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Circulating osteoprotegerin as a biomarker in coronary heart disease and heart failure

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“Out of clutter, find simplicity. From discord, find harmony.
In the middle of difficulty lies opportunity.”

Albert Einstein

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Abbreviations

ACE1	Akershus Cardiac Examination study 1
ACE2	Akershus Cardiac Examination study 2
ACS	acute coronary syndrome
AUC	area under the curve
BNP	brain natriuretic peptide
CAC	coronary artery calcium
CAD	coronary artery disease
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
ECG	electrocardiogram
ELISA	enzyme linked immunosorbent assay
ESC	European Society of Cardiology
GISSI	Gruppo Italiano per lo Studio della Sopravvivenza nell'Insufficienza Cardiaca
HF	heart failure
HFmrEF	Heart failure with mid-range ejection fraction
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
JPD	Juvenile Paget's disease
kDa	kilo Dalton
LVEF	Left ventricular ejection fraction
MeSH	Medical Subject Heading
MERLIN	Metabolic efficiency with ranolazine for less ischemia in non – st elecation acute coronary syndrome
miRNA	micro RNA
MMP	matrix metallo-proteinase
MPI	myocardial perfusion imaging
NRI	net reclassification index
NSTE-ACS	non st elevation acute coronary syndrome
NT-proBNP	N-terminal pro B-type natriuretic peptide
OCIF	osteoclastogenesis inhibitory factor
OPG	osteoprotegerin
PCI	percutaneous coronary intervention

PET	positron emission tomography
PUFA	polyunsaturated fatty acids
PROCAM	Prospective Cardiovascular Münster
RCT	randomized controlled trail
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
SCORE	systematic coronary risk evaluation
SDS	summed difference score
SPECT	single-photon emission computer tomography
SRS	summed rest score
SSS	summed stress score
STEMI	st elevation myocardial infarction
TIMI	Thrombolysis in myocardial infarction
TIMP	tissue inhibitor of matrix proteinases
TNFR	tumour necrosis factor receptor
TNFRS11B	tumour necrosis factor receptor superfamily member 11b
TR1	tropine reductase 1
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
TR-IFMA	time-resolved immunofluorometric assays
VSCM	vascular smooth muscle cells

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List of papers

Publication I

Røysland, Ragnhild; Røsjø, Helge; Høiseth, Arne Didrik; Gullestad, Lars; Pirouz, Bader; Kravdal, Gunnhild; Omland, Torbjørn

Osteoprotegerin concentrations in patients with suspected reversible myocardial ischemia: Observations from the Akershus Cardiac Examination (ACE) 1 Study.

Cytokine 2015; 73(1), 122- 127

Publication II

Røysland, Ragnhild; Bonaca, Marc P; Omland, Torbjørn; Sabatine, Marc; Murphy, Sabina A; Scirica, Benjamin M; Bjerre, Mette; Flyvbjerg, Allan; Braunwald, Eugene; Morrow, David A.

Osteoprotegerin and cardiovascular mortality in patients with non-ST elevation acute coronary syndromes.

Heart 2012; 98(10), 786-791

Publication III

Røysland, Ragnhild; Masson, S.; Omland, Torbjørn; Milani, Valentina; Bjerre, M; Flyvbjerg, Allan; Di Tano, G; Misuraca, G; Maggioni, Aldo P.; Tognoni, G; Tavazzi, L; Latini, R.

Prognostic value of osteoprotegerin in chronic heart failure: The GISSI-HF trial.

American Heart Journal 2010; 160(2), 286-293

Publication IV

Røysland, Ragnhild; Pervez, Mohammed Osman; Pedersen, Marit Holmefjord; Brynildsen, Jon; Høiseth, Arne Didrik; Hagve, Tor-Arne; Røsjø, Helge; Omland, Torbjørn.

Diagnostic and prognostic properties of osteoprotegerin in patients with acute dyspnea: observations from the Akershus Cardiac Examination (ACE) 2 Study

PloS One 2016; 11(7), e0160182

1. Introduction

This thesis is based on four publications that evaluate associations between the circulating protein osteoprotegerin (OPG) and cardiovascular disease (coronary heart disease and heart failure). Coronary heart disease and heart failure (HF) are included in the broader group of cardiovascular diseases, which is defined by the WHO (1) as a group of disorders of the heart and blood vessels that include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease and deep vein thrombosis and pulmonary embolism. Cardiovascular disease is the leading cause of mortality worldwide. In 2012, 17.5 million people died from cardiovascular diseases, representing 31% of all global deaths. Of these deaths, 7.4 million were caused by coronary heart disease (1).

Due to increased knowledge of risk factors, better understanding of the pathobiology and improved prevention and treatment strategies for coronary heart disease and HF, age adjusted cardiovascular disease associated deaths have declined (2). Nevertheless, there has been an upward trend of hospitalization discharge rate for cardiovascular disease in Europe (3), and globally risk factors for atherosclerosis increase (4-7). Moreover with an aging population, the prevalence of HF has increased (8), and HF is the leading cause of hospitalization among adults above 65 years of age (9). Thus, providing good quality care for the people suffering from coronary heart disease and HF will be a challenge on our healthcare system in the years to come. Increasing our understanding of the process involved in atherosclerotic disease and in HF development, and developing and improving strategies and tools to identify and treat the right person at the right time, is important to meet those challenges.

2. Biomarker research and personalized medicine

2.1 What is a biomarker?

According to Vasan (10) the term biomarker (biological marker) was first introduced in 1989 as a Medical Subject Heading (MeSH) term: "measurable and quantifiable biological parameters (e.g. specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances) which serve as indices for health- and physiology-related assessments, such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse, pregnancy, cell line development, epidemiologic studies, etc.". In 2001, the formal definition of a biomarker was standardized by a National Institute of Health (NIH) working group, and was stated as follows: "a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (11). This definition includes a wide variety of measurable quantities, however in the context of this thesis, the word biomarker will refer to circulating substances measurable in bodily fluids, mainly in the blood.

2.2 General rationale for cardiac biomarker research

Cardiac biomarkers have helped refine our understanding of the pathobiological mechanisms of cardiovascular disease and improved patient care (12). For instance, measuring cardiac troponin is essential for diagnosing myocardial infarction (13) and knowing the levels of natriuretic peptides improves clinicians' ability to identify patients with HF (14). Our current knowledge of risk factors for cardiovascular disease helps us in detecting patients at high risk of developing coronary heart disease. However traditional risk factors do not fully explain the incidence of cardiovascular disease in the general population. Moreover global risk assessment scores like the Framingham Score (15), the PROCAM Score (16) and the European Society of Cardiology SCORE (17) report a c-index of 0.76-0.82, i.e. when randomly selecting two individuals from a population, these prediction models will correctly assign the highest risk to the right person 76-82% of the time. Hence, when using these risk tools, a considerable number of patients will still not be classified correctly. Moreover silently progressing cardiovascular disease will not be discovered before developing of overt disease. In patients with known coronary heart disease Khot et al. reported that 15-20% lack any of the

traditional risk factor (18). Myocardial infarction causes irreversible damage to the heart, and one way of decreasing morbidity, HF development and mortality due to this condition, is by preventing the first heart attack. In other words, if we improve our ability to predict cardiovascular disease or recognize subclinical changes that eventually lead to cardiovascular disease, we might postpone or avoid morbidity and mortality caused by this condition. Novel and emerging biomarkers are of considerable interest to improve risk estimation beyond traditional risk scores, to improve decision-making and guide therapy, and to increase our understanding of pathobiological mechanisms involved in cardiovascular disease.

2.3 Characteristics of a clinically useful biomarker

The dramatic increase in the number of research articles focusing on biomarkers of HF have been described as a tsunami (19). The large number of articles published and novel biomarkers introduced, have made this research field complex and unclear and have urged the need for quality standards for the reporting of biomarker research, as well as strategies to evaluated the usefulness of novel biomarkers. In a review of biomarkers for cardiovascular disease, Vasan described a set of general characteristics that are important for an ideal biomarker regardless of its intended use (10). The biomarker should be accurate, reproducibly obtained in a standardized fashion, acceptable to the patient, easy to interpret, have a high sensitivity and specificity for the outcome, in addition predictive value, likelihood ratio, low cost, explain a reasonable proportion of the outcome, consistently, and data to suggest that knowledge of the biomarker level changes management. Morrow and de Lemos emphasized in an editorial in 2007 (20) that for a biomarker to have potential as a clinical tool, three questions needs to be answered: (1) Can the clinician measure the biomarker? (2) Does the biomarker add new

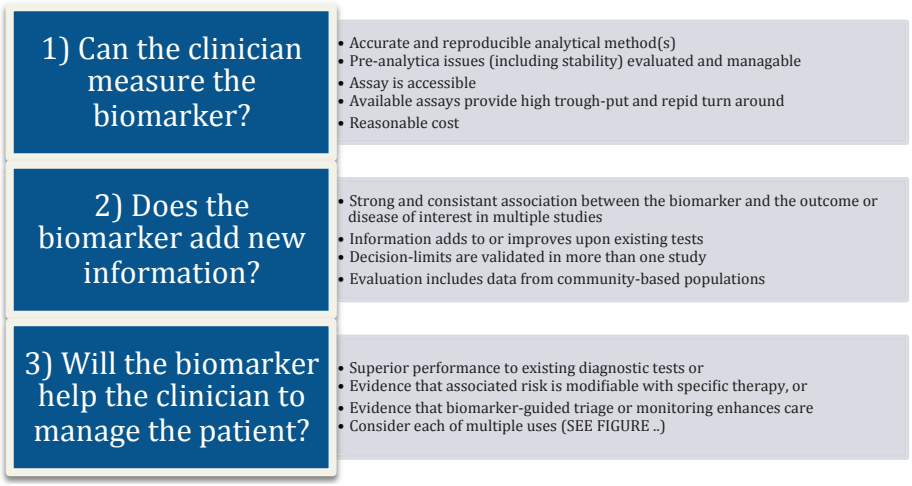


Figure 1 Criteria for assessment of novel cardiovascular biomarkers for clinical use. Figure is adapted from Morrow et al. Circulation 2007.

information? (3) Will the biomarker help the clinician to manage the patient? (Figure 1, page 11)

2.4 Phases of biomarker research

How do we make the move from a promising novel biomarker to useful clinical tool? It has been estimated that more than 150,000 publications document thousands of claimed biomarkers, but fewer than 100 have been validated for routine clinical practice (21). In 2001 Pepe suggested a pipeline for biomarker research for the early detection of cancer with the final goal of developing biomarkers for cancer screening (22). Vasan adapted this for cardiovascular biomarker research, and introduced five phases of biomarker development, from discovery to delivery (Figure 2) (10). This is a good overview to keep in mind when reading articles about different biomarkers. And it shows the considerable research effort needed for a biomarker to reach clinical use. Most biomarker known at the present will probably not reach phase 5.

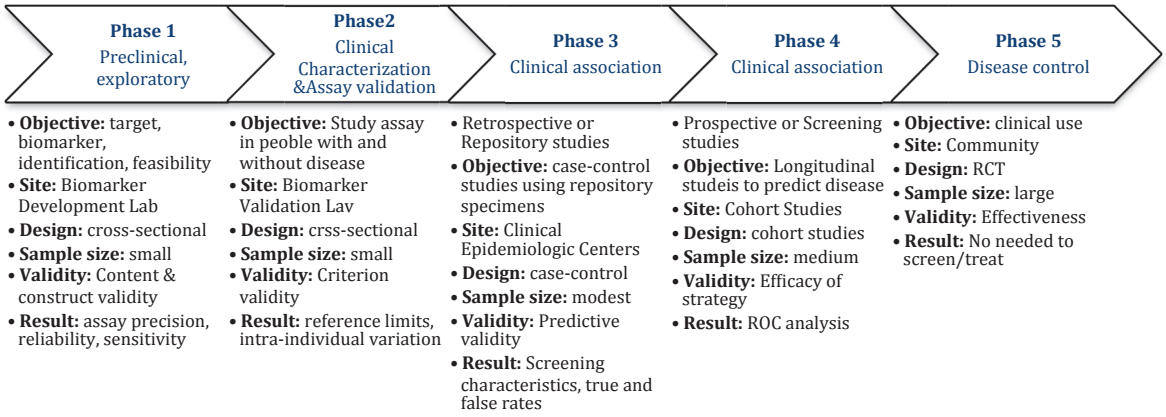


Figure 2 Phases of cardiovascular biomarker research. Figure is adapted from Vasan Circulation 2006.

3. Coronary heart disease and heart failure

3.1 Atherosclerosis

Atherosclerosis is the principal cause of coronary heart disease, a main cause of HF, and a leading cause of cardiovascular disease. It occurs in the medium- and large-sized elastic and muscular arteries throughout the body, and is a slow, progressive process (Figure 3). Stiffening, narrowing and obstruction of the arteries can eventually lead to myocardial ischemia and infarction, causing coronary heart disease, stroke or peripheral vascular disease. Endothelial dysfunction is thought to be the initial step of the atherosclerotic process (23).

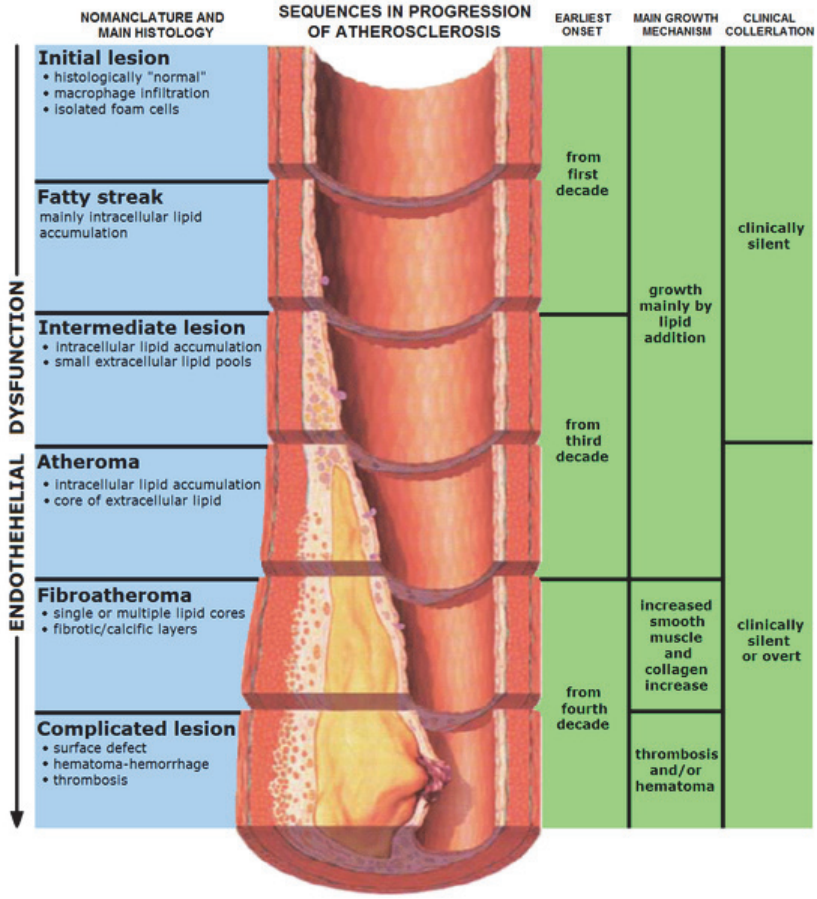


Figure 3 The progression of atherosclerosis. Used under CC BY-SA 3.0, from Wikipedia.

The endothelium is the single layer of cells lining the vessel wall. These cells represent a barrier between the blood and the different tissues in the body and play a key role for regulating vascular homeostasis (24). Endothelial dysfunction refers to abnormal behaviour of this barrier and is thought to arise as a consequence of different stressors. Disturbances in the

homeostasis of the endothelium leads to increased permeability and adhesiveness of inflammatory cells. In addition, the endothelium's anticoagulant ability is changed to a procoagulant state with the increased production of vasoactive molecules, cytokines, and growth factors. If the stress on the endothelium continues, inflammatory cells will accumulate in the intima layer of the vessel wall and smooth muscle cells will start to proliferate, signalling that the atherosclerotic processes have started (25).

For a long time atherosclerosis was mainly considered a fat/cholesterol deposit disease, but the understanding of atherosclerosis as a chronic inflammatory disease has received more support in the last decades (23). Classical risk factors for atherosclerosis include age, high blood pressure, smoking, high cholesterol and diabetes (26). These traditional risk factors, however, do not explain all the aspects of the development of atherosclerosis, and in 1999, Ross discussed inflammation as an important driver of the atherosclerotic process, especially for the 50% of patients with cardiovascular disease without hypercholesterolemia (23). Ross concluded that "if we can selectively modify the harmful components of inflammation in the arteries and leave the protective aspect intact" this might give new avenues of diagnosis and management of atherosclerotic disease (23).

3.2 Heart failure and ventricular dysfunction

3.2.1 Definition

HF is a complex clinical syndrome and there is continuing debate on how best to define HF (27). In the European Society of Cardiology (ESC) guidelines for the diagnosis and treatment for acute and chronic HF from 2016, a clinical definition is used: "HF is a clinical syndrome characterized by typical symptoms (e.g. breathlessness, ankle swelling and fatigue) that may be accompanied by signs (e.g. elevated jugular venous pressure, pulmonary crackles and peripheral oedema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/ or elevated intracardiac pressures at rest or during stress." (28).

3.2.2 Terminology

There is no single diagnostic test for HF, but historically the main terminology used to describe HF is based on measures of the left ventricular ejection fraction (LVEF) (28). In the 2016 ESC guidelines HF is divided into three groups: HF with preserved (HFpEF), mid-range

(HFmrEF) and reduced ejection fraction (HFrEF) (28). The Table 1 sums up criteria HFrEF, HFmrEF and HFpEF, and is adapted from the 2016 ESC guidelines (28). It is important to differentiate patients according to LVEF because HFpEF have different aetiology and different response to therapy
than HFrEF (29). At the moment no specific treatment exists for HFpEF (29), and it is only in HFrEF that therapies have been shown to reduce both morbidity and mortality (28).

Table 1. Types of heart failure.

Types of HF		HFrEF	HFmrEF	HFpEF
CRITERIA	1	Symptoms±Signs	Symptoms±Signs	Symptoms±Signs
	2	LVEF < 40%	LVEF 40-29%	LVEF ≥ 50%
	3	-	1. Elevated levels of natriuretic peptides* 2. At least one additional criterion: a. relevant structural heart disease (left ventricular hypertrophy and/or left atrial enlargement) b. diastolic dysfunction	1. Elevated levels of natriuretic peptides* 2. At least one additional criterion: a. relevant structural heart disease (left ventricular hypertrophy and/or left atrial enlargement) b. diastolic dysfunction

* Signs may not be present in the early stages of heart failure (especially in HFpEF) and in patients treated with diuretics.
** BNP > 35 pg/ml and/ or NT-proBNP > 125 pg/ml

3.2.3 Aetiology and models of heart failure development

In people >65 years of age presenting with dyspnoea during exercise in primary care, one in six will have unrecognized HF (30). In subjects aged 55 years, women have 28% and men 33% lifetime risk of developing HF (31). Hypertension, coronary heart disease, valvular disease and cardiomyopathy are the most common causes of HF, with hypertension and coronary heart disease being attributable to more than 70 % of cases (32). According to Braunwald, several perspectives or models can be used to describe how HF develops (33). A short summary of the different models is presented below.

Models of HF development:

- The **haemodynamic model** refers to the observations that in failing hearts an increase in haemodynamic load on the ventricle is followed by a reduction in the contractility of the cardiac muscle. This is opposite to what is found in normal functioning hearts. Ventricular remodelling is an important cause of the hemodynamic changes, and the lack of compensatory increased contractility in HF.
- The **extracellular matrix model** refers to changes in the architecture of the ventricles and how myocardial muscle cells might be replaced by scar tissue and stiff fibrous

tissue after damage caused by e.g. myocardial infarction. The imbalances of enzymes that break down e.g. matrix metallo-proteinase (MMP) and inhibits breakdown e.g. tissue inhibitor of matrix proteinases (TIMP) of extracellular matrix are important for remodelling of the ventricle after injury.

- The **cardiorenal model** refers to renal sodium and water retention that causes the classical symptoms of HF, namely oedema and dyspnoea. Treatment with diuretics and sodium restriction are crucial to the management of congestion in HF.
- The **neurohumoral model** refers to the vicious cycle caused by prolonged activation of the sympathetic nervous system and the renin-angiotensin-aldosterone system. In acute HF these mechanisms maintain arterial pressure and cardiac function. In chronic HF however, these mechanisms cause maladaptive hypertrophic remodelling and apoptosis. The increased survival seen in HF patients treated with beta-blockers and angiotensin converting enzyme inhibitors/angiotensin II type 1-receptor blockers and aldosterone receptor blockers, have underscored the importance of this model.
- **Abnormal Ca^{2+}-cycle model** refers to dysfunction in mechanisms controlling Ca^{2+} influx and release from the sarcoplasmic reticulum in the heart muscle cells. Pathology in this systems may both cause decreased contractile strength of the ventricle leading to systolic dysfunction, but may also influence the relaxation of the ventricle causing decreased filling of the ventricle and leading to diastolic dysfunction.
- The **cell-death model** refers to the observation that all types of HF are characterized by an increased rate of myocardial cell death. Many different stressors are thought to be responsible of increased apoptosis including up-regulation of neurohormonal systems, inflammation, oxidative stress, toxins and infiltrative processes. In addition, HF develops after myocardial necrosis caused by myocardial infarction, but severe incidents of myocardial ischemia and toxins like doxorubicin may also cause myocardial necrosis.
- The **genetic model** refers both to monogenic disorders causing cardiomyopathies that lead to HF, but also to the genetic variants for specific diseases that lead to HF e.g. coronary artery disease (CAD), hypertension, hyperlipidaemia and diabetes mellitus. A relatively young research field looks into the small (~22-nucleotide) RNAs, or micro RNAs (miRNAs) and their role in health and diseases leading to HF.

3.3 Biomarkers of atherosclerosis and heart failure

Blood borne substances present during different steps of inflammation can represent clinically useful biomarkers if they provide non-overlapping information regarding diagnosis, management or risk stratification. For instance, inflammatory substances can be used as diagnostic biomarkers reflecting different mechanisms and stages of the atherosclerotic process, namely (1) endothelial dysfunction, (2) the initiation of atherosclerosis, (3) stable CAD and progressing atherosclerosis, (4) unstable plaque and the acute event of myocardial infarction (5) cardiac ischemia and ischemia-reperfusion injury and (6) ischemic cardiomyopathy and HF development. According to Braunwald, biomarkers that are currently available reflect at least seven pathobiological processes operative in HF, namely myocardial stretch, myocyte injury, matrix remodelling, inflammation, renal dysfunction, neurohumoral activation and oxidative stress (33). At the moment only biomarkers of myocardial stretch/stress (BNPs), myocyte injury (cardiac troponins), renal dysfunction (creatinine, cystatine c) and generalized inflammation (CRP) are commonly measured in the clinical setting when evaluating patients with HF. Figure 4 shows presently used biomarkers, and biomarkers potentially useful in the future, that are in the pipeline of biomarker development in HF (34).

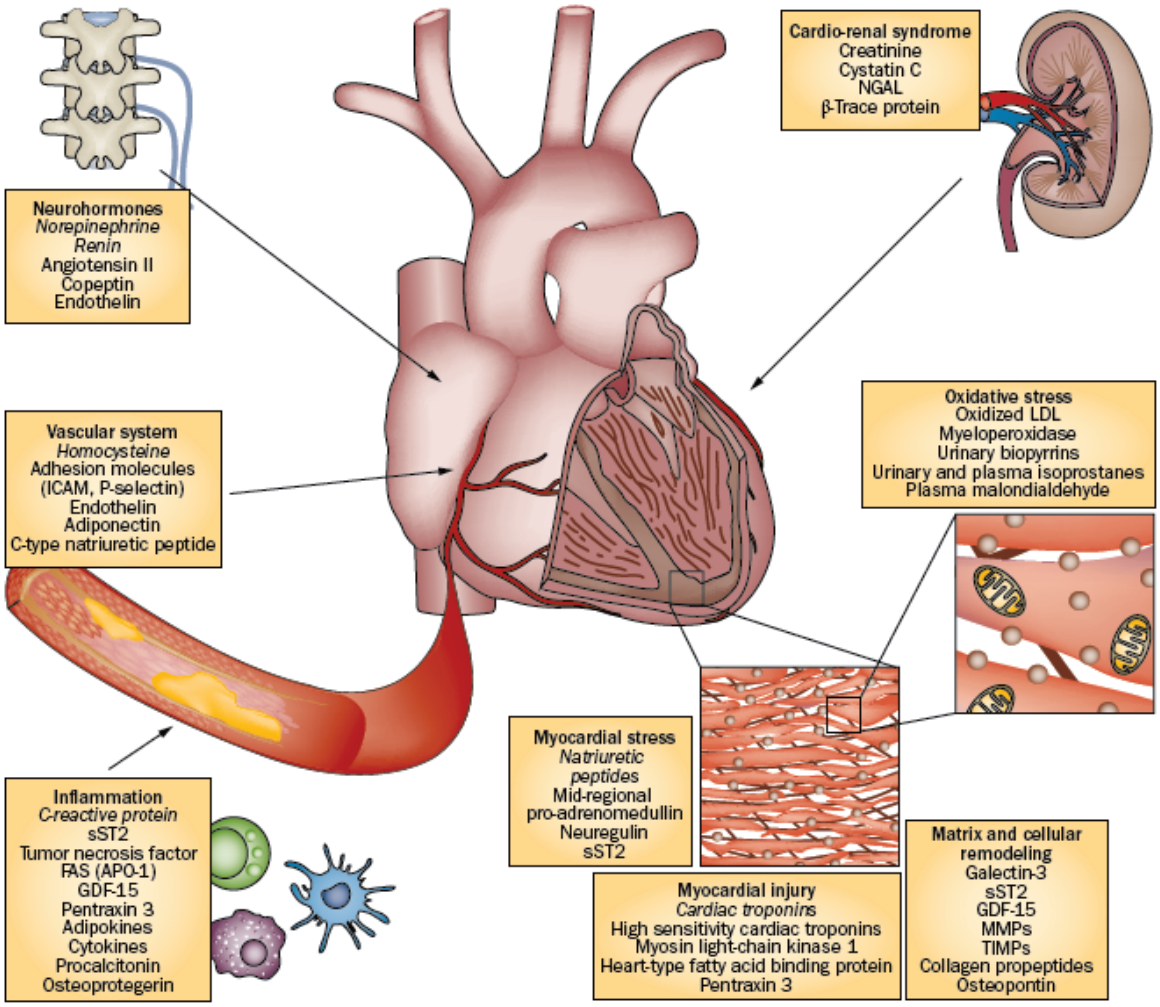


Figure 4. Biomarkers in chronic heart failure. From Ahmad et al. Nat Rev Cardiol 2012. Reprinted with permission from RightsLink.

4. Osteoprotegerin – an introduction

OPG is an extracellular protein that can be found and measured in almost all bodily fluids and in many human tissues. Giving a complete overview of the roles OPG plays in health and disease is beyond the scope of this thesis. In this chapter some important properties of OPG in relation to heart and cardiovascular disease will be discussed, and a main focus will be on OPG in the circulation.

4.1 Discovery of OPG

OPG is a glycoprotein of the tumour necrosis factor receptor (TNFR) superfamily and was first described in 1997. In contrast to most of the other TNFR super family members, OPG is not membrane bound, and is thought to exist only as a secreted molecule (Figure 5). OPG was discovered almost at the same time by different groups (35-38), but the discovery was first published by Simonet et al.(38). They demonstrated that OPG is an important protein in bone metabolism by showing that transgenic mice with an overproduction of OPG developed osteopetrosis, a condition with densification and hardening of the bone structure. In 1997, a functional effect of a TNFR member on bone, would not have been predicted, and the revelation of the OPG/RANKL/RANK-system is an example of a successful story based on "discovery driven" research (39). The uncovering of OPG was a result of the general research focus of the industry at that time, on developing new therapeutic agents from novel secreted proteins (40), based on knowledge from extensive sequencing of cDNA libraries.

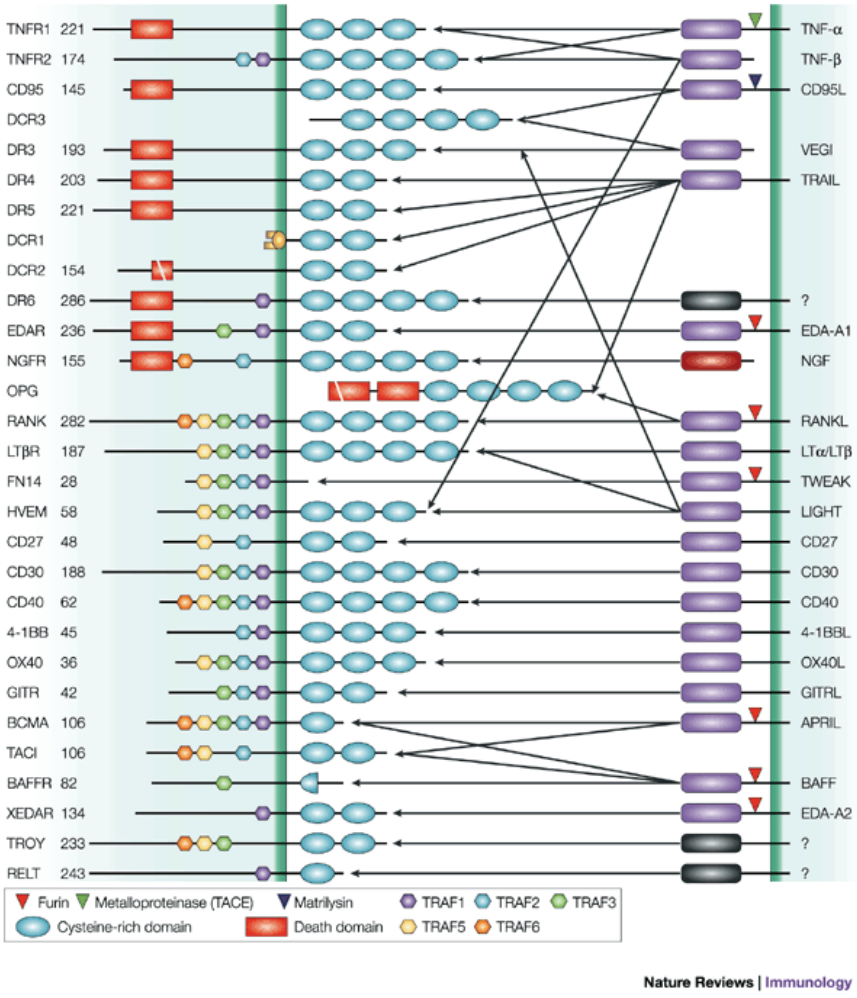


Figure 5. A diagrammatic representation of the ligands of the TNF superfamily and their receptors. From Aggarwal et al. Nat Rev Immunol 2003. Reprinted with permission from RightsLink.

The elucidation of the OPG/RANKL/RANK-signalling system has been described as an important milestone in the history of osteology (41), because it led to the finding of the long sought-after osteoclast differentiating factor (ODF, now called RANKL). In addition, it also led to the development of the recombinant RANKL-targeted antibody denosumab (40), a therapeutic agent, a bone resorption inhibitor, for treating lack of sex hormone induced osteoporosis. Nevertheless, research on this system soon uncovered that bone was not the only tissue affected by a disturbance in OPG production. Already in the second article published by the same group, Simonet and colleges, demonstrated that mice in which the OPG gene expression was "knocked out", not only developed osteoporosis but also calcification of the large arteries (42). This finding was a trigger of the great effort of research during the last fifteen year of understanding the true relationship between the OPG/RANKL/RANK-system and cardiovascular disease in human subjects.

Table 2. Cytokines, growth factors and hormones that can regulate OPG expression.

Cytokine/ growth factor/ hormone	OPG levels	Cell type/tissue	Referanse
IL-1α	↑	ECs	(44)
IL-1β	↑/↓	ECs, fibroblasts, SMCs, osteoblasts, DCs, bone marrow stromal cells	(53), (54), (55), (56), (57), (45)
IL-6	↑/↓	Calvaria, bone marrow stromal cells	(54), (58)
IL-11	↑/↓	Osteoblasts, calvaria, bone marrow stromal cells	(59), (60), (54)
IL-17	↓	Bone marrow stromal cells	(54)
IL-18	↑	Bone marrow stromal cells, osteoblasts	(61)
TNFα	↑	ECs, Smooth muscle cells, fibroblasts, DCs	(44), (56), (55), (45), (57)
BMP-2	↑/↓	Smooth muscle cells, osteoblasts,	(53), (62)
BMP-4	↑	Bone marrow stromal cells	(63)
BMP-7	↓	Smooth muscle cells	(62)
TGF-β	↑/↓	Bone marrow stromal cells, osteoblasts, Smooth muscle cells	(62), (64), (65)
bFGF	↑/↓	Smooth muscle cells, osteoblasts	(66), (45)
PDGF	↑	Smooth muscle cells	(45)
IGF-1	↓	Bone marrow stomal cells	(67)
Estrogen	↑	Smooth muscle cells	(68)
Estradiol	↑	Osteoblasts	(69)
Vitamin D3	↑	Osteoblasts	(53)
PTH	↓	Osteoblasts	(70), (71)
PTHrP	↓	Osteoblasts	(70)
PGE_2	↓	Osteoblasts	(72)
Glucocortico -steroides	↓	Osteoblasts	(73), (74),

IL = interleukin; EC = endothelial cells; SMC = smooth muscle cells; DC = dendritic cells; TNF = tumor necrosis factor; BMP = bone morphogenic protein; TGF = transforming growth factor; bFGF = basic fibroblast growth factor; PDGF = platelet-derived growth factor; IGF = insulin-like growth factor; Parathyroid hormone = PTH; Parathyroid hormone–related protein = PTHrP; PG = prostaglandin.

4.2 Synthesis, metabolism and degradation of OPG

OPG is an extracellular protein and the highest levels of OPG mRNA are found in the lung, heart, kidney and placenta, but there are also detectable levels in various hematopoietic and immune organs (38). In vascular tissue, gene expression of OPG is found in the heart as well as in arteries and veins (43). Both endothelial cells and vascular smooth muscle cells expresses OPG, (44-46) and strong immunostaining for OPG protein was seen in myocardial

tissue (47). Table 2 is adapted from tables presented by Reid et al. (48) and Venuraju et al. (49), and gives an overview of cytokines, growth factors and hormones that can regulate OPG expression. High amount of OPG protein is found in the vascular wall. Tissue extracts from human aorta have shown a concentration of OPG as high as that found in human bone extracts (50). When it comes to circulating levels of OPG, the concentration in blood is more than 100 times lower than in bone and arterial tissue (51). The main source of circulating OPG, however, is not known (52). Whether OPG has hormonal properties in the circulation and is absorbed by a target tissue, or whether circulating OPG "overspills" from tissues where OPG has paracrine functions is not clear.

In the skeletal and the immune system, OPG is mainly secreted by bone stromal cells, osteoblastic lineage cells and dendritic cells (75). OPG is a glycoprotein with 401 amino acids per monomer, giving a monomeric molecule weight of approximately 60 kDa (Figure 6). In vivo OPG is found both in the monomeric and the dimeric form. The dimeric form has been considered the most biologically active, and natural OPG exists predominantly as disulphide-linked dimers of approximately 120 kD (Figure 6). Little information is available regarding the mode of degradation, clearance and elimination of OPG from the circulation. OPG levels increase with decreasing glomerular filtration rate (76), and OPG cannot cross a haemodialysis membrane (77). As OPG is a relatively large protein of 60 kD (monomer), it is unlikely to be able to cross the kidney barrier in the physiological state. The OPG half-life and function in the circulation has not been well characterized (78), but Tomoyasu et al. found that the half-life of OPG in the circulation, both as a monomer and a homodimer, was 30 min or less.

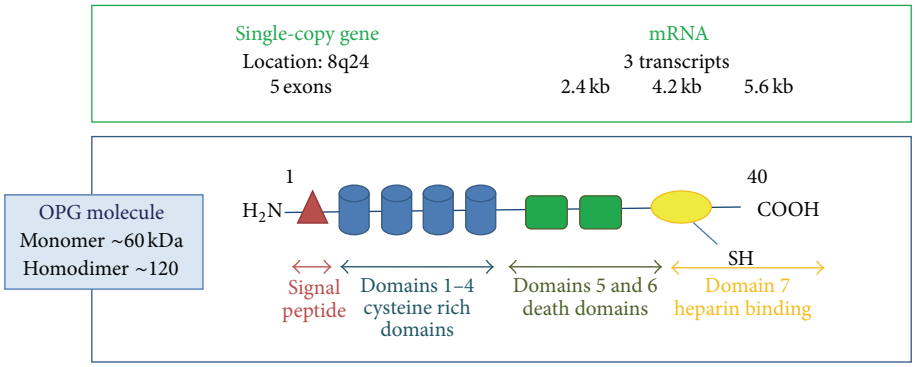


Figure 6. OPG structure. Modified from figure presented by Perez de Ciriza et al. Int J Endocrinol 2015 and used under CC BY.

4.3 Physiological role of OPG

From early on, OPG was also known as osteoclastogenesis inhibitory factor (OCIF) (37), tumour necrosis factor receptor superfamily member 11b (TNFRS11B) and tropine reductase 1(TR1) (36), but in 2000 a committee decided that OPG was the preferred name (79). OPG has been implicated in bone remodelling, immune functioning and vascular biology (43). OPG acts as a decoy receptor and blocks the binding of two known ligands to their receptors, namely receptor activator of nuclear factor-kappaB ligand (RANKL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Figure 7). The physiologic role of OPG is not fully elucidated, but is closely connected to the physiological role of RANKL and TRAIL.

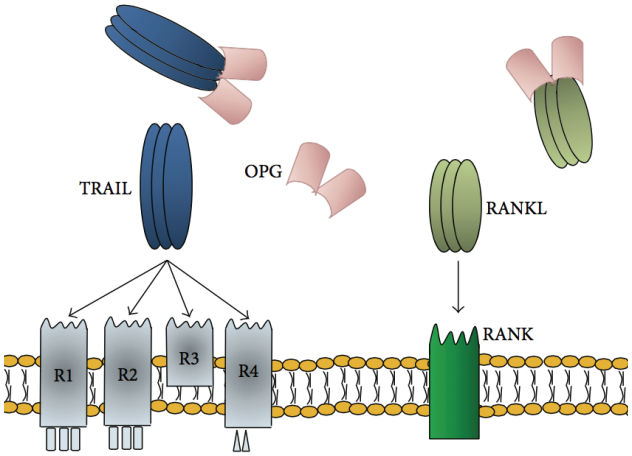


Figure 7. OPG, TRAIL and RANKL and their receptors. Modified from Bernardi et al. Biomed Res Int 2016 and used under CC BY.

4.3.1 Bone physiology

Osteoprotegerin means a "protector of bone", and the physiological role of OPG in bone metabolism have been most widely studied. The OPG/RANKL/RANK-system is important for coordinating the balance of bone "production" by osteoblasts and bone degradation by osteoclasts (Figure 8). When OPG inhibits the RANKL-RANK interaction, the consequence is (1) maturation of osteoclasts is slowed down, and (2) osteoclast bone degradation is slowed down due to a lack of direct stimulation. The RANKL/RANK binding is considered a final step in osteoclast differentiation and activation, giving OPG a crucial role in the regulation of this fine tuned orchestra of bone remodelling (80).

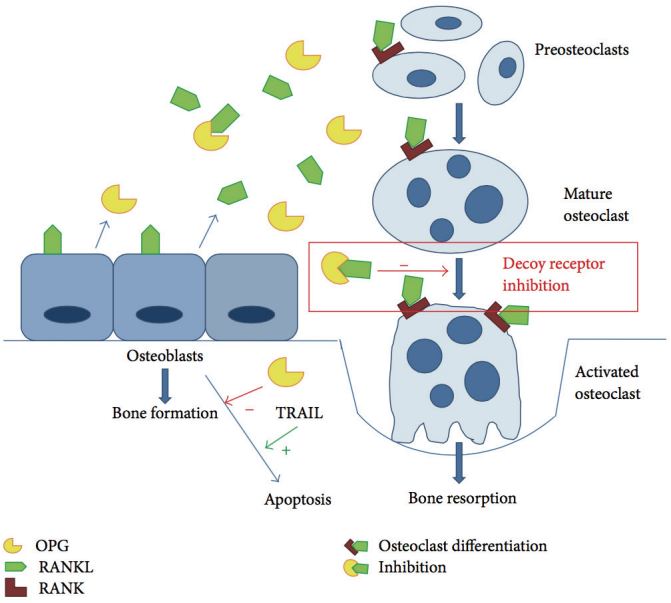


Figure 8. The OPG/RANKL/RANK-system and bone metabolism. Modified from Perez de Ciriza et al. Int J Endocrinol 2015 and used under CC BY.

4.3.2 Vascular physiology

Physiological effects of OPG on the vascular system are not well characterized. However, OPG and RANKL have been indicated to play a role in extra-skeletal calcium handling, and OPG has been suggested to possess protective properties against vascular calcification (81). In vascular tissue OPG is produced in endothelial and smooth muscle cells. Within the endothelial cells, OPG is synthesized and stored in secretory granules and is co-localized with von Willebrand factor (vWF) (46). In vitro, OPG can make complex with vWF and be secreted in response to TNF-α and interleukin-1β (IL-1β)(46). The OPG-vWF complex is present and can be measured in the human circulation, but the physiologic role of OPG in this setting is not known (46). Several studies have demonstrated that OPG can promote cell survival both for endothelial cells and vascular smooth muscle cells (82-84), and might thereby play a role in vascular homeostasis. The significance of OPG/TRAIL interaction on vasculature is even less understood than the OPG/RANKL/RANK-system. Endothelial cells and vascular smooth muscle cells (VSCM) both express TRAIL receptor 1 and 2. TRAIL activates vascular cell apoptosis, and OPG might influence cell survival and vascular remodelling by blocking the action of TRAIL (83, 84).

4.3.3 Immune physiology

OPG have also important functions in the immune system. OPG and RANKL have been found to be essential for dendritic cell functioning, lymph node organogenesis and

lymphocyte development (43). OPG deficient mice have altered B-cell maturation and lack the ability to develop an efficient antibody response (85). Mice lacking RANKL or RANK have defective T-cell and B-cell maturation, and lymph nodes do not develop (43). TRAIL is expressed on the surface of activated immune cells, such as natural killer (NK) cells, T-cells, macrophages and dendritic cells (86). It functions as an immune effector molecule, mediating antitumor cytotoxicity and immune surveillance (86). TRAIL can bind to four TRAIL receptors (Figure 6,
page 23). Two of the receptors are decoy receptors like OPG, but the two receptors (TRAIL receptor 1 and 2) contain a cytoplasmic death domain and binding of TRAIL to these receptors induce apoptosis in the cells (87).

4.4 OPG and bone disease

In 2002 Whyte et al. discovered that a homozygous deletion of the gene that encodes OPG causes hereditary hyperphosphatasia or Juvenile Paget's disease (JPD) (88), an extremely rare condition with approximately 60 reported cases worldwide (89). "OPG deficiency" due to a mutation in the OPG gene is not the only cause of JPD, but it is the most common cause (90). It is usually diagnosed in early childhood and causes bone deformity, fracture and bone pain, in addition to premature loss of teeth and deafness (88). In addition to inherited skeletal diseases, the OPG/RANKL/RANK- system is involved in metabolic bone diseases, immune-mediated bone diseases and malignant diseases affecting bone. A summary of different bone diseases mediated by the OPG/RANKL/RANK-system is presented in the Table 3 (previous page), which is adapted from a review by Hofbauer et al. (75).

Table 3. OPG/ RANKL/ RANK – mediated bone diseases

Metabloic bone diseases
Postmenopausal osteoporosis
Glucocorticoid-induced osteoporosis
Hyperparathyroidism
Sporadic Pagets disease
Immune-Mediated Bone diseases
Rheymatoid arthritis
Peridontal infection
Malignant diseases
Myeloma Bone disease
Osteolytic bone metastases
Humoral hypercalcemia of malignancy
Inherited skeletal disease
Familial expansile osteolysis/ Familial Pagets Disease
Expansile Skeletal Hyperphosphatasia
Juvenile Pagets Disease

4.4 OPG and cancer

In vitro studies have found that breast, prostate and colon carcinoma cell lines are able to produce sufficient amounts of OPG to protect them against TRAIL-induced apoptosis (91). In human studies, increased serum levels of OPG have been found in for instance colo-rectal, pancreatic, squamous cell head and neck cancer (92, 93). Moreover increased OPG levels were associated with the presence of bone metastasis in breast, lunge and prostate cancer (94).

In contrast, in advanced stages of myeloma cancer, patients had lower levels of serum OPG (95). Recently, in a population-based cohort from Tromsø in Norway, circulating OPG was associated with increased risk of incident gastrointestinal cancer, inversely associated with breast cancer, and predicted cancer-related mortality (96).

4.5 OPG and vascular disease

Circulating levels of OPG has from early on been associated with vascular disease in human studies. Tissue levels of OPG have been linked to inflammation and stability of atherosclerotic plaques, calcification of the atherosclerotic plaques, and calcification and bone formation in vasculature in general including cardiac valves (51). Early studies reported OPG to be present in atherosclerotic lesions (97, 98), and it was suggested that OPG gene expression might vary with plaque type and the stability of plaques (99). Although theoretically OPG may protect against vascular calcification and bone formation on the tissue level, most human studies have linked higher circulating OPG levels to increased disease activity and adverse prognosis in cardiovascular disease. In 2001, in a prospective study of elderly women, higher OPG levels were associated with the presence of diabetes and with the risk of cardiovascular mortality (100). In a population-based study, in 2004 Kiechl et al. studied OPG levels in 915 people from the Italian Bruneck study. OPG levels were associated with 10-year progression of carotid atherosclerosis, as well as incident cardiovascular disease and vascular mortality (101). Several studies have linked OPG levels to the extent of CAD in patients referred to angiography due to suspect CAD (102, 103). In 2010, Lieb et al. found OPG levels to be associated with cardiovascular mortality and cardiovascular risk factors like age, smoking, diabetes, systolic blood pressure and prevalent cardiovascular disease, in 3250 participants from the Framingham study (104).

4.5.1 OPG and risk factors of atherosclerosis

OPG levels are associated with risk factors of atherosclerosis like age, hypertension, fasting glucose levels and renal impairment in many studies (49, 51). Moreover OPG have been of particular interest in conditions associated with increased media calcification like diabetes mellitus and kidney failure (105), because mouse deficient of OPG developed media calcification (42). Media calcification increases also with aging (106). A close connection between aging and circulating OPG levels were established early (107). Yano et al. demonstrated that serum levels of OPG increase with age, and accelerate both in Japanese

men and women around the age of 50-60 years (107). Kudlack et al. in 2003 reported similar findings in 1134 Austrian men and woman free of chronic disease (108), except that OPG levels accelerated in women around age 60 and in men around age 70 years. Yano et al. suggest that a high serum OPG level in older age reflects a high bone turnover state associated with the loss of sex hormone function after menopause (107). Kudlack et al. however, did not find an association between OPG levels and markers of bone mass in the study of 1134 Austrians without chronic disease.

Patients with diabetes mellitus have higher risk of accelerated atherosclerosis. In 510 asymptomatic patients with type 2 diabetes OPG was associated with atherosclerotic plaque burden (109). Increased amount of OPG protein was found in the tunica media of diabetic patients compared to non-diabetic individuals (50), and circulating OPG levels are increased in diabetic patients in several cohorts (100, 103, 110). Moreover OPG levels correlated with HbA1C levels (110, 111) and was associated with the development and progression of diabetic complications (112). Several studies also relate OPG levels to prognosis in patients with diabetes. OPG is an independent predictor of cardiovascular complications (112) as well as an independent marker of mortality (113)

Many studies link the OPG/RANKL/RANK-system to the vascular calcification and bone disorders seen in patients with chronic renal disease (114). Kidney function deteriorates with age, and serum OPG levels have been found to increase along with the impairment of the glomeral filtration rate (76). In patients with kidney failure, circulating OPG levels are elevated (115, 116), but return to normal levels in patients undergoing renal transplantation (117). The mechanisms responsible for the increase in circulating OPG in uraemia are, however, unknown. Accumulation due to decreased renal clearance is unlikely. Recently, OPG levels were found to predict 5- and 10 year renal decline in elderly woman (118). Moreover OPG levels are associated with cardiovascular disease extent (119, 120), cardiovascular events and mortality (121-123) in patients with chronic and end-stage kidney disease.

4.5.2 OPG and mechanisms of atherosclerosis

Several mechanisms have been suggested as to how OPG might be involved in atherosclerosis through its action of blocking RANKL. Although circulating levels of RANKL was not related to cardiovascular disease risk in some observational studies in the general population (104, 124), the proinflammatory/ proangiogetic properties of RANKL, has made RANKL a suggested villain in atherogenesis. The Figure 9 is taken from a review by Kiechl et al. 2006 and shows how RANKL stimulates monocyte chemotaxis by activating endothelial cells, causes increased MMP release from vascular smooth muscle cells, stimulates dendritic cell survival and enhances T-cell maturation. OPG has been thought to exert protective mechanisms in acting as a decoy receptor for RANKL, thereby blocking the pro-inflammatory response stimulated by RANKL. However some studies also suggest that OPG might have direct unfavourable effects on e.g. vascular matrix remodelling by directly stimulating MMP (Figure 9), and by stimulating smooth muscle cell and endothelial cell apoptosis. This effect might be directly linked to OPG binding to TRAIL (51).

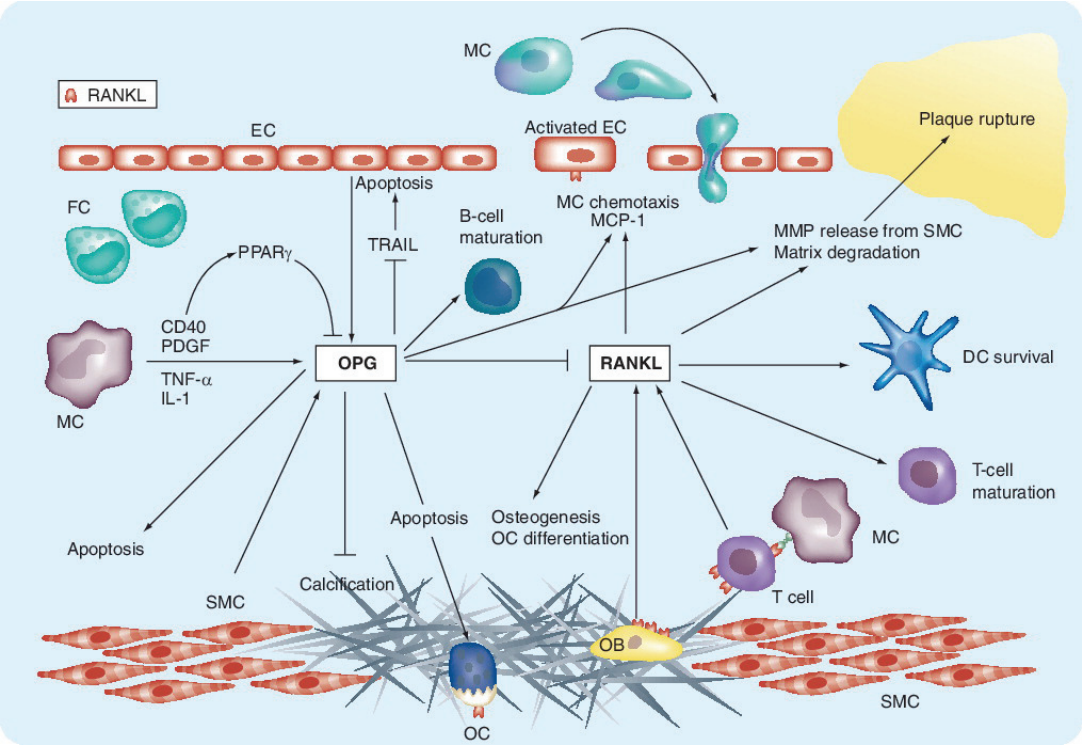


Figure 9. Putative mechanisms of OPG in atherosclerosis and vascular diseases: a schematic overview. From Kiechl et al. Expert Rev Cardiovasc Ther 2006. Reprinted with permission from RightLink.

4.6 OPG and heart failure

4.6.1 OPG is increased in HF

Inflammation is important in the progression of many forms of HF (125). OPG is a member of the TNF-receptor super family and circulating levels of another cytokines of this family, TNF alpha, are linked to matrix remodelling and HF development (126, 127). Experimental studies have found increased OPG expression in myocardial tissue of HF patients (47, 128). Several early studies have also suggested that plasma OPG levels are higher in patients with HF (47, 129, 130). Fore instance, in 2004, Ueland et al presented one of the first studies to specifically address the OPG/RANKL/RANK-system in a cohort of patients with myocardial infarction complicated with HF (129). OPG levels were higher and stayed higher in patients who developed HF in the acute phase of myocardial infarction, compared with age and gender matched controls. In addition, OPG levels decreased to a lower level within one month after the acute event, but stayed elevated at a stable higher level than in the healthy controls throughout the 2 years follow-up period (129). Another study reported higher OPG levels in patients with aortic stenosis referred for consideration of valve replacement. OPG levels were higher in patients with aortic stenosis and left ventricular pressure overload HF, than in patients with only aortic stenosis (130). In addition, OPG levels decrease after valve replacement in the HF patients, while pre- and postoperative OPG levels remain unchanged in patients with only aortic stenosis, suggesting that if the stimulus of HF is removed, circulating OPG decreases.

Another indication of OPG playing a role in HF comes from findings in general population study where plasma OPG levels are associated with indices of left ventricular function. In 2715 participants of the Dallas Heart Study, higher OPG levels were associated with higher end systolic volume and lower ejection fraction (131). In addition, OPG levels were associated with left ventricular mass in males but not in females (131). An association between circulating OPG and atherosclerosis as discussed previously, also imply that OPG levels might be higher in HF patients due to extensive coronary atherosclerosis. Many studies indicate that higher levels of plasma OPG are associated with the extent of CAD (102, 103, 132, 133), and atherosclerosis might be a main cause of the OPG increase seen in HF patients. However, higher cardiac tissue levels of OPG protein in HF patients with both ischemic dilated cardiomyopathy as well as in patients with idiopathic dilated cardiomyopathy than in

control subjects (47), suggest a more generalized link between OPG and matrix remodelling in HF.

4.6.2 Mechanism of increased OPG in HF

The studies mentioned above connect circulating OPG levels to possible pathophysiology in the myocardium. However, the biological mechanisms and the understanding of potential sources of and causes of higher circulating levels of OPG in HF cannot be determined in clinical studies. The number of experimental studies evaluating the possible underlying biological mechanism is sparse. In 2005, Ueland et al. studied OPG in experimental and human HF, and found OPG gene expression to be upregulated in HF tissue. Moreover circulating OPG levels correlated with functional, hemodynamic and neurohormonal parameters of disease severity (47). These findings suggest that circulating OPG levels might be produced in the heart, thereby directly linking circulating OPG levels to HF development. Liu et al. 2008 studied the effect of experimental autoimmune myocarditis on IL-17, a T-cell cytokine with proinflammatory properties, and on the OPG/RANKL/RANK system in cardiac fibroblasts. They suggested that the OPG/RANKL/RANK system might be linked to cardiac remodelling by induction of MMP-2 and MMP-9 activity (134, 135).

4.7 OPG, a cardiac biomarker?

In summary, in general population cohorts circulating OPG have been implicated as a marker or mediator of cardiovascular disease (100, 101, 104). Several studies suggest that plasma OPG levels increases with increasing burden of coronary artery calcification (119, 132, 136) and with increased severity of cardiovascular disease (101). Moreover, an association between higher circulating OPG levels and adverse prognosis in patients with acute myocardial infarction has been shown in medium-large sized studies (129, 137). The performance in larger, more contemporary studies and the incremental value of OPG to more established biomarkers, are less well studied. Moreover, whether the association between OPG and cardiovascular mortality can be ascribed to one or more of the main determinants of mortality after ACS e.g. electrical instability, left ventricular dysfunction or recurrent ischemia, is unclear.

OPG levels are increased in experimental and clinical HF (47) and in patients with acute myocardial infarction complicated with HF (129). In addition higher OPG levels are associated with left ventricular dysfunction in the general population (131). Moreover, higher levels of OPG during ACS are associated with mortality and hospitalization for HF (137). Our knowledge of the prognostic value of OPG in acute and chronic HF, however, is limited, and whether OPG is a useful biomarker for acute HF in patients with acute dyspnoea has not been evaluated previously.

5. Aim

The overall aim of this thesis was to evaluate the prognostic and diagnostic properties of circulating OPG in coronary heart disease and HF.

Specific aims of this thesis are:

- To evaluate whether plasma OPG is a biomarker of coronary ischemia.
- To assess the prognostic value of circulating OPG in patients with acute coronary heart disease.
- To evaluate the usefulness of OPG as a diagnostic and prognostic biomarker in HF.

6. Methods and material

This section will sum up important aspects of the participants, the study design, laboratory methods and statistical methods used in the four different publications. Detailed information on inclusion and exclusion criteria and variables used in the different studies are found in the separate articles.

6.1 Participants – four different cohorts

Publication I: OPG serum levels were measured in 200 patients with chest pain and suspected CAD referred for myocardial perfusion imaging (MPI) at Akershus University Hospital and included in the Akershus Cardiac Examination (ACE) 1 study. Inclusion was consecutive into three predefined risk strata. Patients were classified according to the probability of reversible myocardial ischemia (0-100%) by a cardiologist before testing. We included 50 patients with low pre-test probability (<33%), 100 patients with intermediate pre-test probability (33-67%), and 50 patients considered to be at high risk (>67% pre-test probability) of reversible myocardial ischemia. Two patients were excluded due to disseminated malignant disease diagnosed shortly after the time of inclusion; thus the final cohort comprised of 198 patients.

Publication II: Circulating OPG levels at enrolment were measured in all available EDTA plasma samples (n = 4463), in a biomarker sub-study of the MERLIN-TIMI 36 study. The MERLIN-TIMI 36 study was a Phase III, randomized controlled, multicentre study of 6560 patients with NSTE-ACS treated with ranolazine or placebo. Eligible patients had at least 10 min of ischaemic symptoms at rest and presented with one of the following: elevated biomarkers of myonecrosis, ST-segment depression ≥0.1 mV, a history of diabetes mellitus, or an intermediate to high (≥3) TIMI risk score.

Publication III: Blood samples for OPG analysis were collected at randomization and after 3 months. In the biomarker sub-study a subset of 1229 patients recruited from 51 clinical centres were included. The original GISSI-HF trial was a randomized controlled, multicentre study lasting from 2002 till 2005. 6975 patients with clinical evidence of chronic and stable HF (NYHA II-IV), of any aetiology and level of left ventricular ejection fraction were

included. Patients were randomized to n-3 polyunsaturated fatty acids (PUFA) (1 g/d), and if eligible, to rosuvastatin (10 mg/d) vs. matching placebos, added to conventional treatment in a nested design.

Publication IV: 308 patients with acute dyspnoea as the main cause for admittance to Akershus university hospital were included in the Akershus Cardiac Examination (ACE) 2 study. Inclusion was consecutive and eligible patients for the study were older than 18 year. The physician examining the patient in the Emergency Department considered whether dyspnoea was the primary cause of hospitalization. The time from hospital admission to study inclusion was less than 24 h. Exclusion criteria included dementia or other cause precluding informed patient consent, disseminated malignant disease, a history of acute myocardial infarction, coronary intervention, or major surgery within the last 2 weeks, or inadequate blood sampling.

6.2 Study design

Three studies evaluate prognostic properties of measuring OPG at baseline: publication II, III and IV. Two of the studies are prospective observational biomarker sub-studies of large multicentre randomized controlled trails (RCT). In all studies bio-banking was pre-specified. The statistical analysis plans was made after conduction of the main studies, but blinded to biomarker results. More complete descriptions and details of study design and rationale of the original RCT can be found elsewhere (GISSI-HF (138), MERLIN-TIMI36 (139)). Publication IV based on the ACE2-cohort, is a single centre observational study on patients admitted to Akershus university hospital due to acute dyspnoea.

Two studies evaluate OPG levels in relation to pathophysiological properties and diagnosis: publications I and IV. In publication IV, the part of the study evaluating the diagnostic usefulness of OPG in the emergency department, has a cross-sectional design. Publication I is based on the ACE1 cohort, a single centre study with a cross-sectional design.

6.3 Data collection

6.3.1 Blood sampling procedures, storage and shipment

In the two multicentre studies, publication II and III, collection of blood was performed at the different participating clinical centres. In publication III, venous blood samples were drawn into EDTA tubes after overnight fasting, at randomization and after 3 months of follow-up. This was the only study included in this thesis in which fasting blood samples were collected. In all the four studies baseline blood samples were drawn by venepuncture of an antecubital vein after the patient had been in a supine position for at least 15 minutes. In publications II and III, plasma aliquots were shipped on dry ice to a central laboratory. Samples were stored at −70°C until analysis. In publication I, the baseline sample collection was done in the morning, non-fasting. An intravenous line was inserted in an antecubital vein and blood samples were obtained before (baseline), immediately after and 1.5 and 4.5 hours after stress testing. In publication IV, blood samples were obtained as soon as possible, and not longer than 24 hours after admission to hospital. A second set of blood samples was acquired from patients staying in hospital for 24-48 hours, drawn approximately 24 h after the first set of samples. In a subset of patients staying longer than 48 h a third set of blood samples were drawn on the day of discharge. In publication I and publication IV, blood samples were put on ice and processed within 60 minutes after collection, and stored locally at Akershus University Hospital at −80°C until analysis.

In publication II and III, EDTA plasma samples were shipped to the University of Aarhus, Denmark, where samples were analysed for OPG by personnel blinded to clinical data and not involved in data analysis. In publication I and IV, serum samples were shipped on dry ice to Biomedica's laboratory in Slovakia for analysis, and was analysed by personnel blinded to clinical data including outcomes. The samples had undergone a maximum of two thaw–freeze cycle before analysis.

6.3.2 Biochemical assays

As discussed in the introduction, OPG exists in the circulation as a monomer, a homodimer and bound to its ligands (RANKL and TRAIL). In addition OPG has a heparin-binding domain and might therefore be linked to other circulating substances like von Willebrand factor and proteoglycans (140). Yano et al. (107) presented one of the first assays to systematically detect OPG serum levels by an ELISA system. They found two different

monoclonal antibodies for OPG, one for recognizing the monomeric form and one for recognition of the homodimeric form. They discovered that the monomeric form of OPG was the most abundant in the circulation (107). The assay used in the study by Yano was also used by Jono et al. 2002 (102), one of the first studies to evaluate OPG and the relationship to cardiovascular disease.

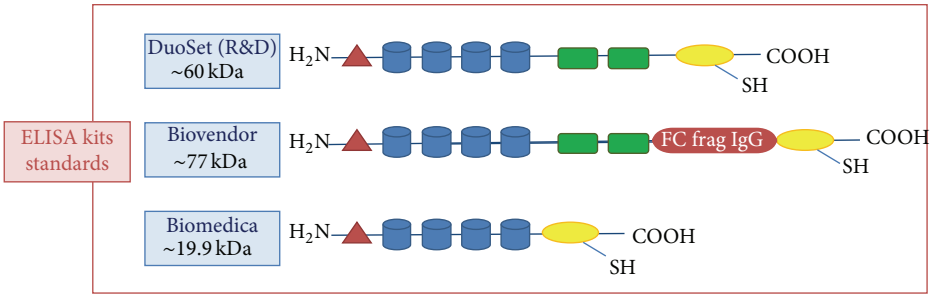


Figure 10. Different OPG ELISA kit standards. Modified from Perez de Ciriza et al. Int J Endocrinol 2015 and used under CC BY.

Presently, there are many different commercially available assays measuring OPG in serum or plasma, provided by e.g Immundiagnostik (141), Biomedica (108), DuoSet by R&D systems (101), BioVender (142). The different manufacturers use different ELISA standards and different molecular weights resulting in differences in OPG concentration presented in different studies (Figure 9). According to Venuraju et al. 2010 (49) the most recent studies use the ELISA technique to detect total serum levels of OPG. However, information about whether or not the different assays actually measures total OPG (free OPG, monomeric and dimeric form as well as OPG bound to its ligands) has been hard to find by examining assay information provided by the manufacturers. In 2006, Clancy et al. compared results from Biovender, Biomedica and DuoSet in a cohort of patients with abdominal aortic aneurisms (143) and suggested that the variation between measured levels of OPG with these assays largely were due to differences in the standards used in the ELISAs (143).

Antibodies for measuring OPG in publication II and III was purchased from R&D systems ((DY085E), manufactured from Abingdon, United Kingdom), who also provides the DuoSet assay kit (DY805 in the DuoSet). However, the method was an in-house time-resolved immunofluorometric assay modified from a previously described enzyme-linked immunosorbent assay (110, 111). Details about measurement procedure are presented in the different articles and elsewhere. In publications I and IV, OPG was analysed using the

Biomedica assay kit and the analysis were done at a Biomedica laboratory in accordance with the assay procedure provided by Biomedica. The Biomedica assay is a sandwich ELISA and the monoclonal antibodies against OPG are commercially available (BI-20403, manufactured by Biomedica,Vienna, Austria). The assay measures both free OPG and complexed OPG-RANKL, and the assay detect both the monomeric and the dimeric form of OPG. (The conversion factor provided by Biomedica, 1pg/ml =0.05 pmol/l (Molecular weight: 19.9 kDa).)

6.3.3 Collection of clinical variables

Procedures for collection of variables like blood pressure, height, weight, risk factors like smoking, and the presence of comorbidity are presented in the different publications and will not be repeated here.

For the bicycle stress testing symptoms, heart rate, blood pressure, and a 12-lead ECG were recorded before the test, midway through each stage, and during recovery. The criterion for an adequate stress test was >85% of the expected maximal heart rate [220 − age (years)]. Stress testing was terminated if there was physical exhaustion, severe chest pain or other symptoms of ACS, >2 mm horizontal or down-sloping ST-segment depression, ≥20 mmHg fall in systolic blood pressure or sustained ventricular arrhythmias. Results of the exercise stress test were determined by a cardiologist and categorized as positive, intermediate or negative based on symptoms and ECG alterations.

6.4 Endpoint assessment:

6.4.1 Detection of cardiac ischemia

Stress ECG is a non-invasive method to assess exercise induced myocardial ischemia, and is widely used in the assessment of patients with chest pain and suspected significant CAD. Stress testing has limitation both in sensitivity and specificity of CAD, and in a meta-analysis of 24 047 patients the pooled sensitivity and specificity for detection of CAD was 68% and 77% respectively (144). To improve detection of CAD, myocardial perfusion imaging (MPI) is performed.

In clinical studies MPI is often used as a reference standard for non-invasive assessment of myocardial ischemia, and changes causing abnormalities on MPI are thought to be visible earlier in the ischemic cascade than ECG changes and before angina occurs (145). There exist several different radioactive substances that are used as tracers and administered intravenously, and the radioactive signal detected by specialized cameras such as single-photon emission computer tomography (SPECT) and positron emission tomography (PET) is used to detect the gamma photons. To evaluate whether stress causes cardiac ischemia due to obstructive CAD, a stress rest protocol can be used. Stress can either be induced by physical exercise or pharmacologically induced with vasodilators or inotropic/chronotropic drugs.

In publication I, a stress-rest protocol with maximal bicycle exercise was used for MPI. The radionuclide tracer, 99m Tc (technetium-99m)-tetrofosmin was administered at peak exercise, and images were taken 45 minutes after administration. 3-4 hours later, the same tracer was administered at rest and pictures were taken 45 min later with SPECT using a two-headed gamma camera (DST-XL; GE Healthcare Technologies). A 17- segment myocardial model was used for semi-quantative analysis rating each segment with a visual perfusion rating ranging from 0-4 (146). Summed stress score (SSS), summed rest score (SRS) and summed difference score (SDS) were calculated, and patients with SRS score ≥4 were considered to have significant fixed perfusion defect on MPI. In publication I, a specialist in nuclear medicine blinded to biomarker data did the visual perfusion rating. The cut-of for a positive MPI test was defined as SDS score ≥3. Automatically determined semi-quantitative analysis of myocardial perfusion using the commercially available software QPS (Quantitative Perfusion Spect) was used as a supplement. Left ventricular ejection fraction (LVEF) was calculated with the Quantitative Gated Spect software.

6.4.2 All-cause mortality, cardiovascular mortality and heart failure diagnosis

In the studies evaluating prognosis the endpoint was either all cause mortality (publication III and IV) or cardiovascular mortality (publication II). An endpoint committee determined whether patients died of cardiovascular causes. In publication IV, two independent physicians determined the acute HF diagnosis retrospectively. The physicians had access to medical information, including follow-up data (median follow-up 464 days before the process was completed in December 2012). The diagnosis was based on criteria from the 2012 ESC

guidelines for diagnosing acute HF (147). Disagreement regarding the diagnosis was determined by consensus.

6.5 Statistical analysis

All statistical analysis was performed according to a pre-planned analysis plans. According to the general policy of the TIMI and GISSI study groups, in publication II and III, statistical analysis was performed by biostatisticians in Boston and Italy who were associated with the TIMI and GISSI study groups according to prospective analysis plans developed by the investigators. In publication I and IV, statistical analysis was performed at Akershus University Hospital by the author of this thesis. Details about the different statistical software used, are provided in the different publications.

6.5.1 Examination of data

Continuous variables were evaluated for normality by plotting the distribution, plotting q-q plots, and performing Shapiro-Wilk or Kolmogorov–Smirnov test for normal distribution. OPG was not considered normally distributed in any of the studies and was log transformed or parametric statistical analysis was used.

In general, in all the four studies, patients in whom data were missing were excluded from the relevant analysis. We did not apply methods for imputation of missing data. If missing values was considered a problem for the interpretation of results, it was reported in the different studies.

6.5.2 Examination of baseline variables

In all the articles a baseline table of the data was presented. In publication II variables were presented according to OPG tertile III vs. I and II combined, and all tertiles for publication III. In publication I, variables were presented according to results on myocardial perfusion imaging, and in publication IV according to the presence or absence of acute HF. In publications I, II and IV, baseline variables were compared using $\chi 2$ test for categorical variables, Student's t test for normally distributed variables, and Mann–Whitney U test for non-normally distributed variables. In publication III, the $\chi 2$ test for categorical variables and analysis of variance, or Kruskal-Wallis test for non-normally distributed continuous variables.

6.5.3 Examination of associations

Examination of variables associated with circulating OPG levels were done with regression analysis. In publication II and III multivariable logistic regression analysis was performed, to identify independent predictors of higher OPG levels at baseline. In publication II, OPG was dichotomized at the third tertile and in publication III at the median. In publications I and IV, multivariate linear regression was used to identify variables independently associated with baseline log-transformed OPG concentrations. In publication I, backward selection with Akaike's information criterion as selection rule was used to identify the best model.

6.5.4 Diagnostic assessment

In publication I, we addressed the diagnostic merit of OPG to detect patients with reversible ischemia. OPG concentrations were measured at 4 different time point and non-parametric methods for assessing repeated measurements were used, due to non-normal distribution of OPG levels. Since results were negative by crude analysis, we did not perform multivariable analysis or evaluate other measures of clinical usefulness. In publication IV, OPG as a diagnostic biomarker was evaluated by logistic regression analysis and calculation of the area under the curve (AUC) after adding OPG to a logistic regression model that already included ED physician prediction and NT-proBNP. In addition, the AUC's of univariable logistic regression models for predicting acute HF that included OPG and NT-proBNP and ED physician separately were presented.

6.5.5 Prognostic assessment and evaluation of clinical usefulness of a prognostic biomarker

To address the association between OPG levels and different outcomes, Cox proportional hazards models were used for survival analysis in publication II, III and IV. Multivariable Cox models were generated to determine independent predictors of the different pre-planned endpoints. In patients with acute coronary syndrome, variables from the TIMI-risk score were included as covariates in the multivariable models. In chronic HF or in patients admitted to hospital with acute dyspnoea, there was no established risk score that would fit the purpose. The strategy of choosing covariates for the models in those two cohorts were based on knowledge of covariates associated with mortality in general, and by examining the data. In

publication IV, OPG was only evaluated as a continuous log transformed variable. In publication II and III OPG was evaluated both as a continuous variable and as a categorical variable by dividing into OPG tertiles. When evaluating the combined endpoint in publication III, the assumption of proportionality was not met for the combined endpoint (mortality or hospitalization for cardiovascular causes) when baseline OPG was a continuous variable. This problem was addressed by evaluating this endpoint considering OPG only as a categorical variable for this endpoint.

Rothman and Greenland describes Bradford Hill's first criterion and states: *"a strong relationship is neither necessary nor sufficient for causality in the same way as weakness is neither necessary nor sufficient for the absence of relationship"* (148). However, as pointed out by Hlatky et. al (149) for a biomarker to be considered an important prediction tool it needs to provide information above and beyond established risk markers. And this is regardless of whether or not a causal link is established. In other words, when a biomarker is an independent predictor in multivariable models, this criterion is necessary but not sufficient to call a biomarker a novel risk marker for an endpoint (150). Additional statistical measures have been suggested to evaluate incremental value of biomarkers, including measures of discrimination, calibration and reclassification (149).

Clinical usefulness can mean many things, but here it will be used as a statistical model's ability to improve the classification of patients compared to a default statistical model, by adding information about the biomarker to the model. In publication III categorical net reclassification index (NRI) was evaluated by adding OPG to the different survival models. Tertiles of risk were used as categories for NRI analysis. In publication II, continuous NRI was reported for the endpoints where OPG was an independent predictor in the multivariable Cox analysis. In publication IV, AUC or NRI was not evaluated because OPG was not an independent predictor in the multivariable Cox analysis.

6.6 Legal and ethical considerations

All the sub-studies included in this thesis, as well as the original clinical trails GISSI-HF (151) and MERLIN-TIMI36 and the ACE1 and ACE2 studies have been conducted in accordance with the Declaration of Helsinki (152). The Regional Ethics Committee approved

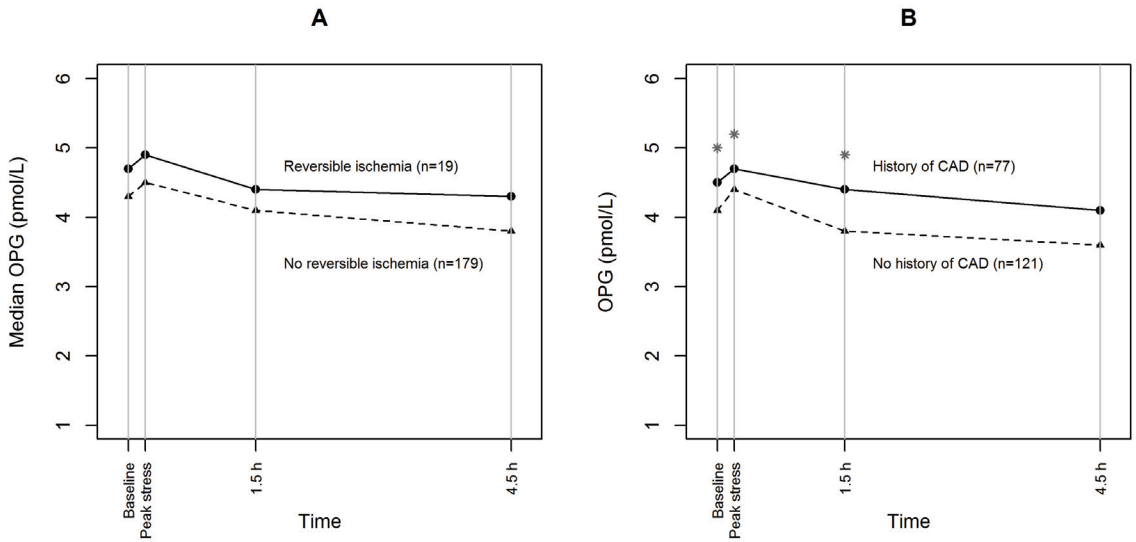
the studies conducted in Norway. Patients provided written informed consent prior to study commencement.

7. Results – summary of papers

Publication I – OPG concentrations in patients with suspected reversible myocardial ischemia

In 198 patients with suspected obstructive CAD, OPG levels were measured before, immediately after, 1.5 h and 4.5 h after exercise stress testing with MPI. OPG levels were not different in the patients with or without significant reversible ischemia on MPI. However OPG levels were higher in the 198 patients than in 8 healthy control subjects. Both in the patients and the controls OPG level increased to a maximum level at peak stress and returned to normal within 1.5 h. Change in OPG levels during exercise was not related to results on MPI. Evaluating variables associated with change in OPG levels observed, we found an inverse association with diastolic blood pressure and having a history of hypertension.
To conclude, OPG levels increase acutely and transiently during stress testing, but are not associated with coronary ischemia.

Figure: Median OPG level at the different time points stratified by MPI results (A), and history of coronary artery disease (B).



*p-value<0.05 for the comparison OPG levels the different groups.

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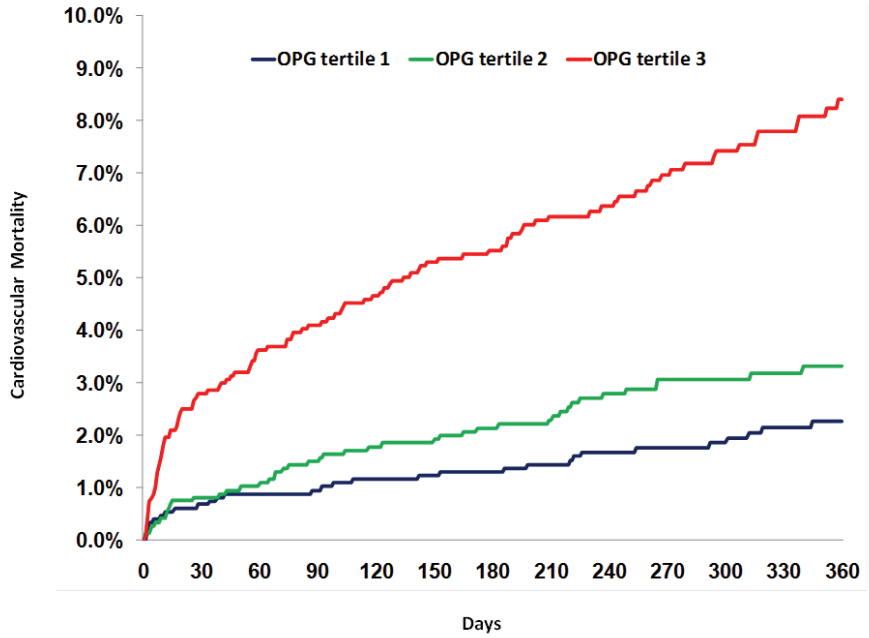
Publication II – OPG and cardiovascular mortality in patients with NSTE-ACS

In 4463 patients with non-ST-elevation acute coronary syndrome, circulating venous OPG levels were measured within 24 hours of hospital admittance. Higher OPG levels were associated higher age, diabetes and decreased kidney function. In addition patients with increased OPG were more likely to have multivessel disease and reduced LVEF. During a median follow-up time of 341 days, 208 patients died of cardiovascular causes and 177 patients were hospitalized due to HF. Both after 30 days and 1 year, OPG was associated with cardiovascular mortality and hospitalization for HF after adjusting for the TIMI risk score covariates, and other established biomarkers of cardiovascular disease.

In conclusion, OPG is independently associated with 30 day and 1-year risk of cardiovascular mortality and HF development after NSTE-ACS. No independent relationship between OPG levels and the risk of recurrent ischemia or myocardial infarction was observed, suggesting that myocardial dysfunction may be a more important stimulus for OPG production than ischemia in ACS.

Figure: Kaplan-Meier curves of cardiovascular mortality by tertiles of osteoprotegerin (OPG) in patients with NSTE-ACS.

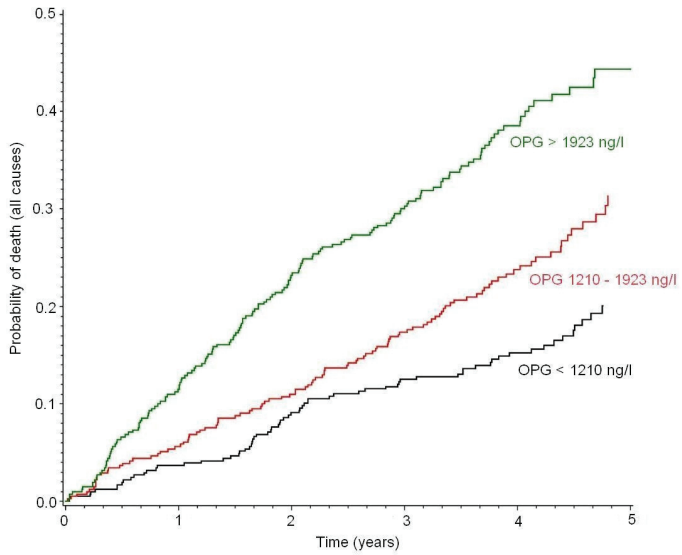


Publication III – Prognostic value of OPG in chronic heart failure

In 1229 patients with chronic stable HF of any aetiology, circulating venous OPG levels were measured at baseline and after 3 months. Variables associated with higher OPG levels included higher age, reduced kidney function, diabetes and higher BNP levels. During a median of 3.9 years, 332 patients died and 791 patients died or were hospitalized for cardiovascular causes. OPG measured on inclusion predicted mortality independently of important clinical variables; however, by reclassification analyses OPG did not provide additional information to BNP and CRP. OPG was also associated with risk of incident AF, but this association was attenuated when adjusting for clinical variables. OPG levels did not change during 3 months of statin treatment, and there were no interaction between OPG levels and rosuvastatin or n-3 polyunsaturated fatty acid treatment. In the patients in whom OPG did change, this change was not associated with future risk of mortality.

To conclude, circulating OPG is associated with mortality independently of conventional cardiovascular risk factors, but does not provide additional information to BNP and CRP by net reclassification analysis.

Figure: Kaplan-Meier curves for mortality by baseline tertiles of OPG.



Log-rank test: chi-square = 62.1, P < 0.0001.

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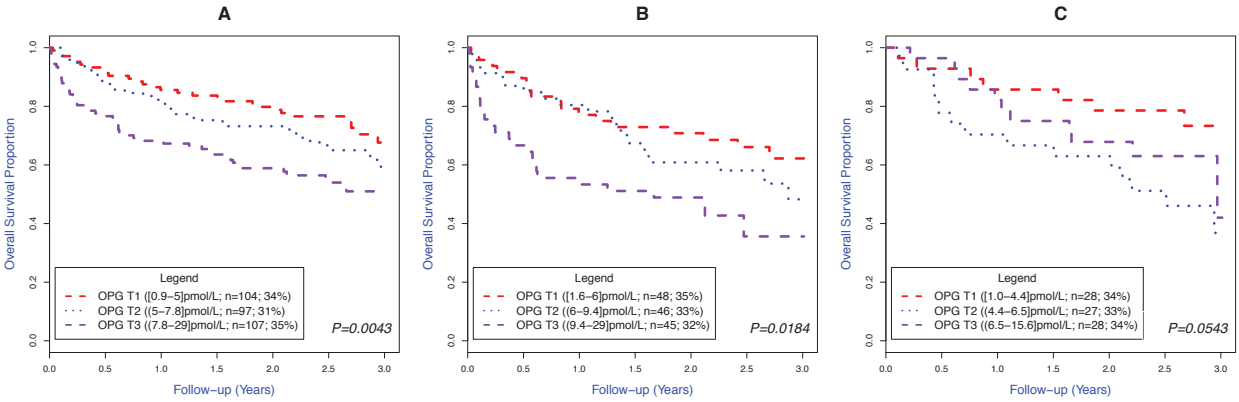
Publication IV – Diagnostic and prognostic properties of OPG in patients with acute dyspnea

In 308 patients with acute dyspnoea, 139 were classified as having acute HF. Serum OPG levels were measured within 24 hours of admission and in a subgroup with 48 hours and at the day of discharge. Higher OPG levels were associated with higher age, NT-proBNP and CRP, whereas a history of COPD was associated with lower OPG levels. During a median follow-up time of 817 days, 112 patients died. OPG were associated with mortality in unadjusted Cox regression analysis, but in a multivariable analysis that included clinical variables and biomarkers, OPG provided no incremental prognostic information. In crude analysis, OPG were associated with mortality in patients with dyspnoea due to acute HF but not in those with dyspnoea due to acute exacerbation of COPD.

To conclude, OPG levels are higher in patients admitted with acute HF than in those hospitalized with dyspnoea from other causes, but OPG does not provide information beyond ED physician assessment for the diagnosis of acute HF or beyond clinical risk variables and established cardiac biomarkers concerning prognosis.

Figure: Kaplan Meier curves according to OPG tertile in patients with A) acute dyspnoea (n=308) B) acute HF (n=139) and C) acute exacerbation of COPD (n=83).



The P values are for the log-rank-test.

8. Discussion

8.1 Discussion of key results

The main findings of this thesis are that OPG provides prognostic information independently of conventional risk markers in patients with acute coronary heart disease. Our findings do not support the theory that coronary ischemia is the underlying pathobiological mechanism contributing to the prognostic value of OPG in ACS because OPG levels were not associated with risk of recurrent ischemia or myocardial infarction. In addition, we did not find evidence for reversible coronary ischemia causing increased plasma OPG levels in patients with suspected CAD examined with MPI. After ACS however, OPG levels predicted HF development. We also found an association between OPG levels and prognosis in acute and chronic HF. Accordingly, our findings support the theory that OPG plays a role in the development of HF. However, our findings do not support OPG as a useful clinical tool for diagnostic and prognostic assessment in HF patients, because it did not provide incremental information to clinically available cardiac biomarkers.

8.1.1 Is circulating OPG a useful biomarker for risk stratification in acute coronary heart disease?

Among the three publications evaluating OPG as a prognostic biomarker presented in this thesis, OPG performed most strongly in patients with NSTE-ACS (publication II) because here it provided information beyond established risk scores, as well as beyond cardiac biomarkers. We found that higher OPG was an independent predictor of all-cause and cardiovascular mortality after 3 months and 1-year follow-up. In addition there was a strong association with new or worsening HF in the entire cohort. The findings in publication II confirms and extend information from prior studies and is by far based on the largest cohort that have investigated OPG level and prognosis in the setting of ACS. In addition our findings add to the previous findings by Omland et al. from 2008 (137), because the patients are more extensively characterised, the study includes more end-points and the cohort is receiving contemporary treatment. Another difference is that we assessed prognosis after 30 days and one year follow-up, while Omland et al. studied long term prognosis with minimum follow-up time of 69 months (137). Compared to the study by Ueland et al. 2004 (129), blood samples were collected in the acute state (within 24 hours after symptom-onset) while Ueland et al.

measure OPG three days after myocardial infarction in patients with post infarction HF only (129).

In the setting of acute coronary heart disease, circulating OPG has been proposed as a marker of the stability of atherosclerotic plaques and a biomarker to represent the inflammatory state triggering rupture of a vulnerable coronary plaque. We found in publication II that patients with elevated OPG were more likely to have multivessel (>2) coronay artery disease and disease involving the left anterior descending artery, and LVEF under 50%. Several other studies that have also evaluated anatomical properties of OPG in ACS, report an association between higher OPG levels and worse cardiac function after ACS (133, 153-161).

For OPG to be an important prognostic biomarker in the setting of ACS there must be a strong and consistent association between the biomarker and adverse outcome. Since publication II was published in 2012 other researchers have also evaluated the prognostic value of OPG in ACS (158, 160, 162-166). Many of the studies are done on patients with STEMI receiving primary PCI, and half of the studies are small to moderate in size including approximately 100-500 patients. There is a consistent association between high OPG levels and poor prognosis in crude analysis. Jansson et al. 2012 found that OPG was an independent predictor of all-cause and cardiovascular mortality as well as HF development, but not myocardial infarction after a median follow-up time of 90 months (164). Pedersen et al. also evaluated prognosis in STEMI patients with similar results (165), while Bjerre et al. 2014 (160), did not find that OPG provided prognostic information beyond final infarction size and salvage index, as evaluated by single-photon emission CT. Hyseni et al 2012 found no relationship between morality and OPG levels in STEMI patients when adjusting for age, sex, CRP and diabetes (163), but in that study they used an insensitive assay for measuring OPG and levels were detectable only in a minority of the patients.

In ACS, existing risk prediction tools include the TIMI-risk score (Table 4) and GRACE risk score (Table 4). In publication II, OPG provided independent information to the TIMI-risk score, while Jansson et al. found that OPG provided additional information to the GRACE score covariates for predicting cardiovascular and all-cause mortality in Cox analysis (164). Pedersen et al. adjusted for many important covariates in the multivariate Cox analysis, and OPG provided independent information to those covariates, but the study did not test the

biomarker to a previously validated risk score (165). Using reclassification analysis, in publication II, we found that OPG in the third quartile improved the discriminative ability of a model including the TIMI-risk score (BMI, BNP and CRP was also included in the model) by significantly improving the NRI. However adding OPG to the model as a continuous variable did not improve NRI. In the study by Jansson et al. the discriminative value of measuring OPG in ACS was evaluated with C-statistics (164). Measuring OPG and the inflammation marker chemokine (C-X-C motif) ligand 16 (CXCL 16) together made a small but significant improvement in the C-statistics to the GRACE score (164).

Table 4. Risk prediction models in ACS

TIMI	Age ≥65 CAD risk factors > 3 Known CAD ASA use in past 7 days Severe angina ECG ST segment changes Positive/elevated cardiac marker
GRACE	Age Heart rate Systolic blood pressure Creatinine Cardiac arrest at admission ST segment deviation in ECG Elevated/abnormal cardiac enzymes Signs/symptoms of HF

In publication II there was a weak but statistically significant positive association between baseline OPG level and time from symptom onset of chest pain. Although interesting, this finding does not support a large dynamic in OPG levels in the pre-hospital phase of NSTE-ACS. Omland et al. 2008 did not find a time-dependent differenced in OPG levels in the first 24 hours after symptom onset in patients with ACS. As an example of a OPG profile during the hospital phase of ACS, Figure 11 is from a recent small study on 42 patients with STEMI treated with primary PCI, presented by Lindberg et al. 2014 (161). As shown in Figure 11, OPG behaves differently during the first 3 days of ACS than the more established cardiovascular biomarkers troponin I and CRP. Consistent with that finding, Gogo et al. 2006 found that OPG levels decreased significantly post-PCI in the patients in the highest tertile of OPG levels, and thereby behaved differently than the other inflammatory markers tested (IL-6, hsCRP and sCD40L) (167). This finding is interesting because it suggests that (1) OPG reflects different pathobiological mechanisms than other inflammatory markers in the setting of ACS and (2) PCI significantly reduces circulating OPG levels. The results of the study by Gogo et al. and Lindberg et al. should be interpreted cautiously, because the blood sampled before PCI were collected from the femoral artery and post PCI blood samples were venous (161, 167). Helske et al. reported significantly higher levels of OPG in arterial blood samples from the aortic root than in blood samples from a peripheral vein (130), and this difference might be the cause of the apparent fall in OPG levels post-PCI. Lindberg et al did not evaluate

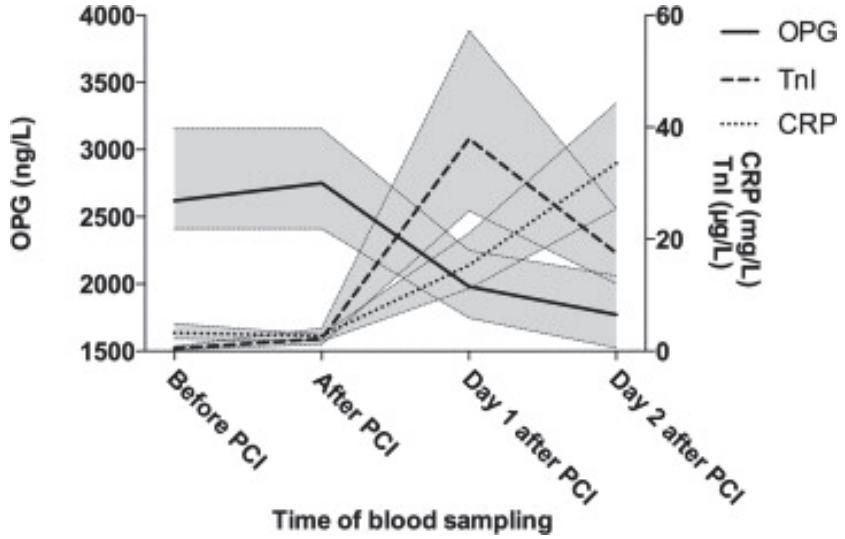


Figure 11. Changes i biomarker levels in the acute phase of STEMI. Figure from Lindberg et al. Can J Cardiol 2014. Reprinted with permission from RightsLink.

whether the decrease in OPG level translated into decreased risk of morbidity and mortality after STEMI (161).

To conclude, OPG is a prognostic biomarker in patients with acute coronary heart disease that provides information beyond the TIMI-risk score and the GRACE risk score in two cohorts. This is in accordance with the results of several studies evaluating prognosis that consistently have reported an association between higher OPG and adverse prognosis in ACS. We also found that higher OPG levels were associated with more extensive atherosclerosis and worse cardiac function. There are no studies evaluating whether any intervention can modify the risk associated with having high OPG levels during the course of ACS, and the pathobiological mechanisms linking OPG to prognosis in ACS is still unclear. Hence, the answer to the question "Is circulating OPG a useful biomarker for risk stratification in acute coronary heart disease?" is presently no. More studies are warranted to understand the involvement of OPG in the pathophysiology of ACS, to further investigate if specific therapies might modify the risk associated with higher OPG levels, and to elucidate if OPG related pathways can be targets for future therapeutic or other interventions.

8.1.2 Is circulating OPG a biomarker of cardiac ischemia?

Biomarkers of cardiac ischemia to early rule out myocardial infarction would be desirable in the setting of ACS (168). OPG is considered to be an inflammatory marker, and some have suggested OPG to be a marker of plaque stability. Few have evaluated OPG as a marker of

myocardial ischemia. In publication II, we found that OPG levels did not predict recurrent ischemic events or recurrent myocardial infarction in the follow-up period after NSTE-ACS. In addition, OPG levels were not associated with findings of myocardial ischemia on the seven days Holter recording. Hence, our findings in patients with NSTE-ACS do not support OPG as a marker of cardiac ischemia. In publication I, we evaluated OPG levels and the relationship to reversible ischemia on MPI in patients with suspected CAD. OPG levels were higher in patients with known CAD, but we found that OPG levels at baseline, or that changes in OPG levels during bicycle stress testing did not associate with MPI results.

A potential transient change in OPG levels during exercise stress testing has not been evaluated previously, and only a few other studies have evaluated OPG in relation to cardiac ischemia measured with MPI (169-174) Avignon et al, Sultan et al. and Guzel et al. evaluated the relationship between OPG and silent myocardial ischemia, defined as significant ischemia on stress ECG or positive MPI. Poulsen et al. evaluated the relationship between OPG levels and ischemia on MPI, and the presence of cardiac ischemia was defined by the summed stress score (SSS). SSS is a score that includes both chronic and reversible ischemia combined. In the ACE1 study a positive stress test was based on the presence of reversible changes, the summed difference score (SDS) (SDS = SSS – summed rest score (SRS)). Results from publication I, can therefore not be directly compared with the other studies, because we only evaluated reversible ischemia on MPI. Still, our findings in publication I are in agreement with the findings by Poulsen et al.(172) who reported no relationship between OPG at baseline and myocardial ischemia in patients with diabetes mellitus type 2. In contrast, Avingnon et al.(170), Sultan et al. (171) and Guzel et al. (174) found that baseline OPG levels were significantly higher in diabetic patients with silent myocardial ischemia than those without. All the above-mentioned investigators evaluated patients without known CAD but with diabetes mellitus. In the ACE 1 cohort 39% had previous CAD and only 13% had diabetes mellitus. Hence, differences in patient characteristics, including distribution of risk factors also associated with higher OPG levels like age, duration of diabetes and kidney function, and the extent of generalized atherosclerotic disease might influence results and be the cause of the discrepant findings.

Publication I was designed as a “proof of concept” study to investigate whether reversible cardiac ischemia as assessed by MPI is associated with measurable changes in blood-borne biomarkers. We did not exclude patients with potentially confounding conditions like

osteoporosis, autoimmune disease or treatment with medications like glucocorticoids and statins. The influence of these conditions and medications may have diluted and obscured a possible association between reversible ischemia and OPG levels at baseline. A brief period of reversible ischemia is not a very strong stimulus, but has been shown to be associated with a rise in high sensitivity troponin I (175). Natriuretic peptides change dynamically in healthy as well as in patients with reversible ischemia during exercise stress testing (176-178). Limited power is a concern for the conclusion of the study in publication I. Only 19 patients had a positive MPI test. Nevertheless, in the same cohort we found higher baseline levels of troponin I (179), troponin T (180) and natriuretic peptides (181) in the patients with positive MPI. This is consistent with findings in other studies (176, 177, 182), lending support to the validity of the results of our studies from this cohort. Moreover it suggests that those biomarkers are more closely related to chronic ischemia than OPG and potentially more useful as biomarkers when evaluating risk of significant ischemic heart disease in the setting of exercise stress testing.

To conclude, based on our findings the answer to the question "Is circulation OPG a biomarker of myocardial ischemia?" is no. In the setting of acute exercise stress testing of patients with suspected CAD, we found no evidence to suggest that OPG is a biomarker for identification of patients with reversible cardiac ischemia. In addition, in patients with NSTE-ACS, OPG levels were not associated with reversible ischemia in the days after ACS and did not predict reversible ischemia or myocardial infarction after 30 days an one year of follow-up.

8.1.3 Is circulating OPG a useful biomarker for diagnosis or risk stratification in heart failure?

The OPG/RANKL/RANK system was suggested to be involved in left ventricular remodelling by Ueland et al. 2005, and OPG has been implicated as a potential biomarker in HF for more than ten years (47). In 2008 Omland et al. found that plasma OPG predicted hospitalization for HF after ACS (137), and this finding was confirmed in publication II. Publication III, however, was the first study that reported a relationship between circulating OPG levels and all-cause mortality in chronic HF. Moreover, in publication IV acute HF was evaluated in an unselected patient group with acute dyspnoea. The diagnostic and prognostic value of measuring OPG in acute dyspnoea has not been evaluated previously.

In chronic HF, we reported a univariable association between higher circulating OPG levels and all-cause mortality (publication III). In addition, OPG provided prognostic information independently of clinical variables including age, diabetes, kidney function and LVEF. However, when evaluating the discriminative value with net reclassification index (NRI), OPG did not add information on risk prediction to a clinical model including BNP and hsCRP. In 2011, the prognostic value of OPG was evaluated in a sub-study of the CORONA cohort (183), a similar cohort to the GISSI-HF cohort (publication III) in that it was originally an RCT for testing the statin rosuvastatin in patients with chronic HF. Neither in publication III, nor in the CORONA study did OPG provide additional information regarding all-cause mortality to biomarkers of inflammation or natriuretic peptides (hsCRP and BNP in publication III, NT-proBNP in the CORONA-study). In the CORONA study, however, OPG independently predicted hospitalization for HF and the combined endpoint all-cause mortality and hospitalization for HF. No adjustment for chronological age, a well-known confounder, was performed, making it harder to interpret the results. OPG needs to provide information beyond chronological age to be clinically useful as a biomarker, because knowledge about age is "simple, inexpensive, and readily available" (149). Consistent with the findings of the CORONA study, OPG was strongly associated with age in all the cohorts evaluated in this thesis, and the finding that OPG and chronological age provide overlapping information regarding prognosis is consistent with our findings.

In the CORONA study the marker of generalized inflammation hsCRP has previously been found to decrease with statin treatment, and patients with higher baseline levels benefited more from the treatment (184). A few studies have evaluated the effect of statin treatment on OPG levels but findings are not consistent, and the studies are small to medium sized. Before publication III was published, no study had evaluated the effect of n-3 PUFA or statin treatment on OPG levels in patients with chronic HF. Accordingly, OPG level increased in patients with diabetes after lovastatin (185) or pravastatin treatment (186), while OPG levels decreased after treatment with simvastatin (187). In patients with carotid stenosis OPG levels decreased in a dose dependent manner with atorvastatin treatment (188). In publication III we found no significant over-all change in OPG levels after 3 months treatment with PUFA or with rosuvastatin (10 mg daily). The latter is consistent with the findings of the CORONA study (183). In addition, in patients with a relative change in OPG concentration over 3 months, this change was not associated to subsequent mortality. In the CORONA study, an

interaction between decreased OPG (lower tertile) and a favourable outcome of statin treatment on all-cause mortality was observed (183). In contrast to OPG, the inflammation marker pentraxin 3 (PTX3) increased and hsCRP decreased in patients treated with rosuvastatin vs. placebo in a combined cohort of patients from the GISSI-HF and the CORONA cohort (189). This suggests that OPG, hsCRP and PTX3, all markers of inflammation, provide different information in the setting of chronic HF.

In patients with acute dyspnoea, as well as in patients with acute HF, OPG was associated with all-cause mortality in crude analysis (publication IV). In the subgroup of patients admitted to hospital with exacerbation of COPD, however, no association with mortality was observed. Moreover, in multivariable analysis, OPG did not add incremental information to the established biomarkers NT-proBNP, cTnT and CRP in the complete acute dyspnoea cohort. Two other studies have evaluated the prognostic value of measuring OPG in the acute setting of HF (190, 191). In publication IV, as well as in the two other studies (190, 191) there is a consistent association between higher OPG levels and adverse prognosis in acute HF in crude analysis. Aramburu-Bodas et al. evaluated patients with HFpEF only, and in multivariable Cox analysis the association with all-cause mortality was still significant when adjusting for NT-proBNP (190). However, OPG did not improve the model for predicting 1-year mortality when calculating continuous NRI (190). In publication VI, we did not evaluate discriminative value of OPG as a prognostic biomarker because it did not provide independent information in Cox regression. Moreover, in publication IV we included patients with both reduced and preserved LVEF, and did not evaluate the prognostic value of OPG in the subgroup of patients with HFpEF separately. Frioes et al. evaluated the prognostic value of measuring OPG at discharge after hospitalization for acute HF (191), and found that OPG levels provided independent information to a model that included BNP and CRP for predicting the combined end-point all-cause death or hospital readmission resulting from HF within 6 months after discharge. Although we did not find that OPG changed significantly during hospital stay in the 81 patients in whom such measures were available (publication IV), we cannot rule out that OPG measured in the acute phase of HF is different from the levels measured at discharge after acute HF. Timing of measurement might be of importance for the prognostic value of measuring OPG in acute HF.

For circulating OPG to be of value as a diagnostic test it needs to perform better than existing diagnostic testes for the diagnosis of HF. Several studies have found that OPG levels are

higher in patients with HF, than in patients without HF or healthy controls (47, 129, 130). However, in patients with acute HF, diagnostic performance of OPG has not been evaluated previously. In publication IV, we found that that NT-proBNP (AUC: 0.860) had a similar ability to predict acute HF as the ED physician. In contrast, the value of OPG (AUC: 0.691) to predict acute HF was of similar strength as patient age. Adding OPG levels to the ED physician prediction and NT-proBNP (AUC: 0.887 [95% CI 0.851-0.924]) did not improve prediction of acute HF.

To conclude, based on our findings the answer to the question "Is circulating OPG a useful biomarker for diagnosis or risk stratification in HF?" is presently no. Our findings support that having higher levels of OPG in acute and chronic HF are associated with adverse prognosis. However, OPG did not provide independent information on risk of all-cause mortality to established biomarkers in neither acute nor chronic HF. Larger studies are warranted to evaluate OPGs predictive ability in the setting of acute HF, especially in patients with HFpEF. We found no evidence indicating OPG as a clinical useful diagnostic biomarker in acute HF. Moreover, we found no indication that statin or n-3 PUFA treatment modified the risk associated with higher OPG levels in chronic HF patients. Overall, our findings favour the theory that OPG might be involved in processes that lead to HF development and progression. However, at the moment only very few studies have evaluated OPG in the acute and chronic setting of HF and our findings must be confirmed by other studies.

8.2 Methodological discussion

8.2.1 Measurement of OPG

Random error refers to the variability and the imprecision between the recorded value and the true value of a measurement. In a recent study (192), pre-analytical and analytical factors influencing OPG measurement were considered (They used the Biovender assay (DY804) purchased from R&D systems). The most important factors discussed were (1) that OPG levels was higher in plasma than in serum; (2) no differences in OPG levels were observed due to different centrifugation force (g forces) (3) no difference when time between extraction and separation was 15, 30 and 60 min has been reported previously, but significant differences at longer times (2 and 6 h) was found (4) no differences in OPG levels when samples were stored during 6, 24 or 48 h at 4 °C. However a significant increase in OPG

values was observed in samples stored at room temperature during 48 h. (5) OPG increased after four freeze-thaw cycles (6) increasing concentrations of haemoglobin decreased OPG levels showing that haemolysis negatively interferes with the assay (7) higher levels of triglycerides above 6.33 mmol/L increased measured OPG levels (192). In all the studies (publication I-IV), bio-banking was predefined, ascertaining a focus on pre-analytical considerations when collecting, storing and analysing blood-samples. In other words, all blood samples should in principle have been handled the same way. In all the individual studies all samples were measured from the same type of specimen, either EDTA plasma or serum. None of the studies included blood samples that had been exposed to more than two freeze-thaw cycles. In publication I, II and IV, patients were non-fasting and high triglyceride level might potentially influence the OPG levels in some of the patients. However, from an overall perspective we believe that the risk is minor, that bias was introduced due to pre-analytic and analytic variation for the OPG measurement.

In publication I-IV there are potentially imprecision in the measurements of OPG. Regarding the analytical precision of the OPG, analyses done in publication II and III, the intra- and inter-assay variations (%) were below 5% and 9%, respectively, at concentration of 1000 ng/l. The limit of detection of the assay was 15 ng/L. In publication II, the range (min-max) of measured OPG was 164-9220 ng/l and in publication III the rage (min-max) was 252-6848 ng/L. Hence, all patients in publication II and III had OPG levels far above the detection limit. OPG analysis was performed by a Biomedica laboratory in Slovakia for publications I and IV. The Biomedica assay has even lower levels of intra and inter-assay variation, ≤3%, and ≤5% respectively, and the limit of detection for the assay is 0.07 pmol/L. They also report a standard range for OPG levels between 0-20 pmol/L. In publication I, the range (min-max) of OPG was 1.4-11.4 pmol/L (one measure had to be reanalysed due to technical difficulties). In other words, all measurements were included in the standard range of the assay. In publication IV the range (min-max) was 4.6-29.0 pmol/L (except for the 4 patients excluded from the analysis where OPG levels were reported to be > 30,000 pmol/L). All patients in publication I and IV had OPG levels far above the limit of detection. In publication IV eight patients had levels above the standard range (>20 pmol/l) of the assay. In general, we consider the analytical precision of the OPG analysis to be good, and considering that the cohorts in publication I-IV were of moderate to large size (N ranging from 198-4463) we do not believe that analytic imprecision in the OPG measurement would change the conclusions based on the results of our studies.

In publication III, fasting OPG levels were measured two times, and OPG did not change during 3 months follow-up between the two measurements. In publication II, OPG was measured only once. We believe that the potential random error introduced due to imprecision in OPG measurement in publication II is overcome by the large sample size. In publication I, we discovered intra subject variation in OPG during exercise stress testing. This observation has to our knowledge not been previously reported in patients undergoing bicycle exercise stress testing. OPG was measured at four time points. We can only speculate on what the causes of transient OPG changes during exercise stress testing are. One possible cause might be that exercise induces a more generalized inflammatory response. Inflammatory markers like IL-α, IL-1β, IL-6 and TNFα have been found to transiently change during strenuous exercise (193). Recently, 12 min of high intensity interval based bicycle exercise was shown to transiently increase OPG and RANKL, as well as the inflammation markers IL-1α, IL-β, IL-6 and TNFα (194). However, the significance of transient cytokine changes during short-term exercise is not known (194). New studies are needed to evaluate whether transient changes in OPG levels during exercise or exercise stress testing are caused by an inflammatory response, haemoconcentration or mechanical stimuli to the bone due to exercise or whether other stimuli are involved. Nevertheless, these findings suggest that if OPG levels are to be measured in a standardized fashion, abstaining from physical exercise before blood is drawn might be wise.

In publication II we found a weak but significant association between the time of onset of symptoms and OPG levels. Other studies also suggest that OPG levels increase slightly in the first hours after myocardial infarction (154, 161). Different medications have been shown to affect OPG levels in some studies and might confound the relationship between OPG levels and acute heart disease. For instance, OPG have previously been shown to transiently increase 2-fold during the first 10 min, and then decrease after short time when heparin (low molecular weight and unfractionated) is administered (195, 196), and a possible source was suggested to be vascular smooth muscle cells (196). In those studies OPG levels declined to baseline values within 1-2h. This might be important in the two studies evaluating OPG in the acute setting of disease namely NSTE-ACS (publication II) and acute dyspnoea (publication IV). According to guidelines all patients with ACS should be treated with anticoagulant therapy as early as possible. In both patients with NSTE-ACS, and patients with dyspnoea this

medication might have been administered to the patient and might therefore influence the OPG levels. In the setting of acute dyspnoea, in addition to anticoagulant therapy, glucocorticoids are commonly used in patients with and index diagnose of acute exacerbation of COPD. Glucocorticoids also potentially affect OPG levels (197), and decrease the acute inflammatory response. In publication IV, there were only small changes in OPG in the patients in whom OPG was measured more than once.

Whether or not OPG levels truly change as a direct consequence of myocardial infarction or acute HF, remains to be determined. We believe that treatment effects on OPG level or different timing of the drawing of blood during the course of disease, might potentially cause underestimation of the true association between OPG and the endpoints measured in publication II and IV. In both studies OPG level predicted all-cause mortality independently of clinical variables, and in publication II independently of other biomarkers, so underestimation would not change the direction of the association. However, in publication IV, OPG levels did not provide prognostic or diagnostic information beyond the more established biomarker NT-proBNP. We cannot rule out that the true relationship between OPG is stronger than what we reported due to variability in the measurement caused by different medication or timing of measurement.

8.2.2 Diagnosis and outcome classification

In publications III and IV the main outcome was all cause mortality. This is an outcome with minimal risk for misclassification, and due to national mortality registries, information about mortality is generally easy to acquire. Higher circulating levels of OPG are associated with mortality of non-cardiovascular causes in different cohorts of cancer patients, in cohorts of patients with osteoporosis and even in a cohort of patient with chronic HF (OPG in CORONA from 2011). One could argue that cardiovascular mortality would be a more appropriate endpoint to study for the overall goal of this thesis, because this would more strongly point to a relationship between OPG levels and the progression of cardiovascular disease.

Using an insensitive method to measure the outcome might potentially lead to under-estimation of effects or drawing of the wrong conclusion. In publication I, the outcome of the study was the presence of reversible ischemia as assessed by myocardial perfusion imaging. This method has been discussed in the Methods section. Difficulties and pitfalls that may

complicate the interpretation of the MPI examination include difficulties with the equipment, the technician or the patient (198). For instance, large body habitus might weaken the signals in general, breasts in females might give perfusion defects most often along the anterior wall of the left ventricle and a large abdomen might give perfusion defects of the inferior wall. Another important issue is that balanced ischemia due to multivessel disease might not be detected as ischemia at all, because SPECT measures relative and not absolute uptake of the radioactive tracer (198). We cannot exclude that some patients have been wrongly classified due to any of these issues.

In publication IV a committee of two physicians decided which patient had the diagnosis of acute HF based on criteria from the most recent clinical guidelines (147). This decision was made based on standard hospital work-up, but in addition they had information about later hospital admissions to Akershus University Hospital. Echocardiography was not done routinely in all patients, so we cannot rule out that some patients might have been misclassified regarding the HF diagnosis. The physicians were blinded to OPG data, but all standard measurements taken during the hospital stay, including NT-proBNP were available to the committee. Adjustment for NT-proBNP in the statistical analysis, when looking for independent predictors of acute HF, might be problematic and make OPG perform worse as a diagnostic biomarker since information about NT-proBNP is part of the reference standard for the diagnosis HF.

8.2.3 Statistical analysis

In all the studies statistical analysis plans were made prospectively and were adhered to to avoid data dredging. When post hoc analysis was done, this was reported in the publications. An important statistical tool for evaluating prognostic markers is multivariable statistical analysis. If too many variables are included, problems might arise in interpreting results of multivariable analyses. This will cause over-correction and thereby increase the risk of doing a type II error, i.e. you conclude that there is no relationship when in fact there is. As rule of thumb there should not be less than 10 events per variable. In all the survival analysis we adhered to this rule for the main analysis.

The risk of low power and making type II error is relevant for publication I, and has been discussed previously. In publication I and IV, we used nonparametric statistical analysis to

evaluate changes in OPG levels due to the skewedness of the OPG distribution in the studies. In publication I, only a few patients had OPG values that could be considered outliers, so an alternative way of analysing the results would have been parametric statistical analysis and excluding the outliers. Nonparametric statistical analyses are in general considered less sensitive than parametric methods, and the disadvantage can be that you do not find an effect that actually exists (type II error). On the other hand, nonparametric statistical analysis is more robust as regards the effect of outliers, and this might reduce the risk of concluding that there is an effect, when in fact there is not (type I error).

In publication II, III and IV measures of discrimination and reclassification were reported. A problem with the c-statistic is that it is insensitive to change and may not increase appreciably even when a new marker is statistically significant and independently associated with the diagnosis or outcome. Net reclassification index (NRI) is relatively new metric (199) that may provide relevant information in biomarker studies (149). One important concern when using NRI that has been subject to some critique is that the models need to be well calibrated. If not, NRI measures might look more advantageous than they actually are (200).

8.2.4 Generalizability of results

"The generalizability of a study's results depends on the researcher's ability to separate the "relevant" from the "irrelevant" facts of the study, and then carry forward a judgment about the relevant facts" (201).

For the results of a study to be representative to a more generalized population than the study sample, both internal and external validity must be evaluated.

- internal validity refers to issues like the suitability of the study design, the carefulness of data collection and the appropriateness of the choice of statistical analyses.
- external validity refers to whether or not the results will apply generally in other study settings or other study samples

To participate in research, due ethical considerations, patients need to give informed consent. The most severely ill patient and patients with dementia were not included and findings cannot be generalized to these patient groups. In addition, in all the prospective studies patients with end-stage kidney failure and <12 months life expectancy were excluded. A

characteristic of many RCTs is strict inclusion and exclusion criteria. RCT's have the advantage that randomization keeps study groups as similar as possible, and differences in both known and unknown confounding variables are due to random variation. When an observational study is embedded in a RCT, the study characteristics are those of an observational study. In publication III patients treated with any of the investigational agents (rosuvastatin or PUFA) within 1 month before randomization were excluded. In publication II, patients using any digitalis preparation (e.g. digoxin), agents that are strong inhibitors of the cytochrome P450 pathway isoform 3A4 and patients using medication that are known to prolong the QT interval were excluded. We have no reason to believe that patients using these agents are different from the patients studied when it comes to OPG levels. Nevertheless, excluding specific patient subgroups reduces the generalizability of our findings to a population of patient eligible for participation in a clinical trial.

Publication I and IV have many different characteristic compared to RCTs when it comes to generalizability. In these two studies, inclusion criteria were wide to ensure that the findings would be representative and close to every day life in the clinic. OPG is, however, an inflammatory marker, and produced in many tissues, so the lack of control of potential confounding factors such as infection, inflammatory diseases like rheumatoid arthritis or markers of bone metabolism might influence the results. However, this limitation is not specific to our study, but to all studies evaluating the diagnostic or prognostic usefulness of any inflammatory biomarker. Moreover, to be clinically useful, biomarkers have to be evaluated in a real life setting.

In the two cohorts that are sub-studies of larger cohorts, OPG was not measured in the full cohorts. In publication III, 1229 of the 6975 patients from the original GISSI-HF trial were included. Clinical characteristics of the biomarker sub-studies have previously been reported to be similar to those of the patients enrolled in the main trail (202). Hence, the findings should be generalizable to the full GISSI-HF cohort. In publication II, 4463 patients of the 6560 patients participating in the original MERLIN-TIMI36 trial were included in the biomarker sub-study. Availability of blood was an inclusion criterion in the sub-study. In theory, availability of blood as an inclusion criterion might introduce bias, if availability of blood is somehow associated with OPG levels or any of the endpoints evaluated. However, we have no indications that such bias is present in the GISSI-HF or MERLIN-36 study. Also,

the large sample size of both studies would tend to increase generalizability. Moreover, we have no reason to believe that biological processes associated with OPG are different in the patient in whom blood samples were available vs. the patients in whom blood was not available. In every day in the clinic, we also generalize and draw conclusions based on findings from randomized controlled trials, even though these studies often have specific inclusion and exclusion criteria, rather than being population probability samples.

7. Conclusions

The key findings presented in this thesis are that

(1) Increased circulating OPG levels in the acute phase of NSTE-ACS are associated with adverse prognosis and development of HF, and provide information beyond clinical information and established prognostic biomarkers (publication II).

(2) Among patients admitted to hospital with acute dyspnoea, patients with acute HF have higher OPG levels than patients with acute dyspnoea of other causes. OPG levels are associated with mortality independently of clinical risk markers, but do not add information regarding prognosis or diagnoses to established cardiac biomarkers or emergency department physician evaluation (publication IV).

(3) In patients with stable chronic HF, OPG levels are similar in patients with HF of ischaemic and non-ischaemic origin, are associated with all-cause mortality, and provide prognostic information independently of important clinical confounders. However, knowledge of OPG level do not provide information beyond established biomarkers of myocardial stretch or inflammation. In addition, OPG concentration is unchanged after 3 months of follow-up, and is not affected by treatment with rosuvastatin or omega-3-fatty acid (publication III).

(4) Circulating levels of OPG are not associated with reversible myocardial ischemia as evaluated by MPI in patients with suspected CAD. However, circulating OPG levels increase acutely during bicycle exercise stress-testing in patients, as well as in 8 control subjects, before rapidly decreasing to the baseline level (publication I).

Our findings have unravelled some new associations between circulating OPG levels and cardiovascular disease and HF, and indicate that OPG plays a role in the pathophysiology of CAD and HF development. Conclusions regarding causality cannot be made based on our studies, however, and whether OPG is a marker, a player, a risk factor, a protector or merely an innocent bystander remains to be determined.

8. Clinical implications and future research opportunities

How can circulating OPG fulfil the criteria for a tool and a biomarker for clinical decision-making in patients with cardiovascular the future? For this to happen the assay needs to be standardized internationally and reference intervals for normal healthy populations must be established. Clear mechanistic understanding of the relation to cardiovascular disease risk, initiation or progression is needed and documentation that knowing the OPG level improves treatment. OPG is not tissue specific and there is great variability and probably a large normal range in circulating OPG levels measured with todays´ assays, especially in older age. Discovering tissue or process specific isoforms of OPG, or developing assays that measures OPG connected to specific ligands might improve the potential of OPG as a clinically useful biomarker in cardiovascular disease.

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