.87, 1.93)</td>
</tr>
<tr>
<td>Age and covariates adjusted</td>
<td>1.47</td>
<td>(1.02, 2.13)</td>
</tr>
<tr>
<td>Nulliparity and oral contraceptive adjusted</td>
<td>1.38</td>
<td>(0.58, 1.96)</td>
</tr>
</tbody>
</table>

All models account for complex sampling design and survey weights in NHANES III.

Table 3
Multivariable adjusted relative hazards for ovarian cancer incidence by total and albumin-adjusted serum calcium concentrations at baseline in the National Health and Nutrition Examination Survey Epidemiological Follow-up Study (NHEFS).

<table>
<thead>
<tr>
<th>Calcium Type</th>
<th>Relative Hazard</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total calcium (per 0.1 mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age adjusted</td>
<td>1.66</td>
<td>(1.16, 2.37)</td>
</tr>
<tr>
<td>Age and covariates adjusted</td>
<td>1.63</td>
<td>(1.14, 2.34)</td>
</tr>
<tr>
<td>Nulliparity and oral contraceptive adjusted</td>
<td>1.75</td>
<td>(1.27, 2.42)</td>
</tr>
<tr>
<td>Albumin-adjusted calcium (per 0.1 mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age adjusted</td>
<td>1.68</td>
<td>(1.16, 2.43)</td>
</tr>
<tr>
<td>Age and covariates adjusted</td>
<td>1.66</td>
<td>(1.15, 2.40)</td>
</tr>
<tr>
<td>Nulliparity and oral contraceptive adjusted</td>
<td>1.78</td>
<td>(1.28, 2.49)</td>
</tr>
</tbody>
</table>

All models account for complex sampling design and survey weights in NHANES.

Discussion

In these nationally representative population-based cohorts, we observed positive associations between total and ionized serum calcium levels and risk of ovarian cancer. These associations remained significant after adjustment for known risk factors for ovarian cancer, including height and BMI and, for ionized serum calcium, remained so after further adjustment for nulliparity and the non-use of oral contraceptives.

Our results from two population-based cohorts differ from those from a case–control study of total serum calcium and incident ovarian cancer as reported by Toriola et al. [15]. These authors studied 172 pregnant cases and 172 pregnant controls nested within the Finnish Maternity Cohort. They reported a significant inverse association between serum calcium and ovarian cancer that resulted from cases and controls in the highest and lowest quartiles of total serum calcium (OR = 0.41; 95% CI 0.19–0.85). An inverse association was not apparent in the other quartiles [adjacent ORs and 95% CIs = 1.0 (reference); 1.04 (0.55–1.96) and 0.84 (0.44–1.61)] and appears to result from the use of a control group with hypercalcemia. The upper limit of the normal range for serum calcium according to the assay used by Toriola et al. is 2.55 mmol/L (10.2 mg/dL) [16]. Of their 172 controls, 44 (26%) had serum calcium levels beyond the normal range, including 17 (10%) with serum values ≥ 2.8 mmol/L (11.2 mg/dL).

We considered how our results could be influenced by chance and by confounding. Although the number of events in each cohort was small, the confidence intervals around the relative hazards excluded the null value and indicated strong to very strong evidence for the observed associations. It is possible that we observed a high proportion of ovarian cancers of a hypercalcemic type by chance. Data on the type of ovarian cancer were not available in these cohorts. However, the type of ovarian cancer most often associated with hypercalcemia, small cell carcinoma, is rare and occurs predominantly in young women (average age of 23.4 in the 132 cases reviewed by Estel et al.) [17]. The median age at death for cases in NHANES III was 67.6 years (range, 49–91 years) and at diagnosis in the NHEFS was 68.9 years (range 61 to 81 years). No case was hypercalcemic. Thus, chance over-sampling of cancers known to be associated with hypercalcemia is unlikely to have influenced our findings.

Because the calcium measurements in the NHANES cohorts were obtained only once, there is a possibility of measurement error, which would tend to bias the results to the null. However, the serum concentrations of ionized calcium is one of the most tightly controlled analytes in laboratory medicine. The group coefficient of variation (CV) for ionized calcium in the normal population is less than 3% [18]. Future prospective studies could benefit from including multiple measurements of serum calcium.

We evaluated possible confounding by several factors. There are numerous studies of dairy/calcium intake and ovarian cancer risk, the results of which are inconsistent [19]. However, it is unlikely that our findings reflect confounding by dietary calcium intake because serum calcium levels in normal individuals are tightly controlled and are little influenced by dietary calcium intake [20,21]. Height and BMI are modestly associated with ovarian cancer [22]. However, the associations we observed remained significant after adjustment for these factors. Other, established risk factors for ovarian cancer include BRCA status, nulliparity, and the non-use of oral contraceptives [23]. NHANES did not contain data on BRCA status; therefore we were unable to evaluate potential confounding by this variable. Total serum calcium levels are reported to be slightly higher in nulliparous women [24] and to be slightly lower among users of oral contraceptives [24,25]. Adjustment for parity and for oral contraceptive use did not materially influence our results. Several studies (but not all) suggest that serum levels of vitamin D may be inversely associated with risk of ovarian cancer [26]. However, adjustment of our model for serum 25-OHD (data only available in NHANES III) caused only a 3–4% change in the association between serum calcium and ovarian cancer. Thus, our observations do not appear to be explicable by confounding by known risk factors for ovarian cancer.

A key question raised by our findings is whether higher levels of calcium in serum cause ovarian cancer or whether the higher calcium levels are a consequence of extant, subclinical ovarian cancer. Although the association between serum calcium and incident ovarian cancer observed in NHEFS might suggest a causal role for serum calcium, the minimum latent period for ovarian cancer, estimated to be 15–20 years, weakens this interpretation [27]. Conversely, we consider that the positive associations between serum calcium and ovarian cancer reflect the influence of subclinical ovarian cancer on serum calcium levels, i.e., a paraneoplastic effect, similar to a phenomenon that we reported for prostate cancer [28].

In NHANES III, we observed a greater relative hazard for increases in ionized serum calcium than for increases in total serum calcium and for albumin-adjusted serum calcium (RH=2.44, 95% CI 1.45–4.09, vs. RH=1.52, 95% CI 1.06–2.19 and RH=1.47, 95% CI 1.02–2.13). This observation may be related to the fact that many women with ovarian cancer have low serum albumin [29]. The low serum
albumin would lower the measured levels of total serum calcium, whereas the levels of ionized ("free") calcium would be unchanged. Although we adjusted total serum calcium levels for low serum albumin, albumin-adjusted serum calcium is known to be less sensitive than ionized serum calcium for detecting mild hypercalcemia [30, 31].

Humoral hypercalcemia of malignancy (HHM) has been described in several types of ovarian cancer, predominantly small cell and clear cell carcinoma [4]. The hypercalcemia in these cancers is caused by the production of the tumor of PTHrP which acts to resorb calcium from bone and inhibit calcium excretion by the kidney [32]. Small cell carcinoma accounts for ~ 1% of ovarian cancers and is associated with HHM in ~ 66% of cases. Clear cell carcinoma accounts for ~ 5% of ovarian cancers in the U.S. and is associated with HHM in 5–10% of cases [33, 34]. Although small cell and clear cell carcinoma of the ovary are uncommon, PTHrP-mediated hypercalcemia also has been described in other types of ovarian cancer [35–39]. Thus, we speculate that there may be a spectrum of higher serum calcium in ovarian cancer in general, with small cell cancer and clear cell cancer inhabiting the far end of that spectrum [36, 40]. In addition to being a biomarker, serum calcium may participate in the pathophysiology of ovarian cancer. For example, the cell type classically believed to be responsible for ovarian cancer, ovarian surface epithelial cells, expresses functional calcium sensing receptors and proliferates in response to extracellular calcium [41].

In summary, in this biomarker discovery study, we found that higher levels of calcium in serum were significantly positively associated with the risk of ovarian cancer in two prospective cohorts. The principal limitation of this study is the small number of cases. Conversely, the study has several strengths: it is prospective, uses population-based data from two nationally representative cohorts, and is the first to study ionized serum calcium. The existence of stored sera from sample sets of women with and without ovarian cancer should facilitate the confirmation or refutation of the association between serum calcium and ovarian cancer.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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