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Serum osteoprotegerin as a long-term predictor for patients with stable coronary artery disease and its association with diabetes and statin treatment: A CLARICOR trial 10-year follow-up substudy

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HIGHLIGHTS

- High serum osteoprotegerin (OPG) predicts mortality and cardiovascular events in coronary artery disease (CAD) patients a decade ahead
- Serum OPG levels were elevated in participants with diabetes *vs* without diabetes
- Statin users without diabetes presented with lower OPG levels than non-statin users

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ABSTRACT

Background and aims: Elevated circulating levels of osteoprotegerin (OPG) are known to add to the prediction of cardiovascular mortality. Our objective was to clarify the long-term risk associated with serum OPG and the possible influence of diabetes and statins on OPG levels in patients with stable coronary artery disease (CAD).
Methods: We assessed the placebo-treated group (n = 1998) from the CLARICOR trial (NCT00121550), a cohort with stable CAD. At entry, 15% of the participants had diabetes and 41% received statins. Serum OPG levels were measured in blood drawn at randomization. Participants were followed through public registers for 10 years.
Results: OPG levels correlated positively with diabetes status, age, CRP and female sex, but negatively with the use of statins. CAD participants with diabetes had significantly elevated serum OPG levels compared to participants without diabetes, $p < 0.0001$. The participants without diabetes treated with statins presented with significantly lower serum OPG levels than the corresponding non-statin-users ($p < 0.0001$). However, statin use showed no association with OPG levels in the participants with diabetes. High OPG levels at entry showed long-term associations with all-cause mortality and cardiovascular events (hazard ratio associated with factor 10 OPG increase 15.9 (95% CI 11.0–22.9) and 6.38 (4.60–8.90), $p = 0.0001$, even after adjustment for standard predictors (3.16 (1.90–5.25) and 2.29 (1.53–3.44), $p < 0.0001$).
Conclusions: Circulating OPG holds long-term independent predictive ability for all-cause mortality and

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cardiovascular events in CAD participants. OPG levels were associated with diabetes, age, and female sex and statin treatment was associated with lower OPG levels in the absence of diabetes.

1. Introduction

Elevated circulating levels of osteoprotegerin (OPG), a cytokine belonging to the soluble TNF-receptor family, adds to the prediction of cardiovascular mortality [1–3]. OPG is secreted by vascular cells and involved in the process of vascular calcification [4,5]. The circulating OPG levels may result from ongoing endothelial damage and inflammatory processes triggered by the vascular damage. Increased OPG levels correlate positively with traditional vascular risk factors, such as age, smoking habits, and diabetes [6]. Women have higher OPG levels than men [7]. Circulating OPG levels are increased in patients with diabetes, even at time of diagnosis, and OPG levels are reported to increase with diabetes duration [8]. High OPG levels are associated with various diabetic complications [7], including coronary calcification, vascular stiffness, and coronary artery disease (CAD) [1,2,5,9,10]. A recent meta-analysis identified 19 studies with 27,450 patients with a mean follow up of 4.2 years. They found that in a population at high cardiovascular risk, including patients with diabetes and patients with previous heart disease, elevated serum OPG levels were associated with risk of future cardiovascular events [11].

Treatment with statins (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors) lowers circulating levels of low-density lipoprotein (LDL) cholesterol and reduces cardiovascular mortality, this being the basis of its preventive and therapeutic use in cardiology [12]. The effect of statin treatment on circulating OPG levels are unclear, as both reduction and increase in serum OPG have been reported in clinical trials [13–17].

Our objective was to explore the influence of diabetes and treatment with statins, recorded at trial entry, on the circulating OPG levels and to investigate the 10-year prognostic potentials of serum OPG for all-cause mortality and cardiovascular events in an existing large cohort of stable coronary artery disease (CAD).

2. Patients and methods

2.1. Study participants

This observational study explores data from the placebo-treated CAD participant group from the CLARICOR (Clarithromycin for patients with stable coronary heart disease) trial [18]. The CLARICOR trial is a randomized, placebo-controlled, multicentre trial including 4372 participants with stable CAD, using central randomization 1:1 [18]. All participants (aged 18–85 years) in Copenhagen with a hospital diagnosis of myocardial infarction (MI) or angina pectoris (ICD codes I 20.9–21.9) during the years 1993–1999 were invited in late 1999 to participate in the trial. Eligibility was based on patient-record diagnoses and interview at randomization. Participants who had MI or unstable angina within the previous three months or coronary interventions within the previous six months or who suffered from other serious diseases such as impaired renal or hepatic function were excluded [18]. At randomization, a blood sample was taken, we registered diabetes mellitus status as well as current medications, but no physical investigations were made.

The main results of the CLARICOR trial were that clarithromycin increased the risk of cardiovascular as well as all-cause mortality. Therefore, to eliminate any effect of clarithromycin influencing our results, we have used only the placebo-treated participants (n = 1998) in this follow-up study [19,20]. The placebo-treated participants may be considered a representative sample of stable CAD patients, not treated with clarithromycin.

2.2. Ethics

Written informed consent was obtained from each participant included in the trial, which complied with the 1975 Declaration of Helsinki. Approvals were given by the local ethics committee (KF 01–076/99 and HB 2009/015), the Danish Medicines Agency (2612-975), and the Danish Data Protection Agency [1999-1200-174and2012-41-0757]. The trial was registered at (NCT00121550).

2.3. Measurement of biomarkers in serum

All measurements have been carried out on serum samples obtained at randomization and stored at −80 °C. Routine laboratory measurements and standard biochemical predictors are described elsewhere [20,21]. The OPG concentration was quantified by an in-house Time-Resolved ImmunoFluoroMetric Assay, using commercially available monoclonal antibodies (DY085E, BioTechne, UK), as described previously [1,22]. The limit of detection was 15 ng/L. The intra- and interassay variation was < 5% and < 9%, respectively. The OPG measurements are stable during multiple freeze-thaw cycles.

2.4. Follow-up of cardiovascular outcomes and all-cause mortality

Participants were followed through hospital and death registries by the trial coordinators, who transmitted patient records and death certificates to formal adjudication by external cardiologists [23] until September 2002 [18]. A later excerpt from the Danish Central Person Register in December 2005 extended the follow-up of all-cause mortality to 6 years [24]. The present study extended the follow-up period to 10 years (December 31, 2009), based on public register data, as described in details elsewhere [19]. The outcomes considered were time to death regardless of cause, and to a composite outcome consisting of acute myocardial infarction (AMI), unstable angina pectoris (UAP), cerebrovascular disease (CeVD), or death [20].

2.5. Statistical analysis

The PREMAC sub-studies of the CLARICOR project follow a unified statistical analysis plan which encompasses several ‘advanced’ biomarkers including OPG [20]. Practically no data were missing from the CLARICOR data set. However, due to shortness of serum samples, 202 of the 2200 placebo records had one or more missing laboratory values. We assumed that data were missing completely at random which was also supported by Little's test (p = 0.93). Hence, we conducted a ‘complete participant’ analysis (n = 1998) instead of a multiple imputation [20].

The prognostic analysis rest on Cox-type proportional hazards analysis described in the statistical analysis plan (PREMAC) [20]. The prognostic implications of the OPG level measured at cohort entry was assessed, in hazard ratio (HR) terms, using two sets of predictors: OPG alone, and OPG together with the full range of standard cardiovascular predictors encompassing clinical predictors (sex, age, smoking status, hypertension, diabetes, previous AMI), medication (aspirin, beta-blocker, calcium-antagonist, ACE-inhibitors, long lasting nitrate, diuretic, digoxin, statins, anti-arrhythmic drugs), standard biochemical predictors (log (CRP), ApoA1, log (ApoB), high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, log (cholesterol), log (tri-glycerides), and glomerular filtration rate (GFR)).

The interpretation of the statistical analyses, however, proved difficult because of an OPG-age correlation. Age has a non-linear and time-dependent impact on certain outcomes, and so may OPG have. For

illustration of the impact of the OPG-related variables (primarily diabetes and statin treatment), we also picked a judiciously chosen third set of predictors with which, to the best of our experience, OPG shows little mutual confounding [i.e., OPG-related HRs show little change when a co-predictor is added or removed, and *vice versa*]. In addition to $\log_{10}$OPG, $\log_{10}$OPG ×{time (years) to event since study entry} and $\log_{10}$OPG × age, the predictor set comprises: age, age squared, age × {time to event since study entry}, sex, smoking habits, history of AMI (*vs*. UAP only, as either was required), diabetes, and statin treatment; here, age is age at entry in decades. All logarithms in the present text are decimal logarithms, and the HR per unit increase therefore refers to a factor 10 increase in marker concentration. The analyses were conducted using SAS version 9.4.

3. Results

3.1. Characteristics at study entry

Table 1 shows selected characteristics of the cohort at entry, describing the OPG levels and survivorship in the entire group and according to diabetes status (Table 1A) and statin treatment (Table 1B). At study entry, 15% of all participants had self-reported diabetes and 41% received statins. Additional characteristics have been presented previously [25]. The geometric mean OPG concentrations was 1781 ng/L and the median OPG concentration was 1758 ng/L as shown in Table 1A.

Female participants had approximately 15% higher OPG levels as compared to males. Accordingly, a positive correlation with OPG levels was found with female sex (r = 0.160), age (r = 0.458), and diabetes (r = 0.097), whereas a negative correlation was observed with use of statins (r = −0.136), all $p < 0.0001$). CRP-levels were significantly higher in participants with diabetes as compared to participants without diabetes (3.1 mg/L (quartiles 1.7–6.9) *vs* 2.7 mg/L (1.3–5.9), $p = 0.005$). A positive correlation was found between OPG and CRP (r = 0.163, $p < 0.0001$). Statin users had significantly reduced CRP levels in the participants without diabetes ($p < 0.0001$), but not in the presence of diabetes ($p = 0.18$, no significant deviation from participants without diabetes).

3.2. OPG-level and survival/observation time

During follow-up, 738 participants died: 47% of the participants with diabetes against 35% of those without diabetes ($p = 0.0002$). Participants with and without statin treatment showed an overall mortality of 29% and 43%, respectively. As detailed in Table 1A, the non-survivors had significantly higher OPG levels as compared to survivors ($p < 0.0001$), and the serum OPG levels were significantly higher amongst those with diabetes (Table 1A) compared to participants without diabetes ($p < 0.0001$).

As regards to treatment with statins at entry, the participants without diabetes showed a lower OPG level (approx. 13% lower) when statin-treated as compared to no statin use ($p < 0.0001$, Table 1B). This difference in OPG levels persisted after adjustment for age or sex or both ($p < 0.007$). Interestingly, this difference was not observed among participants with diabetes ($p = 0.7$), and age and sex adjustments did not change it. The percent survivors were significantly improved in the group of participants treated with statin, overall and in CAD-participants without diabetes ($p < 0.0001$), less clearly in the diabetes minority ($p = 0.06$).

3.3. OPG and 10-year prognosis

During follow-up, 1204 composite events/outcomes (AMI, UAP, CeVD, or death) occurred. The individual outcomes have been described elsewhere [26]. Table 2 summarises our Cox-model analyses. High OPG levels at entry were significantly associated with all-cause mortality as well as composite outcome. OPG level also remains an independently informative predictor after adjustment for standard predictors (described in Table 2) (log-OPG HR for death was 3.16 (95% confidence interval (CI) 1.90–5.25) and for composite outcome 2.29 (1.53–3.44), $p < 0.0001$).

In order to describe the prognostic impact of OPG, illustrative models were generated by adding selected characteristics (lower part of Table 2). OPG levels, age, and cardiovascular risk show a hard-to-unravel covariation that complicates the time-to-event studies. In some models, time since entry combined with OPG levels ($\log_{10}$OPG × time) added a minor, but significant, factor to the risk of composite outcome even after adjustment of the standard predictors; the associated HR being > 1.00 as is the main effect of OPG, one concludes that having

Table 1
Selected patient characteristics. OPG levels in patients with CAD according to (A) diabetes status, (B) use of statins and 10-year survivorship.

A	All CAD patients	CAD patients without diabetes	CAD patients with diabetes
	(n = 1998)	(n = 1698)	(n = 300)
Age (years)	65.2 ± 10.4	65.2 ± 10.6	65.4 ± 9.6
Males (%)	1374 (69%)	1153 (68%)	221 (74)%
CRP (mg/L)	2.7 (1.3, 5.9)	2.7 (1.2, 5.8)[a]	3.1 (1.7, 6.9)
All-cause mortality	738 (37%)	598 (35%)[b]	140 (47%)
OPG (ng/L)	1758 (1342, 2287)	1736 (1330, 2261)[c]	1953 (1443, 2524)
OPG (ng/L) survivors	1580 (1243, 2032)	1562 (1237, 2010)	1675 (1312, 2197)
OPG (ng/L) non-survivors	2092 (1635, 2691)	2075 (1627, 2636)	2162 (1668, 2817)

B	CAD patients without diabetes		CAD patients with diabetes	
	No statins (*n* = 1020)	Statins (*n* = 678)	No statins (*n* = 156)	Statins (*n* = 144)
Age (years)	67.2 ± 10.5	62.2 ± 9.5	66.9 ± 9.9	63.7 ± 9.0
Males (%)	665 (65%)	488 (72%)	116 (74%)	105 (73%)
CRP (mg/L)	3.1 (1.4, 6.7)[d]	2.1 (1.0, 4.4)	3.5 (1.7, 7.1)	2.9 (1.4, 6.3)
All-cause mortality	421 (41%)[c]	177 (26%)	81 (52%)	59 (41%)
OPG (ng/L)	1848 (1384, 2397)[d]	1589 (1257, 2018)	1973 (1466, 2680)	1913 (1437, 2398)
OPG (ng/L) survivors	1645 (1276, 2143)	1507 (1191, 1874)	1728 (1309, 2204)	1645 (1313, 2186)
OPG (ng/L) non-survivors	2172 (1707, 2749)	1813 (1514, 2285)	2171 (1681, 2892)	2151 (1659, 2687)

[a,b,c] *p*-value no diabetes compared to diabetes [a]$p = 0.005$, [b]$p = 0.0002$, [c]$p < 0.0001$.
[d] *p*-value no statin compared to statin $p < 0.0001$.
CAD: coronary artery disease, OPG: osteoprotegerin. Age is reported as mean ± SD, OPG and CRP data as median (quartiles).

Table 2
Three Cox model results, showing the informative properties of OPG levels. Hazard ratios (95% confidence intervals).

Prognostic predictors	All-cause death	**Composite outcome** (first of AMI, UAP, CeVD or death)
OPG only		
logOPG	15.9 (11.0–22.9)[b]	6.38 (4.59–8.87)[b]
logOPG × time (annually)	1.07 (0.95–1.22)	1.27 (1.13–1.42)[b]
OPG, adjusted for standard predictors#		
logOPG	3.16 (1.90–5.25)[b]	2.29 (1.53–3.44)[b]
logOPG × time (annually)	1.09 (0.92–1.28)	1.23 (1.08–1.40)[a]
Illustrative model		
logOPG	5.33 (3.24–8.75)[a]	2.63 (1.78–3.90)[b]
logOPG × time (annually)	1.04 (0.89–1.22)	1.21 (1.06–1.37)[a]
logOPG × age (per decade)	0.55 (0.34–0.90)[a]	0.86 (0.60–1.23)
Age (per decade)	2.09 (1.89–2.32)[b]	1.46 (1.37–1.57)[b]
Age^2 (per $decade^2$)	1.06 (0.97–1.15)	1.10 (1.04–1.16)[a]
Age × time (per decade, annually)	1.03 (0.99–1.06)	1.02 (1.00–1.05)[a]
Male sex	1.28 (1.08–1.51)[a]	1.16 (1.02–1.32)[a]
Smoker (present *vs.* never)	2.05 (1.63–2.57)[b]	1.60 (1.35–1.90)[b]
Smoker (former *vs.* never)	1.44 (1.161–1.79)[a]	1.24 (1.05–1.46)[a]
History of AMI	1.26 (1.07–1.48)[a]	1.20 (1.06–1.36)[a]
Diabetes	1.46 (1.21–1.76)[a]	1.34 (1.15–1.56)[a]
Statin user at entry	0.85 (0.72–1.00)	0.90 (0.79–1.02)

[#]Standard predictors: clinical predictors (sex, age, smoking status, hypertension, diabetes, previous AMI as opposed to angina only), medication (aspirin, beta-blocker, calcium-antagonist, ACE-inhibitors, long lasting nitrate, diuretic, digoxin, statins, anti-arrhythmics), standard biochemical predictors (log(CRP), ApoA1, log(ApoB), HDL-cholesterol, LDL-cholesterol, log (cholesterol), log(tri-glycerides), GFR).
AMI: acute myocardial infarction, CeVD: cerebrovascular disease, UAP: unstable angina pectoris, Log: $\log_{10}$, OPG: osteoprotegerin, Time: time to event since study entry.
[a] $p < 0.05$, [b] $p < 0.0001$.

had an elevated plasma OPG level *at entry* appears to gain prognostic force as blood sampling date glides into the past.

We see this unexpected feature as a sign that other risk factors are less stable and therefore lose predictive force over the years. Furthermore, indications are provided by Fig. 1, which depicts the OPG levels at trial entry against time of death during a 10-year follow-up, and grouped according to statin use at entry. The graph reveals some suggestive features, a tentative interpretation of which will be given in the Discussion.

Whilst participants with diabetes had an about 40% excess risk of death or composite outcome, treatment with statins at randomization was significantly associated with reduced risk of death by nearly 40% (without adjustments: HR 0.60 (0.51–0.70), $p < 0.0001$), by 18% after adjustment for standard predictors (HR 0.82 (0.68–0.98), $p = 0.03$), or, as shown in Table 2, by 15% even after adjustment for the variables of the "illustrative" model (HR 0.85 (0.72–1.00), $p = 0.05$). As to prognostic interaction between diabetes and statin use, none was found.

4. Discussion

The main result of the present study shows that elevated serum OPG levels hold a long-term independent association with all-cause mortality and a composite outcome in patients with stable CAD, even after adjustment for the standard predictors. The highest circulating OPG levels were detected in the participants with diabetes who died during follow-up. These findings are in concordance with previous findings [1,7]. This is also in keeping with the long-term (10–16 years) predictive power of OPG for all-cause mortality in patients with type 1 diabetes (T1D) [27,28] and type 2 diabetes (T2D) [29]. It is essential, when trying to interpret these findings, to note that an increase in serum OPG may be a response to, rather than a cause of atherosclerosis or vascular calcification, perhaps representing an attempt to regulate the processes.

The Cox survival analysis (Table 2) showed that circulating OPG levels appear to gain, rather than lose, predictive force as the time from blood sampling increases. Accordingly, in the graph of Fig. 1 one notes that the 10-year survivors showed a lower mean $\log_{10}$(OPG) than amongst those dying, even amongst those who died after several years of survival. In fact, those who died after 7–9 years, rather than showing a similarity with the 10-year survivors, had OPG levels nearly as high as early deaths. In other words, the OPG level is not so much a signal of approaching death; instead, an association exists with real long-term chances of survival. We have screened a dozen of newer biomarkers in the CLARICOR trial (Winkel et al., 2017 [20] and recently submitted, but as yet unpublished results (Winkel et al., 2020)), and have found cTnT and proBNP to be of most importance, followed by OPG, once the 'standard predictors' (including, e.g., CRP, see footnote Table 2) have been taken into account.

Statins have many beneficial pleiotropic effects, notably the normalizing lipid profile, reducing inflammation and improving endothelial function. In our study, an 18% reduction in mortality after adjustments for standard predictors of cardiovascular event was observed in the patients receiving statin treatment. Statins were prescribed (at study entry in 1999 or early 2000) to 41% of the participants [19], reflecting that 40% of participants without diabetes and 48% of participants with diabetes received statin treatment. In addition to having much better survival, statin users, as also made clear in the graph, have a consistently lower mean OPG level.

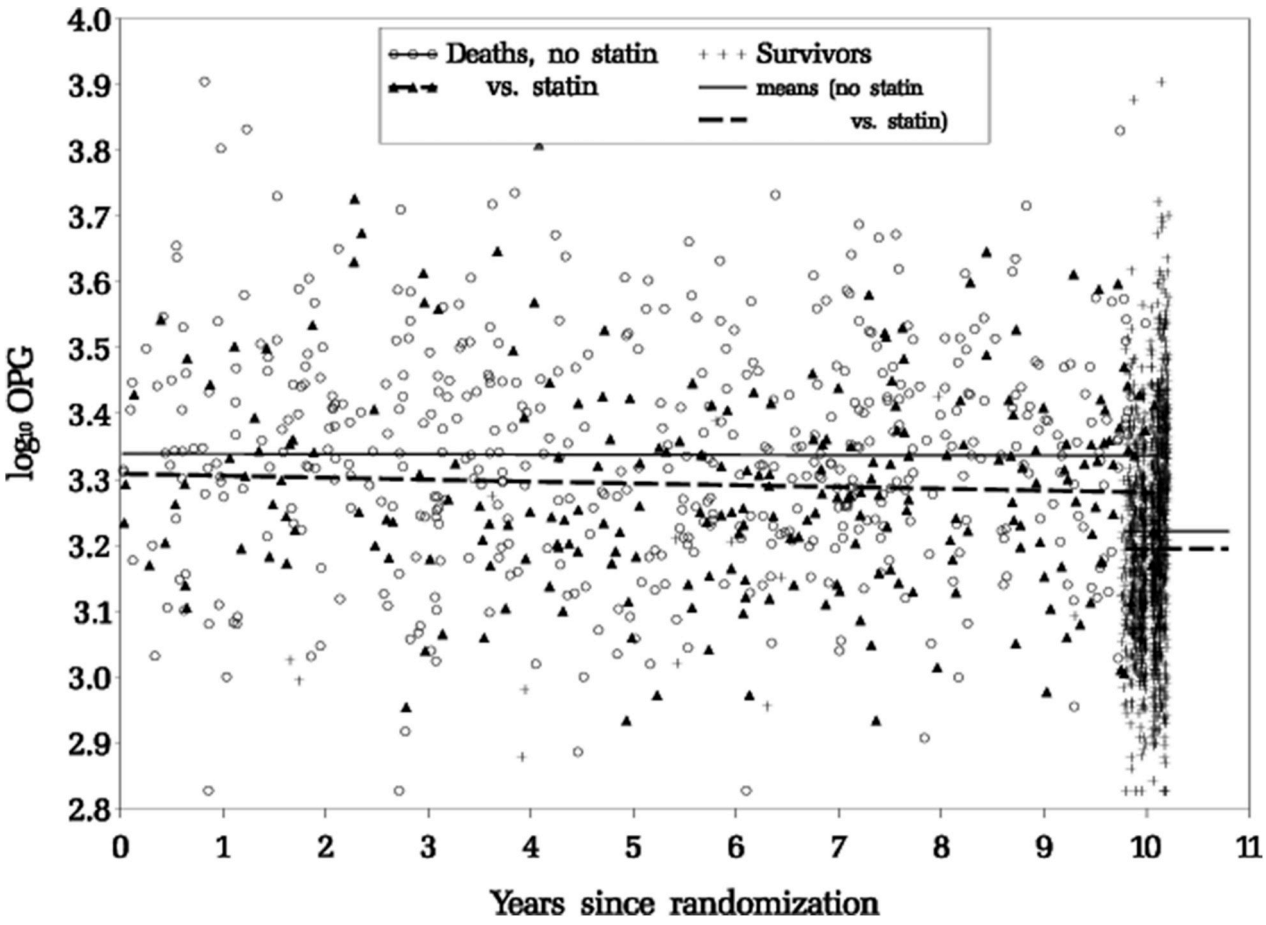

Fig. 1. OPG levels at trial entry against time of death during the 10-year follow-up.
OPG levels in statin users (triangles, mean OPG levels dashed line) and non-statin-users (circles, solid line) plotted against date of death or end of follow-up. Similarly portrayed are the corresponding means of the 10-year (± 3 months) survivors (+'s on the far right*).
*any "+" elsewhere is a dropout, e.g. due to emigration.

In participants without diabetes, a significantly lower OPG level was found in statin users than in non-statin-users. This was not observed in the participants with diabetes. A suppressive effect of statins on circulating OPG levels has been described by a few clinical statin intervention trials, in patients with and without diabetes. Kadoglou et al. reported a 47% reduction in OPG levels after 6 month of simvastatin therapy in ninety-seven statin-free patients newly diagnosed with stable CAD [16]. Davenport et al. showed a significant reduction in serum OPG levels after both 3- and 12-months treatment with atorvastatin in fifty-one patients with T2D [30]. The reduction in OPG levels correlated with a reduction of pulse wave velocity as an indication of reduced arterial stiffness. A small intervention study of 18 weeks treatment with low-dose simvastatin reported reduced OPG levels in eighteen T2D patients with microalbuminuria [13]. In contrast, an increase in OPG concentration after 90 days of lovastatin therapy (20 mg/day) has been reported in thirty patients with T2D, and OPG concentration returned to baseline 30 days after lovastatin withdrawal [31]. Additionally, an increase in circulating OPG levels in thirty patients with T2D and hypercholesterolemia was found after both 3 and 6 months of low-dose pravastatin [14]. In summary, different statins appear to affect OPG levels differently. Our data do not shed light on this question as the study is observational and the drug names, drug doses, or the duration of treatment were not recorded.

Returning to Fig. 1, a tentative interpretation may be that statins are dampening (the processes represented by) the production of circulating OPG; if the death-preventing effect of statins had acted in competition with said processes, one would have expected those who die despite statin use to have particularly high OPG levels. An association between OPG levels and use of statin may be explained by the anti-inflammatory effect of statins [32], such as reduction of CRP and the pro-inflammatory cytokines IL-6 and TNF-α, as the same circulating cytokines are known to enhance OPG production [33]. Ben-Tal Cohen and coworkers showed that statins counter-regulate OPG production in endothelial cells *in vitro* through TNF-α [34].

Together, these data suggest a role for statins in regulation of circulating OPG levels in patients with CAD. However, based on our observations, the mechanism may differ according to the presence or absence of diabetes.

4.1. Limitations

This study is an observational study and blood samples were only collected at study entry. Diabetes was self-reported, and the number of patients with diabetes may be underestimated. No information on the initiation of treatment with statins, formulations prescribed or dose is available. The participants were enrolled from 1999 to 2000, and prescription practice has increased since then. The trial primarily includes Caucasians, and no data on body mass nor longitudinal predictor data is available.

4.2. Conclusions

The present study shows that patients with stable CAD, but without diabetes, who use statins have a decreased level of circulating OPG. A

similar finding was not observed in patients with diabetes. Serum OPG holds a long-term independent association with serious disease manifestations, and all-cause mortality in particular, in CAD patients. The association persists for at least 10 years.

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CRediT authorship contribution statement

Mette Bjerre: Data curation, Writing - original draft. **Jørgen Hilden:** Formal analysis, Data curation. **Per Winkel:** Formal analysis, Data curation. **Gorm Boje Jensen:** Writing - review & editing. **Erik Kjøller:** Writing - review & editing. **Ahmad Sajadieh:** Writing - review & editing. **Jens Kastrup:** Writing - review & editing. **Hans Jørn Kolmos:** Writing - review & editing. **Anders Larsson:** Data curation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Johan Ärnlöv:** Data curation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Janus Christian Jakobsen:** Data curation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Christian Gluud:** Data curation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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