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Influence of pre-analytical and analytical factors on osteoprotegerin measurements☆

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ABSTRACT

Introduction: Osteoprotegerin (OPG), an osteoclastogenesis inhibitor implicated in bone remodelling, has emerged as a potential biomarker for cardiovascular disease. In order to implement OPG determination in the clinical laboratory, it is crucial to identify the most appropriate specimen type, preparation and measurement conditions. The present study focuses on identifying the pre-analytical variables that may influence OPG measurements.

Methods: Serum and plasma (in EDTA, heparin and citrate) were collected from 45 healthy volunteers (men ($n = 21$, 46.7%), women ($n = 24$, 53.3%)). OPG was analysed by ELISA. The influence of the centrifugation speed, the number of freeze–thaw cycles, delay in sample processing, thermo-stability and endogenous interfering agents (haemolysis, triglycerides, bilirubin, cholesterol and RANKL) were studied.

Results: OPG concentrations were significantly lower ($p < 0.0001$) in serum (1015 ± 357 pg/mL) than in all plasma samples (1314 ± 448 pg/mL in EDTA, 1209 ± 417 pg/mL in heparin and 1260 ± 498 pg/mL in citrate). Increasing centrifugation speed (200 g to 3000 g) did not change serum OPG concentration ($p = 0.88$). However, OPG concentration significantly increased when centrifuged serum samples were stored at 48 h at room temperature ($p < 0.0001$). Repeated freeze–thaw cycles did not modify OPG levels until 4 cycles ($p < 0.0001$). Increasing time before processing the samples (2 h and 6 h) raised OPG concentrations both at room temperature ($p < 0.0001$) or 4 °C ($p < 0.001$).
Positive concentration-dependent interference of triglycerides was found in the analysed pooled samples; however, OPG concentrations were falsely diminished with haemoglobin interference. Bilirubin, cholesterol and RANKL did not interfere with OPG measurements.

Introduction

Osteoprotegerin (OPG) is a soluble member of the tumour necrosis factor (TNF) receptor superfamily, originally discovered in 1997 as an inhibitor of osteoclastogenesis by two different research groups [1,2]. Biochemically, OPG is a basic glycoprotein composed of 380 aminoacids and seven structural domains which exist as a more active monomeric form (~60-kDa) and a homodimeric form [3].

OPG is a soluble decoy receptor for receptor activator of NF-κB ligand (RANKL) to prevent binding to the receptor activator of NF-κB (RANK) and as such regulates bone remodelling [4]. More recently the OPG/RANKL/RANK axis has been described to also play a role in carcinogenesis, cardiovascular pathophysiology, inflammation and immune response [5,6]. Several studies highlight a potential role for OPG in cardiovascular disease [7–9], atherosclerosis [10], plaque destabilization [11] and pulmonary hypertension [12]. In addition, increased serum or plasma OPG concentrations have been described associated with increased cardiovascular risk [13], with the presence and severity of cardiovascular disease [14], mortality [15–17] and with survival in idiopathic pulmonary arterial hypertension [18].

Enthusiasm raised by these studies for the potential use of OPG as a cardiovascular disease biomarker has been hampered by differences in the sampling methods and reported concentrations due to the lack of standardization. This has made interpretation of studies complex. Stability of the biomarker under various conditions has not previously been defined and pre-analytical and analytical variables that may affect the analyte or the assay must be considered [19].

Common sources of variability in clinical laboratories occur in the pre-analytical phase and they are related to sample collection (type of tube), transportation (length and environmental conditions), sample preparation for analysis (speed of centrifugation) and storage (length, temperature and freezing-thawing). Interfering substances can also be a significant source of error in clinical laboratory measurements and to date, little information exists regarding the effect of substances that interfere with OPG measurements.

The aim of the present work was to provide information about factors that may influence the concentrations of OPG by (1) comparing

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serum and different plasma types, (2) varying the number of freeze–thaw cycles, (3) changing the centrifugation speed, (4) modifying the time and temperature before processing the samples, (5) studying the thermo-stability and (6) analysing endogenous potential interfering factors (haemoglobin, bilirubin, cholesterol, triglycerides and RANKL) that may affect OPG quantification by enzymoimmunoassay (ELISA).

Methods

Blood samples

Samples were collected by veni-puncture in Vacutainer® tubes. Serum and plasma (collected in citrate, EDTA and heparin) were collected from healthy volunteers ($N = 45$, 46.7% men, median age 32 (28, 38) years). All subjects were free from cardiovascular disease based on the absence of history of coronary artery disease, stroke or peripheral artery disease. Besides, all healthy donors did not present any risk factor associated with cardiovascular disease such as hypertension, diabetes, dyslipidaemia or obesity and were not taking any medication. Other exclusion criteria were: patients with cancer, inflammatory disease, rheumatoid arthritis or osteoporosis. The Local Ethics Committee approved the study protocol and all participants gave written informed consent.

All samples were obtained in the same standard operating procedure. The extraction was performed by three trained nurses with experience and according to the guidelines established in our laboratory that it is accredited by the Joint Commission International. Sample extraction and preparation were supervised by Dr. Pérez de Ciriza to ensure standards and reproducibility was maintained throughout the whole pre-analytical and analytical process. Serum samples were allowed to clot for 30 min before centrifugation.

Unless otherwise stated, samples were centrifuged 2500 *g* for 7 min before storage. To reduce analytical variation, all samples were kept at −20 °C prior to analysis and analysed in the same run with the same lot of reagents and standards. Other conditions were studied in serum samples; each condition was assayed in a subsample of donors ($N = 7$).

Influence of sample preparation on OPG concentration

To determine the stability of OPG under several freeze–thaw cycles, OPG concentrations were measured immediately, or after one, two, three or four freeze–thaw cycles.

To determine the influence of centrifugation on OPG concentration replicate samples were centrifuged for 7 min at increasing speeds: 200 *g*, 400 *g*, 1000 *g*, 2000 *g*, 2500 *g* and 3000 *g*.

To evaluate how the time elapsed from blood collection to sample preparation influenced serum OPG levels, serum samples were centrifuged after 30 min, 2 h or 6 h of storage at room temperature or at 4 °C prior to measurement of OPG concentration.

Finally, to evaluate the stability of OPG in isolated serum, serum was maintained for 6, 24 or 48 h at room temperature or 4 °C after centrifugation prior to assaying for OPG concentration.

Analytical interferences on OPG measurement

Analytical interference of endogenous substances that can be found at high levels in human specimens such as haemoglobin, cholesterol, triglycerides, bilirubin and RANKL was subsequently determined in the OPG assay.

All the analytical interference studies were performed in serum pools from healthy volunteers ($n = 20$) as previously described [20].

Haemoglobin. For the haemoglobin assay two different protocols were used: 1) EDTA samples from healthy volunteers were used to prepare an erythrocytes lysate. Plasma was removed and cells washed twice using sodium chloride 0.9%. Supernatants were discarded after centrifugation and the cells were frozen at −80 °C, and thawed twice to lyse cells. After centrifugation, haemoglobin concentration was measured in an ABX Pentra 60 (Horiba ABX Diagnostics). Haemoglobin concentration was adjusted in the pool sample (level 5: 20 g/dL (12.4 mmol/L)) using the lysate. 2) Two 10 mL serum tubes were drawn from the same patient ($n = 3$). One was centrifuged at 2500 *g* for 7 min, serum removed and frozen at −20 °C until analysis. The other tube was frozen at −80 °C and thawed twice and vortexed to induce haemolysis. Haemoglobin concentration was measured and adjusted in the pool sample (level 5: 10 g/dL (6.2 mmol/L)) using the lysate.

Table 1
Concentration of the interfering agents.

Interferent	Level 1	Level 2	Level 3	Level 4	Level 5
Bilirubin (μmol/L)	6.8	68.4	130	190	251.4
(mg/dL)	0.4	4.0	7.6	11.1	14.7
Cholesterol (mmol/L)	4.6	6.1	7.6	9.1	10.6
(mg/dL)	178	235	293	350	408
Haemoglobin (mmol/L) POOL samples	0	3.1	6.2	9.3	12.4
(g/dL)	0	5	10	15	20
Haemoglobin (mmol/L) Individual samples	0	1.55	3.1	6.2	9.3
(g/dL)	0	2.5	5	7.5	10
Triglycerides (mmol/L)	0.86	2.1	4.17	4.88	6.22
(mg/dL)	76	190	314	432	551
RANKL (pg/mL)	0	1	2	4	8

Bilirubin, cholesterol, triglycerides and RANKL. Pool assay samples were kept frozen until analysis and supplemented with either exogenous bilirubin (Sigma, reference 14370, 0.4 to 14.7 mg/dL (6.84 to 251.4 μmol/L)), cholesterol (Acofarma, reference C27H460, 178 to 408 mg/dL (4.6 to 6.5 mmol/L) or RANKL (Biomedica, reference BI-20452, human recombinant sRANKL, 20 KDa, 0 to 8 pg/mL). A pool containing high triglyceride concentrations was used. Triglyceride concentration as well as cholesterol and bilirubin in the pool was quantified by standard laboratory techniques in a Cobas 8000 analyser (Roche).

Concentrations of all the interfering agents are summarised in Table 1.

A stock solution of each potential interfering agent was prepared using commercial reagents or serum containing high concentration of the agent as described above. Commercial reagents were dissolved according to their solubility. All solvents were tested before and a variability of less than 8% was considered adequate (data not shown). The concentration of all tested substances was determined in the control pool. Haemoglobin concentration was considered null in the level 1 sample.

Each concentration of each interfering agent was analysed three times together and in the same analytical run. Samples were analysed in random order to prevent carry-over or any systematic drift effects.

OPG measurement

Plasma and serum OPG were measured by enzyme linked immunosorbent assay (ELISA, R&D Systems, Duoset DY805) according to the manufacturer's instructions. In short, Maxisorp plates (Nunc Inc.) were coated overnight at room temperature with anti-human OPG antibody. Unbound antibody was washed away with 0.05% Tween 20 in PBS. Then, plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. After washing, samples were added and incubated 2 h at room temperature. After washing to remove unbound antibody, the biotinylated antibody was added and plates were incubated for a further 2 h. After further washing, streptavidin conjugated to horseradish peroxidase was added and plates were incubated 20 min at room temperature. After incubation, the optical density was measured at 450 nm. All the process was performed in an automated ELISA processor TRITURUS (Grifols) to reduce the variability and to

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