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Published in:
Journal of Clinical Endocrinology and Metabolism

DOI:
10.1210/jc.2015-1899
Proportion of undercarboxylated osteocalcin and serum P1NP predict incidence of myocardial infarction in older men

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Context: Undercarboxylated osteocalcin (ucOC) modulates insulin secretion and sensitivity in mice, and higher ucOC is associated with lower prevalence of diabetes in men. The influence of ucOC distinct from other markers of bone turnover on incidence of cardiovascular events is unclear.

Participants: Community-dwelling men aged 70–89 years resident in Perth, Western Australia.

Main outcome measures: Serum total osteocalcin (TOC), N-terminal propeptide of type I collagen (P1NP) and collagen type I C-terminal cross-linked telopeptide (CTX) were measured by immunoassay, and ucOC by hydroxyapatite binding. The ratio ucOC/TOC was calculated. Hospital admissions and deaths from myocardial infarction (MI) and stroke were ascertained.

Results: There were 3,384 men followed for 7.0 years during which 293 experienced an MI, 251 stroke and 2,840 neither. In multivariate analyses, higher ratio of ucOC/TOC (expressed as %) was associated with lower incidence of MI (quartiles Q2–4, ≥49% vs Q1, <49%, hazard ratio [HR] = 0.70, 95% confidence interval [CI] = 0.54–0.91), but not of stroke (0.99, 0.73–1.34). Higher P1NP was associated with higher incidence of MI (Q2–4, ≥28.2 μg/L vs Q1, <28.2 μg/L, HR = 1.45, 95% CI = 1.06–1.97), but not of stroke (0.94, 0.70–1.26). CTX was not associated with incident MI or stroke.

Conclusions: A reduced proportion of undercarboxylated osteocalcin or higher P1NP are associated with increased incidence of MI. UcOC/TOC ratio and P1NP predict risk of MI but not stroke, in a manner distinct from CTX. Further studies are needed to investigate potential mechanisms by which bone turnover markers related to metabolic risk and to collagen formation could modulate cardiovascular risk.

Abbreviations:
Osteocalcin is a peptide secreted by osteoblasts (1). It is present in the circulation in both γ-carboxylated and undercarboxylated forms which are markers of bone turnover and vitamin K availability (2). The γ-carboxylated form binds to hydroxyapatite and comprises part of the bone matrix (1). Undercarboxylated osteocalcin (ucOC) has been identified as an endocrine regulator of glucose metabolism in mice, acting via a G-protein coupled receptor GPRC6A (3, 4). In these studies, the amount of ucOC as a proportion of total osteocalcin (TOC) in the circulation was increased in mice resistant to the development of diabetes (3). Consistent with this finding, administration of ucOC in vitro and in wild type mice increased insulin secretion and sensitivity (4, 5).

We have recently demonstrated that in older men, higher serum ucOC concentrations are associated with lower risk of diabetes, independently of age, BMI, other conventional risk factors and unrelated bone turnover markers (6). However, the long-term implications of this association on major health outcomes remain unclear. Previous studies have documented associations of lower TOC concentrations with adiposity, adverse cardiovascular risk factors and the metabolic syndrome (7–9). In a study of mainly men who were aged ≤ 40 years, men who had experienced premature myocardial infarction (MI) had lower TOC concentrations compared with age-matched controls (10). Smaller studies have reported associations of higher ucOC with improved markers of glucose metabolism (11, 12). However, other reports have shown similar associations of ucOC and carboxylated or total osteocalcin with insulin resistance, markers of β cell function and risk of developing diabetes (13–15). Some studies were limited by relatively small sample sizes and measured ucOC using immunoassays (12, 14, 15), which in the absence of a hydroxyapatite binding step to remove carboxylated osteocalcin may overestimate ucOC (2). Using a hydroxyapatite binding step, Levering et al showed that ucOC was associated with whole body insulin sensitivity both at rest and postexercise in obese men (16). Nevertheless, existing studies remain limited by lack of extended follow-up to examine the relationship of ucOC to long term outcomes such as incidence of cardiovascular events.

Given the association of ucOC with diabetes (6), and the established association between diabetes with vascular risk (17), we aimed to test the hypothesis that ucOC, or the proportion of ucOC in the circulation relative to TOC, would predict the incidence of MI or stroke in a large, population-based cohort of older men in whom ucOC was measured using a hydroxyapatite-binding assay (18). To determine associations of ucOC or the ratio of ucOC/TOC with incident MI or stroke, we compared with the nonosteocalcin bone turnover markers N-terminal propeptide of type I collagen (P1NP) and collagen type I C-terminal cross-linked telopeptide (CTX) (19).

Participants and methods

Study population

The Health In Men Study (HIMS) is a cohort study of community-dwelling older men from Perth, Western Australia, which has been described previously (20). Briefly, men aged 65 years or more were randomly selected from the electoral roll (voting being compulsory for Australian citizens) and invited to participate in the study, from which 12,203 men completed a questionnaire and attended for physical examination in wave 1 (W1, 1996–1999). 4248 men attended for reassessment and reexamination in wave 2 (W2, 2001–2004). Approximately 95% of the men were of Caucasian. The University of Western Australia Human Research Ethics Committee approved the study, and all men gave written informed consent.

Assessment of medical comorbidities

Medical data collected at W2 were utilized to identify men with a history of osteoporosis or bone fracture and Paget’s disease. Medications data were analyzed to identify men receiving bisphosphonates or glucocorticoids. The list of medications included then available oral and parenteral bisphosphonates (alendronate, risedronate and zoledronic acid) and the range of glucocorticoid preparations (cortisone, hydrocortisone, dexamethasone and prednisolone). As γ-carboxylation is a vitamin K-dependent process, we also identified men who were receiving warfarin. Men were considered to have hypertension if they reported this diagnosis at W1 or W2, or used antihypertensive medication or had blood pressure (BP) ≥ 140/90 mmHg at W2. Dyslipidemia was defined as having fasting HDL < 0.9 mmol/L, LDL ≥ 3.4 mmol/L, triglycerides ≥ 1.8 mmol/L or total cholesterol ≥ 5.5 mmol/L, or receiving lipid-lowering therapy at W2. Men diagnosed with diabetes, reporting use of glucose-lowering medication, or with fasting or nonfasting glucose at W2 of ≥ 7 mmol/L or ≥ 11.1 mmol/L, respectively, were considered to have (predominantly type 2) diabetes. Further assessment of morbidity was performed via the Western Australian Data Linkage System (WADLS) which provides electronic linkage to records from death, hospital, and cancer registries and captures admissions to all public and private hospitals in Western Australia (21). Cancer diagnoses were identified from the cancer registry between 1990 and W2. Prevalent cardiovascular disease (CVD) was defined as self-reported history of angina, acute MI, stroke, or abdominal aortic aneurysm (AAA) by questionnaire responses in W1 and W2, or hospital diagnoses of these conditions prior to W2.

Ascertainment of incident MI and stroke

The occurrence of hospital admissions due to MI and stroke, and death due to these causes, was obtained from WADLS. This contains hospital admissions data and complete morbidity coding, and death certificates and the ICD-10 coded record generated from these data and other sources by the Australian Bureau of Statistics (21). We examined the outcome of nonfatal or fatal MI based on hospital admissions and deaths due to MI compris-
ing ICD-9 and ICD-10 codes 410, I21 and I22. For the outcome of fatal or nonfatal stroke codes 430–438, I60-I64 and I69.0-I69.4 were used. Both hemorrhagic and ischemic stroke were included. At the time of linkage, all hospital admissions and deaths occurring in Western Australia up to the end 2010 had been recorded in WADLS. Surviving men were censored 8 years after the collection of blood samples and no later than 31 December 2010.

**Laboratory assays**

Blood samples were collected between 0800h and 1030h at W2. Aliquots of plasma and serum were prepared immediately following phlebotomy and stored at −80°C until assayed. Serum TOC, P1NP and CTX were measured by electrochemiluminescence immunoassay using a Modular E170 analyzer (Roche Diagnostics, Australia), as previously described (18). Coefficient of variation (CV) was TOC 3.7% and 2.9% at 18 and 89 ug/L; P1NP 4.0% and 5.7% at 28 and 191 ug/L; and CTX 4.1% and 3.8% at 0.31 and 0.71 ug/L. Serum samples were incubated with hydroxyapatite (5 mg/mL), mixed and centrifuged to separate out carboxyalted osteocalcin as previously described (18). The ucOC in the supernatant was measured using the same assay as for TOC and was reported as a concentration and as the ratio of ucOC/TOC. For a reference osteocalcin standard with expected fractional hydroxyapatite binding of 0.80, kindly supplied by Professor Caren Gundberg (Yale School of Medicine, New Haven, CT), mean fractional hydroxyapatite-bound osteocalcin was 0.77 and between-run imprecision was 6.0%. Vitamin D was measured using a chemiluminescent immunoassay, as previously reported (22).

**Statistical analyses**

The statistical package Stata version 12.1 (StataCorp, College Station, Texas, USA) was used. Men receiving glucocorticoid, bisphosphonate, or warfarin treatment were excluded. Baseline descriptive data were shown as mean and standard deviations (SD) or percentages (%). Comparisons of means were performed using two sample t tests with equal variances, which are robust for parametric and modestly skewed distributions with sufficiently large sample sizes (23). Nelson-Aalen plots of incident nonfatal or fatal MI and stroke according to quartiles of TOC, ucOC, the ratio of ucOC/TOC, P1NP and CTX were constructed. For the primary longitudinal analysis, Cox proportional hazards regression was performed to assess associations of TOC, ucOC, the ratio of ucOC/TOC, P1NP and CTX with incident MI or stroke. Adjustments were made for variables that might potentially confound associations of bone turnover markers with cardiovascular events. Models were age-adjusted, with subsequent additional adjustment for education, smoking, body mass index (BMI), waist:hip ratio (WHR), then for hypertension, dyslipidemia, diabetes, creatinine and vitamin D, and finally for history of cancer or existing CVD. A two-tailed p value of <0.05, or 95% confidence intervals that did not cross 1.0, were considered significant.

**Results**

**Baseline characteristics of study participants**

Assay of bone turnover markers was performed in serum aliquots collected at baseline (wave 2) from 4010 men. Of these, we excluded 24 men with a history of Paget’s disease of the bone, 127 with osteoporosis and 192 with bone fracture. An additional 11 men reported receiving bisphosphonate therapy, 60 glucocorticoid therapy and 212 reported warfarin use. Exclusion of these men left 3384 men for inclusion in the analysis. Baseline characteristic of these men are shown in Table 1. Compared to men who experienced neither MI nor stroke during follow-up, men who experienced either were older, more likely to have prevalent diabetes and CVD, and had higher

<table>
<thead>
<tr>
<th>Variable</th>
<th>Neither MI nor stroke n = 2840</th>
<th>MI n = 293</th>
<th>P1 value</th>
<th>Stroke n = 251</th>
<th>P2 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 70–74 yr</td>
<td>1169 (41.2)</td>
<td>74 (25.3)</td>
<td>&lt;0.001</td>
<td>65 (25.9)</td>
<td>0.004</td>
</tr>
<tr>
<td>75–79 yr</td>
<td>1210 (42.6)</td>
<td>133 (45.4)</td>
<td>0.826</td>
<td>118 (46.5)</td>
<td>0.266</td>
</tr>
<tr>
<td>80–84 yr</td>
<td>400 (14.1)</td>
<td>64 (21.8)</td>
<td>&lt;0.001</td>
<td>64 (25.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>85+</td>
<td>61 (2.1)</td>
<td>22 (7.5)</td>
<td>&lt;0.001</td>
<td>14 (5.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Completed high school</td>
<td>1393 (48.1)</td>
<td>141 (48.3)</td>
<td>0.796</td>
<td>114 (45.4)</td>
<td>0.266</td>
</tr>
<tr>
<td>Smoker - Never</td>
<td>1019 (35.9)</td>
<td>84 (28.7)</td>
<td>0.009</td>
<td>78 (31.1)</td>
<td>0.165</td>
</tr>
<tr>
<td>Past</td>
<td>1690 (59.5)</td>
<td>199 (67.9)</td>
<td>0.009</td>
<td>158 (62.9)</td>
<td>0.175</td>
</tr>
<tr>
<td>Current</td>
<td>131 (4.4)</td>
<td>10 (3.4)</td>
<td>0.825</td>
<td>15 (6.0)</td>
<td>0.175</td>
</tr>
<tr>
<td>BMI ≥25 kg/m²</td>
<td>1859 (65.6)</td>
<td>194 (66.9)</td>
<td>0.651</td>
<td>175 (70.3)</td>
<td>0.133</td>
</tr>
<tr>
<td>WHR ≥0.90</td>
<td>2412 (85.1)</td>
<td>250 (85.6)</td>
<td>0.806</td>
<td>221 (88.4)</td>
<td>0.156</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2182 (76.8)</td>
<td>230 (78.5)</td>
<td>0.519</td>
<td>194 (77.3)</td>
<td>0.868</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>2049 (72.1)</td>
<td>223 (76.3)</td>
<td>0.149</td>
<td>193 (76.9)</td>
<td>0.107</td>
</tr>
<tr>
<td>Diabetes</td>
<td>393 (13.8)</td>
<td>60 (20.5)</td>
<td>0.002</td>
<td>49 (19.5)</td>
<td>0.058</td>
</tr>
<tr>
<td>CVD</td>
<td>869 (29.8)</td>
<td>162 (56.6)</td>
<td>&lt;0.001</td>
<td>116 (47.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cancer</td>
<td>509 (17.9)</td>
<td>45 (15.4)</td>
<td>0.274</td>
<td>43 (17.1)</td>
<td>0.754</td>
</tr>
<tr>
<td>Creatinine, mmol/liter</td>
<td>0.97 ± 0.30</td>
<td>0.104 ± 0.40</td>
<td>&lt;0.001</td>
<td>97.2 ± 32.9</td>
<td>0.008</td>
</tr>
<tr>
<td>Vitamin D, nmol/liter</td>
<td>68.5 ± 23.5</td>
<td>67.2 ± 21.7</td>
<td>0.364</td>
<td>67.1 ± 20.7</td>
<td>0.375</td>
</tr>
<tr>
<td>Total OC, µg/liter</td>
<td>20.8 ± 12.1</td>
<td>23.9 ± 15.0</td>
<td>&lt;0.001</td>
<td>21.7 ± 16.5</td>
<td>0.301</td>
</tr>
<tr>
<td>Undercarboxylated OC, µg/liter</td>
<td>11.0 ± 5.0</td>
<td>12.2 ± 6.9</td>
<td>&lt;0.001</td>
<td>11.3 ± 6.6</td>
<td>0.462</td>
</tr>
<tr>
<td>Undercarboxylated/total OC, %</td>
<td>54.7 ± 8.8</td>
<td>53.0 ± 9.7</td>
<td>0.001</td>
<td>54.2 ± 8.9</td>
<td>0.404</td>
</tr>
<tr>
<td>P1NP, µg/liter</td>
<td>42.8 ± 28.7</td>
<td>46.1 ± 29.8</td>
<td>0.060</td>
<td>41.7 ± 27.0</td>
<td>0.562</td>
</tr>
<tr>
<td>CTX, µg/liter</td>
<td>0.32 ± 0.17</td>
<td>0.35 ± 0.23</td>
<td>0.002</td>
<td>0.33 ± 0.19</td>
<td>0.347</td>
</tr>
</tbody>
</table>

Characteristics of the study participants at baseline (n = 3384); stratified according to whether men experienced a myocardial infarction (MI) or stroke during follow-up. Data are shown as number (%) for categorical variables and mean ± SD for continuous variables. BMI = body mass index, WHR = waist:hip ratio, CVD = cardiovascular disease. P1 compares men with incident MI to those with neither MI nor stroke; P2 compares men with incident stroke to those with neither MI nor stroke.
creatinine concentrations. Men who experienced an MI were also more likely to have smoked compared to men who experienced neither MI nor stroke. TOC, ucOC and CTX were higher in men who experienced an MI, but the ratio ucOC/TOC was lower. Men who experienced a stroke had similar bone turnover markers at baseline compared to men who experienced neither MI nor stroke.

Incidence of MI and stroke during follow-up

The mean (±SD) duration of follow-up was 6.97 ± 1.99 years. During this time, 293 men experienced a death or hospitalization due to MI and 251 due to stroke. Cumulative incidence of MI according to quartiles of TOC, ucOC, ucOC/TOC, P1NP and CTX are shown in Figure 1, and the corresponding results for cumulative incidence of stroke in the Supplementary Figure. Higher TOC and CTX concentrations were associated with increased incidence of MI (Figure 1A and 1E respectively). Men with ucOC/TOC in the lowest quartile had the highest incidence of MI, and men with P1NP in the lowest quartile had the lowest incidence of MI (Figure 1C and 1D respectively). UcOC was not associated with risk of MI (Figure 1B). There was no apparent association of any of these markers with incidence of stroke (Supplementary Figure).

Multivariate analyses of bone turnover markers vs incidence of MI

The results of the longitudinal analyses examining baseline bone turnover markers against the outcome of incident MI are shown in Table 2. In the univariate model, higher TOC was associated with increased risk of MI. However, this association was abrogated by adjustment for age, and was not present in the fully-adjusted model. A higher ratio of ucOC/TOC was associated with reduced incidence of MI in univariate and full-adjusted models (Quartiles Q2–4, ≥49% vs Q1, <49%: hazard ratio (HR) [HR] 0.70, 95% confidence interval (CI) [CI] 0.54–0.91). Higher P1NP was associated with increased incidence of MI (Q2–4, ≥28.2 µg/L vs Q1, <28.2 µg/L: HR 1.45, 95% CI 1.06–1.97). CTX was not associated with incident MI.

Multivariate analyses of bone turnover markers vs incidence of stroke

The results of the longitudinal analyses examining baseline bone turnover markers against the outcome of incident stroke are shown in Table 3. There was no association of TOC, ucOC, the ratio of ucOC/TOC, P1NP or CTX with incident stroke in either univariate or adjusted analyses.

Figure 1. Nelson-Aalen plots showing the cumulative incidence of nonfatal or fatal MI according to quartiles of A: total osteocalcin (TOC), B: undercarboxylated osteocalcin (ucOC), C: the ratio of ucOC/TOC, D: serum N-terminal propeptide of type I collagen (P1NP), and E: collagen type I C-terminal cross-linked telopeptide (CTX) in 3384 community-dwelling men aged 70–89 years.
Higher incidence of MI (Q2–4, 0.57–0.99), while higher P1NP remained associated with quartile (Q2–4). Results are shown for men in the highest three quartiles compared with men in the lowest quartile (Q2–4). Proportional hazards regression of total OC, ucOC, ucOC/total OC, P1NP and CTX for the outcome of non-fatal and fatal myocardial infarction (MI). Results are shown for men in the highest three quartiles compared with men in the lowest quartile (Q2–4 vs. reference Q1). Q = quartile, HR = hazard ratio, CI = confidence interval.

Table 2. Associations of ucOC and other bone turnover markers in quartiles with non-fatal and fatal myocardial infarction in older men. Proportional hazards regression of total OC, ucOC, ucOC/total OC, P1NP and CTX for the outcome of non-fatal and fatal myocardial infarction (MI). Results are shown for men in the highest three quartiles compared with men in the lowest quartile (Q2–4 vs. reference Q1). Q = quartile, HR = hazard ratio, CI = confidence interval.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Range</th>
<th>MI N (%)</th>
<th>Univariate HR (95% CI)</th>
<th>Model 1 HR (95% CI)</th>
<th>Model 2 HR (95% CI)</th>
<th>Model 3 HR (95% CI)</th>
<th>Model 4 HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OC (µg/liter)Q1</td>
<td>4.64–14.69</td>
<td>56 (1.1)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OC (µg/liter)Q2–4</td>
<td>14.70–248.30</td>
<td>237 (13.1)</td>
<td>1.44 (1.08–1.93)</td>
<td>1.22 (0.91–1.64)</td>
<td>1.24 (0.92–1.67)</td>
<td>1.55 (1.15–2.09)</td>
<td>1.29 (0.94–1.74)</td>
</tr>
<tr>
<td>UcOC (µg/liter)Q1</td>
<td>3.00–8.04</td>
<td>62 (7.8)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UcOC (µg/liter)Q2–4</td>
<td>8.06–86.02</td>
<td>231 (19.0)</td>
<td>1.20 (0.87–1.67)</td>
<td>1.13 (0.85–1.50)</td>
<td>1.12 (0.84–1.48)</td>
<td>1.33 (1.00–1.77)</td>
<td>1.14 (0.85–1.53)</td>
</tr>
<tr>
<td>UcOC/TOC Q1</td>
<td>5.51–48.36</td>
<td>102 (13.1)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UcOC/TOC Q2–4</td>
<td>48.99–87.30</td>
<td>191 (8.1)</td>
<td>0.60 (0.47–0.76)</td>
<td>0.66 (0.52–0.84)</td>
<td>0.67 (0.52–0.85)</td>
<td>0.64 (0.50–0.82)</td>
<td>0.70 (0.54–0.91)</td>
</tr>
<tr>
<td>P1NP (µg/liter)Q1</td>
<td>9.45–26.19</td>
<td>53 (8.6)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1NP (µg/liter)Q2–4</td>
<td>28.20–710.10</td>
<td>240 (10.2)</td>
<td>1.53 (1.14–2.06)</td>
<td>1.42 (1.05–1.91)</td>
<td>1.42 (1.05–1.92)</td>
<td>1.59 (1.17–2.16)</td>
<td>1.45 (1.06–1.97)</td>
</tr>
<tr>
<td>CTX (µg/liter)Q1</td>
<td>0.04–0.20</td>
<td>67 (8.3)</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX (µg/liter)Q2–4</td>
<td>0.20–2.53</td>
<td>226 (9.6)</td>
<td>1.18 (0.90–1.54)</td>
<td>1.03 (0.79–1.36)</td>
<td>1.02 (0.77–1.35)</td>
<td>1.21 (0.91–1.60)</td>
<td>1.03 (0.77–1.37)</td>
</tr>
</tbody>
</table>

Model 1: Adjusted for age
Model 2: adjustment as in Model 1 and for education, smoking, BMI and WHR
Model 3: adjustment as in Model 2 and for hypertension, dyslipidemia, diabetes, creatinine and Vitamin D
Model 4: adjustment as in Model 3 and for prevalent CVD and cancer.

Trimmed analysis of bone turnover and incidence of MI
To verify that the results were not influenced by the presence of low or high outliers, we repeated the longitudinal analyses after excluding men with concentrations of bone turnover markers in the lowest or highest 1% of values (Supplemental Table S1). Exclusion of low and high outliers (N = 62) did not alter the results. A higher ratio of ucOC/TOC remained associated with lower incidence of MI (Q2–4, ≥49% vs Q1, <49%; HR 0.75, 95% CI 0.57–0.99), while higher P1NP remained associated with higher incidence of MI (Q2–4, ≥28.2 µg/L vs Q1, <28.2 µg/L: HR 1.54, 95% CI 1.09–2.17).

Sensitivity analysis
We repeated the longitudinal analyses after excluding men who experienced an MI within the first twelve months of follow-up (N = 56). Results are shown in Supplemental Table S2. Exclusion of events occurring within the first year of follow up did not alter the association of higher ucOC/TOC ratio with lower risk of MI (Q2–4 vs Q1: fully-adjusted HR 0.70, 95% CI 0.54–0.92). Higher P1NP remained associated with higher risk of MI (Q2–4 vs Q1: fully-adjusted 1.49, 95% CI 1.07–2.06).

Supplementary analyses
We tested whether ucOC/TOC ratio and P1NP remained associated with incident MI after excluding men with diabetes at baseline (Table 4). Excluding men with diabetes gives a fully-adjusted HR for incident MI for Q2–4 vs Q1 ucOC/TOC of 0.75 compared to 0.70, and Q2–4 vs Q1 P1NP of 1.38 compared to 1.45 in the original analyses (Table 2). We performed a second supplementary analysis excluding men with metabolic syndrome (Table 5). Excluding men with metabolic syndrome gives a fully-adjusted HR for Q2–4 vs Q1 ucOC/TOC of 0.70, and for Q2–4 vs Q1 P1NP 1.43. In both supplementary analyses the HRs are largely unchanged compared to the
original results, but statistical power is less with exclusion of these subgroups. When men with eGFR < 30 ml/min/1.73m² were excluded, the results were similar with HR for Q2–4 vs Q1 ucOC/TOC being 0.77, and for Q2–4 vs Q1 P1NP 1.39 (Supplemental Table S3).

Discussion

In older men without pre-existing osteoporosis or history of bone fracture, who were not receiving glucocorticoids, bisphosphonates or warfarin, a higher ratio of ucOC/TOC predicted a lower incidence of hospitalizations or deaths from MI. By contrast, higher serum P1NP was associated with higher incidence of hospital admissions and deaths from MI. The results could not be accounted for by age or other conventional cardiovascular risk factors. Of note, these divergent associations were outcome- and marker-specific, being present for MI but not stroke and death from MI. The results could not be accounted for by age or other conventional cardiovascular risk factors. Of note, these divergent associations were outcome- and marker-specific, being present for MI but not stroke and not seen with a different marker of bone turnover, CTX. Therefore, lower ucOC/TOC and higher P1NP are biomarkers for, or contributors to, increased risk of MI in older men.

Previous studies have associated reduced TOC with metabolic syndrome (7–9), coronary artery disease (CAD)
(10, 24) and mortality risk (25, 26). None of those studies reported ucOC data, nor were other markers of bone metabolism other than CTX assayed for comparative purposes. Given the association of lower TOC with adverse metabolic parameters, CVD and mortality, in this study we examined as outcomes the two principle manifestations of CVD, namely MI and stroke. Of note, TOC was not associated with either outcome, instead a higher proportion of ucOC predicted lower incidence of MI.

UcOC measured by immunoassay without a precipitation step has been associated with better indices of glucose metabolism in older men (12) and men with type 2 diabetes (27). However, in several studies associations of ucOC and total or carboxylated osteocalcin with metabolic outcomes were comparable (11, 13–15). These studies have been limited by sample size, and the possibility of overestimation of ucOC in the absence of a precipitation step (2). In a cross-sectional analysis of 63 overweight and obese adults with normal or impaired fasting glucose, TOC was associated with insulin sensitivity determined by intravenous (IV) glucose tolerance testing; while the ratio of ucOC/TOC measured using hydroxyapatite binding was associated with β-cell responses to glucose (28). Until now, large longitudinal studies examining the association of ucOC or the ratio of ucOC/TOC measured using hydroxyapatite binding with the outcome of cardiovascular events have been lacking. In the current study we found that a lower proportion of circulating ucOC relative to TOC was robustly associated with incident MI, but not stroke. Therefore, in older men lower ucOC/TOC ratios may be biomarkers for or contributors to the risk of atherothrombotic events in the coronary circulation.

The presence of common and contrasting aetiologies for MI and stroke would accommodate differential associations with distinct biomarkers or modulating factors (29). Our analyses suggest that the observed associations of ucOC/TOC, and also P1NP, with incident MI are largely not accounted for by the presence of diabetes or metabolic syndrome. With regard to MI, there has been previous interest in the association of markers of collagen turnover and cardiovascular outcomes (10). The combination of elevated type 1 collagen telopeptide (ICTP) and brain natriuretic peptide was associated with all-cause mortality and with a composite of CVD death and heart failure hospitalization in a longitudinal analysis of 476 patients with congestive heart failure (CHF) following MI (30). It has been postulated that ICTP represented a breakdown product of type 1 collagen arising from the action of matrix metalloproteinases involved in myocardial tissue repair after acute coronary syndromes (ACS), whereas CTX reflected cathepsin-mediated breakdown of type 1 collagen in bone (31). However, in men both ICTP and CTX are elevated following either an acute coronary syndrome or a tibial shaft fracture indicating an overlap of these markers in detecting bone and soft tissue collagen degradation (31).

A higher ratio of amino-terminal propeptide of procollagen Type III (PIIINP) to ICTP has been reported following MI (32). PIIINP is structurally different from P1NP, being a marker of type 3 rather than type 1 collagen synthesis. Both type 1 and type 3 collagen are present in the myocardium, thus acute myocardial damage leading to left ventricular (LV) remodelling may manifest with elevations in markers of collagen synthesis and degradation traditionally associated with bone metabolism (33). This differs from our study where baseline P1NP and CTX were measured, with the outcome of MI occurring years after. In other studies higher ICTP and PIIINP as well as the bone turnover marker urinary deoxypyridinoline have been associated with mortality in men (34, 35). We found that higher P1NP, but not CTX, was associated with greater risk of incident MI. Therefore the association of P1NP with incident MI was not a reflection of a generalized increase in vascular matrix collagen degradation as no comparable association was seen with CTX. Instead, our results identify P1NP as a novel biomarker for risk of MI in older men. Further investigation would be required to clarify whether in this setting P1NP may reflect an interaction between bone formation and the vasculature, or a specific pathway involving collagen synthesis.

Osteoblast-lineage cells are present in the circulation making up 1%–2% of mononuclear cells; these cells are positive for osteocalcin and are capable of forming mineralised nodules in vitro (36). Furthermore, circulating endothelial progenitor cells (EPCs) from patients with coronary atherosclerosis are more likely to express osteocalcin compared with EPCs from control patients with normal coronary arteries (37). Thus osteoblast-like cellular activity occurs in the vasculature and it is conceivable that increased P1NP concentrations are indicative of this process. It is not known to what extent these cells contribute to amounts or proportions of ucOC and TOC in the circulation. Increased numbers of osteoblast-like cells are associated with vascular calcification which may be an intermediate step mediating adverse cardiovascular outcomes (37, 38).

The finding that lower ucOC/TOC ratio rather than TOC was predictive of incident MI but not stroke is noteworthy. The ratio of ucOC/TOC has been utilized as a marker of vitamin K status, as vitamin K deficiency impairs γ-carboxylation of osteocalcin and vitamin K supplementation reduces ucOC concentrations (39). Vitamin K deficiency has been associated with higher ucOC/TOC ratios, and this has been proposed as a contributor to
arterial calcification in the coronary circulation (40). By contrast, we found that higher ucOC/TOC ratios were independently associated with lower incidence of MI. Therefore, deficiency of vitamin K is unlikely to account for our findings, as vitamin K repletion would lead to lower rather than higher ucOC/TOC ratios. Instead, our findings are consistent with higher ucOC/TOC ratio representing a novel biomarker for reduced cardiovascular risk, possibly mediated via more favorable metabolic profiles as demonstrated in the original animal studies (3, 4). While a reduced ratio of ucOC/TOC identifies men at risk of MI, further studies are needed to clarify mechanisms underlying this association and whether an optimal hormonal milieu incorporating input from bone and glucose metabolism modulates CVD risk. Ultimately placebo-controlled trials of ucOC would be needed to directly establish cause and effect.

Strengths of this study include the large cohort of community-dwelling men, the measurement of ucOC using a hydroxyapatite-binding assay, the parallel measurement of total osteocalcin, P1NP and CTX to compare and contrast associations of different bone turnover markers with incident MI and stroke, the accrual of large numbers of outcome events adding power to the longitudinal analyses, and the systematic adjustment for potential confounders. The results were not driven by high or low outliers, and the sensitivity analysis excluding men who experienced an event within the first twelve months makes reverse causality less likely. We acknowledge several limitations of the study including its observational nature. Men seen in wave 2 were drawn from the larger original cohort hence a “healthy survivor” effect may be present. We relied on a single blood sample for assay of ucOC, total osteocalcin, P1NP and CTX, and did not have serial measurements of these markers. Outcome events were ascertained using data linkage, however WADLS captures all hospital admissions in the state of Western Australia and few men of this age emigrate interstate or overseas (21). Some cases of stroke may have been missed had they not resulted in hospital admission, but there is no reason to suspect that this would have introduced any bias. Finally, men in HIMS are predominantly Caucasian therefore our results may not apply to other populations of men of differing ethnicities and we cannot comment on associations in women.

**Conclusions**

In older men, a lower ratio of ucOC/TOC predicted incident MI independently of conventional cardiovascular risk factors. By contrast, higher P1NP was associated with increased incidence of MI. These associations were not found with an alternative bone turnover marker, CTX, nor were they present for incident stroke. These findings identify ucOC/TOC ratio and P1NP as robust, specific and divergent biomarkers for risk of coronary events. Further investigations are warranted to clarify whether ucOC/TOC mediates incidence of MI via the intermediary of glucose metabolism or other means, and whether P1NP in this setting links bone formation to cardiovascular risk.

**Acknowledgments**

We thank Prof Caren Gundberg, Yale School of Medicine, for assistance in establishing the ucOC assay, and George Koumantakis and Roche Diagnostics Australia for assistance with the supply of assay kits. We thank the staff of PathWest Laboratory Medicine, Fremantle, Royal Perth and Sir Charles Gairdner Hospitals, Perth, Western Australia, and the Data Linkage Unit, Health Department of Western Australia, Australia, for their excellent technical assistance. We thank the staff and management of Shenton Park Hospital for their support of the study. We especially thank all the men and staff who participated in the Western Australian Abdominal Aortic Aneurysm Program and the Health In Men Study.

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Disclosures: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

This work was supported by Grants and Fellowships: BBY is recipient of a Clinical Investigator Award from the Sylvia and Charles Viertel Charitable Foundation, New South Wales, Australia. This study was supported by Grant-in-Aid G11P 5662 from the National Heart Foundation of Australia. The Health In Men Study was funded by Project Grants 279 408, 379 600, 403 963, 513 823, 634 492, 1 045 710 and 1 060 557 from the National Health and Medical Research Council of Australia. JG holds Practitioner Fellowship 1 019 921 from the National Health and Medical Research Council of Australia. The funding sources had no involvement in the planning, analysis and writing of the manuscript.

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