UNIVERSITY OF TROMSØ UIT

FACULTY OF HEALTH SCIENCES
DEPARTMENT OF CLINICAL MEDICINE
Hematological Research Group

Osteoprotegerin and Cardiovascular Disease

Anders Vik

A dissertation for the degree of Philosophiae Doctor
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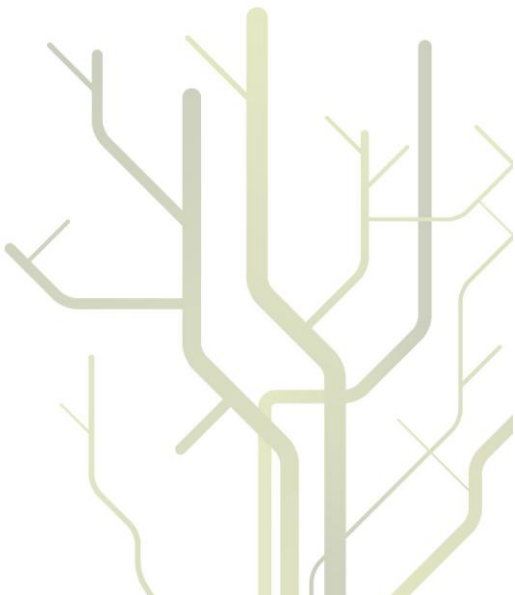

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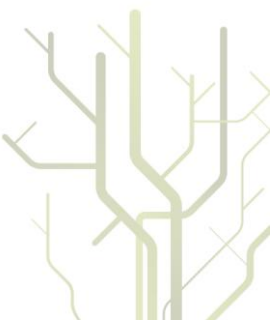

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Tromsø, September 2010

Anders Vik

Contents

List of papers

1. Vik A, Mathiesen EB, Noto AT, Sveinbjornsson B, Brox J, Hansen JB. Serum osteoprotegerin is inversely associated with carotid plaque echogenicity in humans. *Atherosclerosis.* 2007;191(1):128-134.
2. Vik A, Mathiesen EB, Brox J, Wilsgaard T, Njølstad I, Jørgensen L, Hansen JB. Relation between serum osteoprotegerin and carotid intima media thickness in a general population - The Tromsø Study. *J Thromb Haemost* 2010; 8: 2133-9.
3. Vik A, Mathiesen EB, Johnsen SH, Brox J, Wilsgaard T, Njølstad I, Hansen JB. Serum osteoprotegerin, sRANKL and carotid plaque formation and growth in a general population - The Tromso Study. *J Thromb Haemost* 2010; 8: 898-905.
4. Vik A, Mathiesen EB, Brox J, Wilsgaard T, Njølstad I, Jørgensen L, Hansen JB. Serum osteoprotegerin is a predictor for cardiovascular disease and mortality in a general population – The Tromsø Study. Submitted.

Abbreviations

BMD	Bone mineral density
BMI	Body mass index
BMP-2	Bone morphogenetic protein- 2
CAD	Coronary artery disease
Cbfa1	Core binding factor alpha 1
CV	Coefficient of variation
CVCs	Calcifying vascular cells
CVD	Cardiovascular disease
CIMT	Carotid intima media thickness
eNO	endothelial nitric oxide
eNOs	endothelial nitric oxide synthase
FMD	Flow-mediated dilatation
GSM	Gray scale median
HDL	High-density lipoprotein
hsCRP	high sensitive cross reacting protein
ICAM-1	Intercellular adhesion molecule -1
IL-1	Interleukin - 1
IP-10	Interferon inducible protein -10
LDL	Low-density lipoprotein
M-CSF	Macrophage colony stimulating factor
MCP-1	Monocyte chemoattractant protein 1
MGP	Matrix Gla protein
MMP-9	Matrix metalloproteinase-9
NFκB	Nuclear factor kappa B

OCIF	Osteoclastogenesis- inhibitory factor = OPG
OPG	Osteoprotegerin
PAI-1	Plasminogen activator inhibitor 1
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
SMCs	Smooth muscle cells
sRANKL	soluble receptor activator for nuclear factor kappa B ligand
TAT	Thrombin-antithrombin complexes
TFPI (ag og ac)	Tissue factor pathway inhibitor antigen and activity
TNF	Tumor necrosis factor
TSP-1	Thrombospondin 1
TRAIL	TNF-related apoptosis-inducing ligand
Tromsø IV	The forth Tromsø Study (1994-95)
Tromsø V	The fifth Tromsø Study (2001-02)
VCAM-1	Vascular cell adhesion molecule-1
VTE	Venous thromboembolism
vWF	von Willebrand factor
WPB	Weibel Palade bodies

Introduction

1.1 Atherosclerosis

Cardiovascular disease (CVD) is the leading cause of death globally [1]. Atherosclerosis is the most frequent underlying cause of coronary artery disease, carotid artery disease and peripheral artery disease. Age, total cholesterol and LDL concentrations are risk markers for future cardiovascular events [2]. Nearly 100 years ago it was observed that rabbits fed a diet rich in eggs developed fatty lesions resembling atheroma [3]. Further dietary experiments showed that cholesterol and not other non-lipid constituents of hen's eggs caused the arterial lesions [3]. The combination of an individual's genetic constitution and a high cholesterol diet induce changes in the vascular wall. Even in the absence of other risk factors, elevated plasma cholesterol is sufficient to drive the development of atherosclerosis [4]. However, sudden death or myocardial infarction without warning symptoms is the first manifestation of coronary atherosclerosis in up to one-half of individuals [2]. Furthermore, many individuals who experience myocardial infarction have cholesterol concentrations at or below thresholds of 5.2 mmol/l (200 mg/dl) for total cholesterol and 3.4 mmol/l (130 mg/dl) for LDL cholesterol indicating that the importance of various risk factors differs between subjects [5].

Atherosclerosis is a disease of large and medium-sized arteries nourished by lipid [4, 6, 7]. The early atherosclerotic lesions develop in a topographical pattern that strongly suggests involvement of hemodynamic forces in their pathogenesis [8]. Predilection sites are in areas where turbulent blood flow occurs. Plasma molecules and lipoprotein particles extravasate through leaky endothelium.

The response-to-injury hypothesis was introduced with the discovery of cytokines (cell hormones) and their receptors to explain the changes in the vessel wall associated with lipid

accumulation. Inflammation is considered to be involved in all cardiovascular diseases, including the earliest steps in atherogenesis. Both in animal models and in humans, leucocytes are localized within the earliest lesions [9]. An atherogenic diet promotes the expression of selective surface adhesion molecules in endothelial cells such as vascular cell adhesion molecule -1 (VCAM-1). VCAM-1 binds monocytes and T-lymphocytes which typically are present in early experimental and human atheroma [9]. Increased expression of other leukocyte adhesion molecules such as intercellular adhesion molecule -1 (ICAM-1) have been reported in the presence of disturbed flow in the arteries [10]. Plasma cells and B-lymphocytes may be present in adventitia nearby lesions [11], whereas neutrophils are present in trombosed coronary plaques, probably as a response to plaque rupture [12].

The migration of leukocytes (monocytes and T-cells) into the intima is facilitated by chemoattractant molecules such as monocyte chemoattractant protein-1 (MCP-1) and IFN-inducible protein 10 (IP-10) [13, 14]. The monocytes differentiate to macrophages within the intima and take up atherogenic lipoprotein (modified LDL) via scavenger receptors [4]. The expression of scavenger receptors in macrophages are stimulated by inflammatory mediators such as macrophage colony stimulating factor (M-CSF) [9] which promotes the ingestion of lipids and in addition to MCP-1 promote the transition to foam cells [9]. Foam cells containing increasing amount of cholesteryl esters is a characteristic for both early (fatty streaks) and late atherosclerotic lesions. The scavenger receptors are not downregulated by increasing intracellular accumulation of modified LDL, leading to apoptosis and necrosis of the macrophages, and formation of a lipid-rich core within the atherosclerotic plaque. In addition to degraded foam cells, atherogenic lipoproteins may accumulate within the intima without passing through foam cells [15].

As the lesion develops, the inflammatory process leads to release of fibrinogenic mediators that can promote proliferation of smooth muscle cells (SMCs) and production of a dense extracellular matrix [16]. Deposition of fibrous tissue characterize the more advanced atherosclerotic lesion [2], which may contribute to stabilize plaques, and thereby reducing the risk of plaque rupture and thrombosis [17]. The proliferation and secretion of extracellular matrix proteins, including collagen by SMCs is stimulated by various growth factors, especially transforming growth factor-β and platelet-derived growth factor, [2] promoting the transition from a lipid-rich plaque to a fibrotic and, ultimately, a calcified plaque.

The lipid-rich core of an atherosclerotic plaque is avascular and hypocellular. Increased apoptosis lead to depletion of vascular smooth muscle cells and decreasing collagen supporting the plaque [18]. Furthermore, inflammatory mediators can inhibit collagen synthesis and evoke the expression of collagenases by foam cells within the intimal lesion [19, 20]. This leads to thinning of the fibrous cap, rendering it susceptible to rupture. In human plaques, matrix metalloproteinase 9 (MMP-9) is catalytically active and may contribute to the dysregulation of extracellular matrix that leads to plaque rupture [21]. Patients with stable coronary disease have increased serum levels of MMP-9 compared to controls [22], and during acute coronary syndromes plasma MMP-9 concentrations are increased 2-to 3-fold compared to controls [23]. A strong concordance between plaque size and morphology in different locations of the vasculature has been reported, indicating that systemic factors are important in the development [24]. Neovascularisation from the artery's vasa vasorum represent another route for leukocyte entry into atherosclerotic lesions [25]. Intraplaque haemorrhage from neovessels can generate thrombin which activates endothelial cells, monocytes/macrophages, SMCs and platelets [26]. Apoptosis contributes to redistribution of phospholipid on the cell surface and the formation of microparticles rich in

negatively charged phospholipid, which enhances tissue factor activity, and thereby the thrombogenicity of the lipid-rich core [27].

The most common cause for artery thrombosis is plaque rupture where there is a defect or gap in the fibrous cap, thereby exposing the thrombogenic lipid-rich core followed by adhesion of platelets and activation of the coagulation system [4]. Key histological characteristics of plaques that have ruptured are a thin fibrous cap, abundant macrophages, and a low number of SMCs. In acute coronary syndroms the culprit lesions are usually less calcified than plaques in patients with stable angina pectoris [28, 29]. In stable angina pectoris plaques often are associated with severe luminal narrowing because of constrictive remodelling. In contrast, plaques responsible for acute coronary syndromes are usually relative large and characterized by expansive remodelling, preserving a normal lumen [24, 30]. Inflammatory activation rather than degree of stenosis renders the plaque vulnerable [20]. Rupture of a plaque is followed by thrombus formation which is the main cause of acute coronary events [31]. Tissue factor is expressed in macrophages/foam cells in atherosclerotic plaques [32]. Tissue factor initiates blood coagulation by binding to factor VII/VIIa with subsequent proteolysis of factor IX and X leading to thrombin generation with subsequent fibrin formation and platelet activation [33, 34]. The thrombogenicity of the plaque is associated with intramural tissue factor expression which is most pronounced in lipid rich plaques [35]. Whether plaque rupture leads to an occlusive thrombus depend on the balance between pro-coagulant and anti-coagulant factors. The formation of fibrin is the final step of the coagulation cascade. The fibrinolytic system evokes the resolution of thrombi. An imbalance between clot-dissolving enzymes and their endogenous inhibitors, primarily plasminogen activator inhibitor-1 (PAI-1), may impair fibrinolysis [36].

1.2 Vascular calcification

Calcification is a common feature of atherosclerotic plaques. Accumulating evidence suggest that atherosclerotic calcification shares features with bone calcification, a process which is carefully regulated [37]. Calcification of arteries may be localized in the media or intima. Media calcification occurs independently of atherosclerosis and is observed frequently in end-stage renal disease [38], diabetes mellitus [39], hypervitaminosis D [40] and in Mønkebergs's sclerosis [41].

Several models of intimal arterial plaque calcification have been proposed. 1; The passive model of arterial calcification postulates that arterial mineral deposition occurs when inhibitors are not able to prevent the precipitation [42]. 2; The active osteoblast-like arterial cell model: Pluripotent arterial cells called calcifying vascular cells (CVCs) are colocalized with bone related proteins and transcriptional factors such as bone morphogenetic protein- 2 (BMP-2) and core binding factor alpha 1(Cbfa1) in atherosclerotic plaques [43, 44]. According to this model matrix Gla protein (MGP) inhibits calcification and Cbfa1 promotes differentiation of pluripotent arterial cells into an osteoblast-like cell [45]. 3; The "arterial OCL (osteoclast-like cell) model" suggests that arterial calcification is due to lack of activity of OCL cells [46].

1.2.1 Osteoporosis and atherosclerosis

Osteoporosis and atherosclerosis, especially vascular calcification, are commonly found together, mainly in elderly people and in individuals with autoimmune diseases [47-51]. An inverse association between bone mineral density (BMD) and calcification in the coronary arteries has been demonstrated, with the highest burden of calcified plaques in women with the lowest bone mineral density [52]. In patients with an acute ischemic stroke the presence of

hyperechogenic carotid plaques, the plaque type with highest level of vascular calcification, was an independent marker for osteoporosis [53]. In the Tromsø Study (n=5269) low bone mineral density (BMD) was associated with increased risk of echogenic atheroscerotic plaques of the right carotid artery [54]. A possible hypothesis for coexistence of osteoporosis and cardiovascular disease might be common risk factors, such as age, hypertension, diabetes mellitus, smoking, and hormonal modifications [53]. Oestrogen deficiency, abnormalities of vitamin D metabolism, and lipid oxidation in a common pathogenetic pathway for the two disorders have been implicated [38], but the underlying mechanisms that operate in bone metabolism and vascular homoeostasis have not been fully defined.

1.3 OPG and RANKL

Osteoprotegerin (OPG), receptor activator of nuclear factor κB ligand (RANKL) and the cytokine network they are part of, have been proposed to represent the long sought link between the skeletal system and the cardiovascular system [47].

OPG was first identified in 1997 by several groups [55-58]. OPG was reported to be central in the regulation of bone turnover by inhibition of osteoclastogenesis, and OPG was alternatively termed osteoclastogenesis- inhibitory factor (OCIF) [59]. The mouse and human OPG proteins are 85% and 94% identical to the rat protein, respectively, indicating that the OPG gene has been highly conserved throughout evolution [55]. Human milk contains OPG at a level which is 1000- fold higher than in serum and it has been suggested that it might be of importance for bone mineral density and the immunological system of the child [60]. Possible sources for OPG are suggested to be both cells in milk, and mammary epithelial cells [60]. OPG is a secreted glycoprotein consisting of 401 amino acids, 21 amino acids is a signal peptide resulting in a mature form of 380 amino acids containing seven domains [55].

The N-terminal region consists of four tandem cysteine-rich TNFR motifs, and the C-terminal half contains a heparin binding region [55]. The synthesized 55-62 kDA monomer is converted to a disulfide-linked homodimeric glycoprotein and secreted [55]. The heparin binding domain is involved in the formation of homodimers [61]. OPG has no transmembrane domain and is in this respect an atypical member of the TNF receptor family. Transcripts of OPG have been detected in several murine tissues including liver, lung, heart, kidney, stomach, intestines, skin and the skull [55]. In humans the highest expression is in the lung, heart, kidney and placenta [55]. The wide tissue distribution of OPG suggests that this molecule has functions in addition to bone turnover.

Overexpression of OPG in mice led to osteopetrosis and splenomegaly (increased hematopoiesis), whereas no other abnormalities were found [55]. OPG knockout mice suffered from severe osteoporosis and vascular calcification [62]. In vitro, recombinant OPG blocked osteoclastogenesis, and the N-terminal portion of OPG containing the TNFR-like domain was necessary and sufficient to do so [55]. This effect was due to binding of OPG to RANKL on the surface of osteblasts, thereby preventing the binding of RANKL to its receptor RANK on precursors of osteoclasts [59]. RANKL is necessary for the maturation and activation of osteoclasts and is expressed on osteoblastic and stromal cells [59, 63]. The dimeric form of OPG exhibits a much higher affinity (two or three log) for RANKL than the monomeric form and also higher heparin-binding capacity [64]. RANKL exist either as a type II membrane protein or as a soluble protein [65]. Two receptors for RANKL have been indentified; soluble OPG and transmembrane RANK [63, 65]. $RANKL^{-/-}$ mice showed severe osteopetrosis and a defect in tooth eruption, and completely lacked osteoclasts [66]. Furthermore, early differentiation of T and B lymphocytes was disturbed and they lacked all lymph nodes, but had normal splenic structure and Peyer's patches. Thus, RANKL is a

regulator of lymph-node organogenesis and lymphocyte development, and is an essential osteoclast differentiation factor in vivo [66].

After binding of RANKL to RANK, intracellular signal transduction pathways such as mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NFκB) are activated [65, 67]. Moreover, RANKL significantly stimulates monocyte chemotaxis via activation of phosphatidylinositol 3-kinase, phosphodiesterase, and Src kinase. The migration is inhibited by OPG [68]. Furthermore, OPG seems to regulate the interactions between T cells and dendritic cells. Dendritic cells isolated from $OPG^{-/-}$ mice more efficiently present antigen in vitro and the release of inflammatory cytokines are enhanced when stimulated with bacterial products [69]. Thus, OPG seems to downregulate the immune response by decreasing dentritic cell survival [70].

1.4 OPG and TRAIL

OPG can also stimulate cell survival by binding TNF-related apoptosis-inducing ligand (TRAIL). TRAIL is also a member of the TNF family, it is a type II transmembrane protein and transcripts are detected in many human tissues [71]. TRAIL induces apoptosis in a wide variety of cells and tumour cells are more sensitive than non-malignant cells [71-73]. OPG produced by monocytes within tumours may be involved in survival of several tumour cell types [74-76]. Many tumour cell lines also express OPG [74, 77, 78], and in vitro studies indicate that OPG may act as a survival factor for tumour cells from both solid tumours [74, 78] and haematological malignancies [79]. Inactivation of the tumour suppressor gene p53 leads to increased secretion of OPG from endothelial tumour cells [80]. Furthermore, a pro-angiogenic effect of OPG by formation of cord like structures and networks has been shown [73]. Several studies have demonstrated that OPG can promote endothelial cell survival. A

study by Pritzker et al suggested that inhibition of TRAIL was involved [81], however, others have proposed that other mechanisms are involved because TRAIL was reported to be absent from endothelial cell cultures [73, 82]. Recently, it was reported that the binding of OPG to TRAIL is in the same order of magnitude as the binding of OPG to RANKL [83].

1.5 OPG and the skeletal system

As mentioned, RANKL is necessary for the maturation and activation of osteoclasts and is expressed on osteoblastic and stromal cells [59, 63]. In patients with primary hyperparathyroidism, a condition associated with increased bone destruction, the ratio of RANKL/OPG expression within the bone microenvironment decreased after parathyroidectomi [84]. As increased osteoclast activity is observed in patients with osteoporosis, metastases and rheumatoid arthritis, the OPG/RANKL/RANK system appears to be a potential therapeutic target for bone diseases [85]. One single dose of recombinant OPG injected subcutaneously in postmenopausal women decreased bone turnover [86]. In mice with osteolytic sarcoma cells injected intramedullary, OPG treatment stopped further bone destruction and reduced ongoing and movement-evoked pain [87]. In humans with multiple myeloma with osteolysis and patients with bone metastases from breast carcinoma, bone resorption measured by urinary N-telopeptide of collagen decreased after one injection of recombinant OPG [88]. A potential concern with the use of OPG was development of anti-OPG antibodies and that binding of OPG to TRAIL might interfere with a natural defense mechanism against tumourgenesis [89]. Denosumab is a human monoclonal antibody to RANKL. Osteoporotic postmenopausal women treated every 6 months for three years had reduced risk of vertebral, nonvertebral, and hip fractures compared to the placebo group [90]. Denosumab treatment in men with prostate cancer treated with androgen blockade was also associated with increased bone mineral density [91].

1.6 OPG and the vascular system

Expression of OPG has been observed in the heart, arteries and veins [92]. OPG is expressed in both endothelial cells and SMCs. In vascular SMCs TNFα, IL-1β, basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and angiotensin II are reported to increase the expression of OPG [93, 94]. SMCs has recently been suggested to be the main source of circulating OPG [95]. In endothelial cells, OPG expression is stimulated by TNFα, IL-1α, IL-1β, and activated integrin $\alpha_v\beta_3$ [93, 96-98]. For some growth factors the expression of OPG is increased by activation of the transcription factor NF-κB, however, PDGF mediated expression of OPG in smooth muscle cells was found to be independent of NF-κB [94], suggesting that alternative mechanisms exist for OPG expression.

At the protein level OPG is localized in endothelial cells within the secretory granules, Weibel-Palade bodies (WPB) [99, 100]. Von Willebrand factor (vWF) and the adhesion molecule P-selectin are colocalized with OPG within the WPB. vWF is considered to be a marker of endothelial dysfunction [101, 102] that performs 2 main functions in haemostasis: it mediates platelet adhesion to the injured vessel wall, and it carries and protects coagulation factor VIII. OPG was associated with vWF both within the WPB and in human serum and plasma [99, 100]. The interaction between OPG and vWF is located to the A1 domain of vWF [100]. This domain can bind to GPIbα, collagen, heparin and sulphatides [103]. Recently, it was reported that OPG is also colocalized with vWF in the alpha granules of human platelets [104].

Risk factors for atherosclerosis, such as hypertension, smoking, hypercholesterolemia and diabetes, are associated with endothelial dysfunction leading to a pro-inflammatory and pro-thrombotic phenotype of the endothelium [105]. As mentioned above, OPG is up-regulated

by pro-inflammatory cytokines in vascular smooth muscle cells and endothelial cells [94, 96, 97]. RANKL has been reported to promote endothelial cell survival [106] and to activate nitric oxide synthase pathways in endothelial cells [97], whereas pre-incubation with recombinant OPG neutralized the activation [97]. Decreased NO (nitric-oxide) production is involved in the clinical course of cardiovascular disease (CVD) and is reported to be a key feature which precedes vascular alterations [105]. Furthermore, OPG has been shown to induce the expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells and to promote leukocyte adhesion [82, 107]. An abnormal increase of leukocyte adhesion to endothelial cells is considered an early step in endothelial cell dysfunction [105].
The inflammatory marker CRP predicts myocardial infarction, stroke and vascular death in a variety of settings [108]. CRP has been shown to down-regulate the production of endothelial nitric oxide synthase (eNOS), to induce the production of cellular adhesion molecules, to inhibit angiogenesis and to promote apoptosis [108, 109]. Thus, in cell cultures, OPG and CRP share some features, however, in contrast to CRP, OPG has been shown to promote survival of endothelial cells [73, 81, 98], suggesting a possible role in maintaining endothelial integrity.

The OPG/RANKL system; expression in atherosclerosis and cardiovascular disease
OPG is considered to neutralize the effect of RANKL. Both animal studies and studies in humans indicate that RANKL/RANK could be mediators in atherogenesis and plaque destabilization. Dhore et al demonstrated presence of OPG and RANKL in SMCs in non-diseased aortas from humans. In plaques, RANKL could only be demonstrated in association with the extracellular matrix surrounding calcium deposits. High expression of OPG was seen in inflammatory cells present in plaques. A regulatory role of these proteins was suggested not only in osteoclastogenesis, but also in atherosclerotic calcification [37]. Golledge and co-

workers studied endartherectomy samples removed from patients with recent (within 6 weeks) focal neurological symptoms or no previous symptoms and demonstrated higher expression of OPG in carotid atherosclerotic plaques in symptomatic subjects than in non symptomatic subjects. Furthermore, treatment with the angiotensin II blocker irbesartan reduced the OPG secreted by explants [110].

In mice RANK and RANKL are only expressed in calcified plaque and not in normal arteries [111], indicating that the calcification process itself could up-regulate RANK and RANKL expression and signaling. In apoE$^{-/-}$ mice strong RANK and OPG immunoreactivity was observed in SMCs and endothelium of nonatherosclerotic areas, whereas RANKL was not detected [112]. Stronger immunoreactivity for both OPG (adjacent to foam cells) and RANK (more evenly distributed in lesions) was demonstrated. Within lesions, RANKL staining was present in areas rich in T-cells and macrophages [112]. More prominent staining for OPG, RANKL and RANK was reported in more vulnerable plaque phenotype partly due to increased INF-γ [112].

In patients with unstable angina pectoris increased gene expression of RANKL in T cells and RANK in monocytes were demonstrated compared to healthy controls. OPG transcripts were not detected in T cells [112]. In PBMCs from patients with stable angina pectoris undergoing PCI the expression of RANKL increased significantly 4 hrs after PCI compared to baseline. No significant change in RANK was detected [112]. In thrombus material from patients with ST elevation myocardial infarction, strong OPG, RANKL and RANK immunoreactivity in monocytes/macrophages and areas with CD41 positive platelets was reported [112].

In patients with unstable angina, RANKL increased the release of monocyte chemoattractant protein 1 (MCP-1) from PBMC, in contrast no significant effect was observed in patients with

stable disease and controls. RANKL had no effect on IL-8, TNFα and MIP-1α in either patients or controls [112].

Matrix metalloproteinases (MMPs) are a class of proteins thought to influence the composition and thereby the vulnerability of plaques, rendering them susceptible to plaque rupture and thrombosis. In patients with acute coronary syndrome increased expression of MMP-9 and MMP-9/TIMP-1 ratio has been reported in debris from coronary plaques compared to plaques from patients with stable angina [113]. OPG treatment induced increased levels of MMP-9 in macrophage-like cells as well as in aortic SMCs from apoE$^{-/-}$ mice. Furthermore, OPG was a potent inducer of MMP-9 activity in bone marrow-derived macrophages [114]. In vascular SMCs, but not in macrophages, RANKL increased total MMP activity (MMP-1,-2,-7,-8,-9,and-13) [112]. OPG increased the RANKL effect on MMP activity in SMC at molar ratios of 0.5 and 3 (molar ratio in serum ≈ 100) [112]. At very high concentrations of OPG, OPG alone induced MMP activity in vascular SMCs [112].

OPG and thrombosis

Limited data on OPG and coagulation factors are available. However, in a study in women with previous gestational diabetes, no significant association between OPG and vWF, PAI-1 and fibrinogen was reported [115]. In a case-control study in young survivors of myocardial infarction, we found no significant associations between OPG and factor VIIa, factor VIIc, fibrinogen, tissue factor pathway inhibitor antigen (TFPIag), TFPIac, and thrombin-antithrombin complexes (TAT) measured 1-4 years after acute myocardial infarction in patients or in controls [116]. Zannettino et al showed that OPG is able to bind vWF reductase, and thrombospondin 1 (TSP-1). They speculated that this may aid in tethering the ultra large vWF multimers at the site of vascular injury, thereby promoting thrombus formation [99].

Some studies indicate that subjects with CVD have increased risk of venous thromboembolism (VTE) and vice versa [117, 118]. Risk factors responsible for this association are poorly understood. In the Physicians' Health Study, hypertension, elevated cholesterol, diabetes, and smoking were associated with increased rates of CHD and stroke, but had no association with VTE. Conversely, higher BMI was more strongly associated with risk of VTE than of either CHD or stroke, and taller men had a significantly increased risk of VTE, but a lower risk of CHD [119]. A link between elevated OPG levels and occurrence of both venous thrombosis and bleeding was reported in patients with the chronic myeloproliferative disease polycythaemia vera [120]. One unit change in OPG was associated with 33% (p=0.03) increased risk of venous thrombosis and with 37% (p=0.013) increased risk of bleeding in a retrospective analysis. OPG was also related to the development of the combined outcome of venous thrombosis and bleeding in a prospective analysis [120].

1.7 OPG and other diseases associated with cardiovascular disease

Serum levels of OPG are increased in subjects with diseases characterized of inflammation and associated with increased risk of atherosclerosis and cardiovascular death.

OPG in renal failure

Theoretically, reduced elimination of OPG could be involved in patients with renal failure. However, studies in rats showed that ^{125}I-labelled protein was rapidly and predominantly distributed to the sinusoids of the liver after intravenous injection [121]. The hepatic uptake was partly regulated under a saturable process, pre-dosing with sulfated glycans such as dextran sulfate and heparin markedly inhibited the uptake [121]. In patients with renal failure OPG is associated with mortality. In subjects undergoing hemodialysis elevated plasma OPG

predicted all-cause mortality and cardiovascular mortality after adjustment for cardiovascular risk factors. OPG was a particularly strong predictor for mortality in patients with increased CRP [122]. In patients (without diabetes) followed for 8 years after renal transplantation, serum OPG measured within 10 weeks post transplant, was independently associated with all-cause mortality and cardiovascular death [123].

OPG and diabetes mellitus

In subjects with diabetes mellitus, Browner et al reported increased serum OPG [124], a finding confirmed in several studies. Anand and coworkers have published two prospective studies in patients with type 2 diabetes mellitus. In the first study, plasma OPG was significantly elevated in patients (n=510) with increased coronary artery calcification both in crude and adjusted analyses. In a multivariable model, only coronary artery calcification score was an independent predictor for cardiovascular disease and mortality [125]. In their second study in 390 subjects without cardiovascular symptoms, OPG was significantly associated with increased coronary artery calcification score in univariable analysis, but not in multivariable analysis [126].

OPG and heart failure

In 234 patients with acute myocardial infarction complicated with heart failure (LVEF < 35%) serum OPG remained elevated compared to controls during 27 months of follow up, and OPG at baseline was associated with adverse outcome and remained an independent predictor for cardiovascular events and mortality [127]. The same research group has shown that serum OPG increases in patients with heart failure, irrespective of the etiology of heart failure [128]. In men with nonischemic dilated cardiomyopathy, plasma TRAIL were elevated compared to controls and positively correlated with left ventricular end-diastolic

diameter. In this study, plasma OPG did not differ between patients and healthy controls. In endomyocardial biopsies, TRAIL and OPG protein were detected by immunohistochemistry, but not in controls. In patients, TRAIL mRNA was upregulated in peripheral blood lymphocytes. OPG mRNA was up-regulated in the myocardium, possibly representing a compensatory mechanism to limit systemic activation of TRAIL in patients with congestive heart disease [129].

Rheumatic disease, CVD and OPG

OPG concentrations were increased in patients with rheumatoid arthritis and associated with inflammation [130]. OPG concentrations were higher in patients with long-standing rheumatoid arthritis and in this group associated with coronary-artery calcification independently of cardiovascular risk factors and disease activity [130]. In patients with inflammatory rheumatic diseases and coronary artery disease (CAD) increased plasma levels of markers of endothelial cell activation such as VCAM-1, vWF and OPG was found compared to patients with CAD without inflammatory rheumatic disease. Acute coronary syndrome was a significant predictor of OPG in the group with inflammatory rheumatic disease. OPG and several other markers of inflammation, but not lipids, predicted CAD in patients with inflammatory rheumatic disease, in contrast to the group with CAD alone [131].

1.8 Ultrasonographic examination for carotid artery plaque assessment

The carotid arteries are readily accessible for ultrasonographic examination, and it is a safe, low-cost method for the assessment of atherosclerosis, both in terms of the presence of plaques and plaque morphology. It is based on two principles; high resolution B-mode imaging to visualize the arterial wall and any local changes, and Doppler flow studies to study

blood flow and changes in blood flow velocity associated with stenosis or the absence of flow associated with occlusion. A brief description of B-mode imaging will be given.

Ultrasound waves are partly absorbed by the tissue and partly reflected. In the ultrasound transducer an oscillating piezoelectric element sends pulses of ultrasonic waves into the tissue. In a certain period after the transmission of waves, the transducer is able to receive reflected ultrasound and convert it to electrical signals, which are processed into a two-dimensional grey-scale image by a computer. The acoustic impedance, which is defined as the product of the density of the tissue and the velocity of the sound, determines how much of the emitted ultrasound that are reflected. The higher the difference in density between the tissue layers, the more energy is reflected and the brighter will the object appear on the B-mode image (B; brightness). Fibrous and calcified tissue will appear bright, whereas moving blood cells will appear black.

Examination of the carotid arteries with ultrasound is a more sensitive method for detecting carotid plaques than angiography, and there is a high correlation between plaque in the carotid arteries and other vascular territories such as the coronary arteries [132]. The earliest visible change in the carotid artery associated with atherosclerosis is an increase in the intima media thickness. The atherosclerotic process mainly involves intima of arteries; however, it is not possible to distinguish well between the intima and media by ultrasound. Carotid intima media thickness (CIMT), measured precisely and noninvasively by B-mode ultrasonography, is a marker of early atherosclerosis [132] associated with cardiovascular risk factors such as age, smoking, hypertension, obesity, dyslipidaemia, diabetes and metabolic syndrome [133-135]. CIMT increases nearly linearly with age [135], and has also been shown to predict cardiovascular events both in the myocardium and brain [136, 137]. Evaluation of plaque

burden can be done by several methods. The simplest method is to determine whether plaques are present or not and to report the number of plaques. Secondly, the thickness of each plaque can be measured; a total atherosclerotic score can be calculated by summarizing the thickness of all plaques detected. Thirdly, the area of plaques can be calculated offline by tracing around the perimeter of each plaque on stored B-mode images with a cursor. Finally the most accurate method would be a three dimensional assessment which can be done by computer tomography or magnet resonance imaging. However, it is an expensive method and not well suited for larger epidemiological studies.

Plaque morphology can be assessed by how the carotid plaques appear on the B-mode image. Based on echogenicity, defined as reflectance of the emitted ultrasound signal, Gray-Weale et al proposed four categories of plaque echogenicity; echolucent, predominantly echolucent, predominantly echogenic and echogenic [138]. The echolucent plaques are lipid rich, whereas the echogenic plaques contain more dense fibrous and calcified tissue. Histological studies of endarterectomy samples have shown a high concordance with plaque morphology assessed by ultrasound [138-140].

Plaque morphology in terms of echogenicity may also be assessed by objective measurements by recording all examinations and measurements of plaques. The echogenicity of digital plaque images may be expressed as a continuous variable on a grey scale, averaged for all pixels in the picture [141]. The inter-observer reproducibility of GSM analysis is good [141-144]. Echolucent plaques with low GSM score are lipid rich whereas echogenic plaques contain more fibrous tissue and calcium. Examinations of carotid endarterectomy samples have shown a high concordance between GSM (included colour mapping) and the histological picture [145, 146].

2. Aims of the study

The overall aim of the project was to study the relation between serum OPG and carotid atherosclerosis as assessed by CIMT, plaque echogenicity, novel plaque formation, plaque progression, and cardiovascular diseases and mortality.

Specifically, the aims of the subprojects were to study:

1. The association between serum concentrations of OPG and carotid plaque echogenicity in subjects with carotid plaques.
2. The association between OPG serum concentration and carotid intima media thickness (CIMT), a surrogate marker for early atherosclerotic disease, in a large population-based study with a wide age span of participants.
3. The association between OPG and cardiovascular risk factors, and the impact of serum levels of OPG and sRANKL on de novo atherosclerotic plaque formation and plaque growth in the right carotid artery during seven years of follow-up, in a general population.
4. The association between OPG and cardiovascular disease (myocardial infarction, ischemic stroke, hemorrhagic stroke) and mortality (total, cardiovascular and non-vascular mortality) during twelve years of follow-up in a population-based cohort study.

3. Study populations and methods

Description of study design, inclusion and exclusion criteria, laboratory and clinical measurements and statistical methods are given in detail in the separate papers. The present chapter gives a brief description of the study populations and discusses briefly some epidemiological and statistical aspects.

3.1 The Tromsø Study and participants

The Tromsø Study is a single centre prospective follow-up study of the population of Tromsø, Norway The studies have been carried out by the Department of Community Medicine at the University of Tromsø, in collaboration with the Norwegian Institute of Public Health (formerly the National Health Screening Service), the University Hospital of Northern Norway (UNN) and Tromsø City Council. The main focus of the Tromsø Study has been on cardiovascular disease. The first survey was carried out in 1974 (Tromsø I), followed by surveys in 1979-80 (II), 1986-87 (III), 1994-95 (IV), 2001-02 (V) and 2007-08 (VI).

The participants in paper 1 were recruited from Tromsø V and the participants in paper 2, 3 and 4 were recruited from Tromsø IV. Participants included in the study reported in paper 3 were examined both in Tromsø IV and V.

The IVth survey consisted of two screening visits 4-12 weeks apart. All registered inhabitants of Tromsø 25 years or older were invited to the first screening visit. The invitation letter also contained a questionnaire about cardiovascular risk factors and disease and declaration of consent (Appendix A). Of 35 443 invited, 27 168 (76.6%) attended the first visit. To the second visit (phase 2) all subjects aged 55-74 years and 5-10% samples in the other 5-year birth cohorts were invited. The total number invited to the second visit was 8732 subjects of

whom 6889 (78.9%) attended. A more comprehensive examination, including ultrasound examination of the right carotid artery was performed in 6727 subjects of those who attended, i.e. 77.0% of the eligible population. The participants were given a second questionnaire covering such issues as dietary habits, use of drugs, life style factors etc (Appendix A). A shorter questionnaire was used for participants older than 70 years (Appendix A).

Paper 1: Participants for this case-control study was recruited from Tromsø V (2001-02) Subjects included were randomly selected among 56–80 years old participants who had one plaque or more in the right carotid bifurcation or internal carotid artery at the screening visit with a plaque thickness of ≥2.5mm and plaque morphology classified as echolucent (grade 1) or echogenic (grade 4) according to the Gray–Weale criteria [138]. Persons in the same age groups without plaques in their carotid arteries were used as controls. As only the right carotid artery was examined at the screening visit, a new ultrasound examination including both carotid arteries was performed.

Paper 2: All participants in this cross sectional study had taken part in the carotid ultrasound examination in Tromsø IV. Valid measurements of CIMT were available in 6677 subjects. Fifty-seven persons were excluded due to lack of written consent to future medical research. Subjects with frozen serum samples available for OPG measurement were included (n=6516).

Paper 3: Subjects examined by carotid ultrasound both in Tromsø IV and V were included. Of the 6727 examined with carotid ultrasound in Tromsø 4, 956 subjects did not attend Tromsø V, 110 attended, but were not examined with ultrasound due to logistic problems, 271 subjects had moved from Tromsø, and 532 had died. Serum samples for OPG measurements were lacking for 100 subjects, and 219 subjects were excluded due to low ultrasound image

quality. Consent was lacking for 19 subjects. Thus, 4520 subjects with valid measurements were included in the study.

Paper 4: Eligible subjects were those who attended both the first and second visit of Tromsø IV (n=6899). Subjects not officially registered inhabitants of the municipality of Tromsø at baseline (n=13), without valid measurement of OPG (n=87), with a known history of myocardial infarction (n=378), ischemic stroke (n=79) or both (n=20) at baseline, and subjects without valid written consent (n=57) were excluded from the cohort participating in Tromsø IV phase 2, leaving 6265 subjects who were followed up from the day of enrolment in 1994/95 to 31st of December 2005.

3.2 Ultrasound examination

The ultrasound methods are described in detail in the papers. The protocol for the ultrasound procedures is included in appendix B. The right carotid artery was scanned with the subject in the supine position with the head slightly rotated to the left. The ultrasound examinations in were carried out by three sonographers (different in each survey). To standardize measurements the sonographers completed a 2-month training program. As previously reported, the intra- and interobserver reproducibility on measurements of carotid intima-media thickness, plaque occurrence, plaque thickness and plaque echogenicity was good in each of the study examinations [147, 148]. Subjects included in paper 1 underwent a repeated scanning of both the left and right carotid arteries.

3.3 Biochemical analyses

Non-fasting blood samples were collected from an antecubital vein and serum was prepared by centrifugation after one hour respite at room temperature. Serum aliquots were stored at -

70 °C. OPG and sRANKL were analysed in thawed serum stored for 12 years. The analyses were performed on coded samples without knowledge of status regarding atherosclerosis in the carotid arteries by the person performing the assays. All samples were analyzed in duplicate.

3.4 Clinical end points assessment

In paper 4 we studied the relation between OPG measured at baseline and incident myocardial infarction, ischemic stroke, hemorrhagic stroke, total mortality, death of myocardial infarction, death of stroke and death of non-vascular causes during up to 12 years follow-up. Adjudication of hospitalized and out-of hospital events was performed by an independent endpoint committee and based on data from hospital and out-of hospital journals, autopsy records, and death certificates. The national 11-digit identification number allowed linkage to national and local diagnosis registries. Cases of incident myocardial infarction and ischemic stroke were identified by linkage to the discharge diagnosis registry at the University Hospital of North Norway (UNN) with search for ICD 9 codes 410-414 and 430-438 in the period 1994-98, and thereafter ICD 10 codes I20-I25 and I60-I69. UNN is the only hospital in the area serving the Tromsø population. The hospital medical records were retrieved for case validation. Slightly modified WHO MONICA/ MORGAM criteria for MI were used and included clinical symptoms and signs, findings in electrocardiograms (ECG), values of cardiac biomarkers, and (when applicable) autopsy reports [149]. An ischemic stroke was defined according to the WHO definition [150] only when CT or MRI scans had ruled out brain haemorrhage. Further, linkage to the National Causes of Death Registry at Statistics Norway allowed identification of fatal incident cases of myocardial infarction and ischemic stroke that occurred as out-of-hospital deaths, including deaths that occurred outside of Tromsø, as well as information on all-cause mortality. Information from the death certificates

was used to collect relevant information of the event from additional sources such as autopsy reports and records from nursing homes, ambulance services and general practitioners. The Norwegian Registry of Vital Statistics provided information on emigration and death.

4. Main results

4.1 Paper 1

Serum osteoprotegerin is inversely associated with carotid plaque echogenicity in humans.

The purpose was to study the relation between serum OPG levels and plaque morphology in subjects with subclinical carotid atherosclerosis and controls. Participants were recruited from a population health study (Tromsø V) and OPG serum levels were compared in 29 persons with echogenic (fibrotic and/or calcified) carotid plaques, 30 persons with echolucent (lipid rich) plaques and 41 persons without carotid plaques. Computerized assessment of plaque echogenicity was done by use of the gray scale median (GSM).

Participants with echogenic carotid plaques (defined as GSM above median, GSM >64.45) had lower serum OPG level (1.23 ng/ml; 1.02-1.48) (geometric mean; 95% CI) than persons with echolucent plaques (GSM ≤ 64.45) (1.76 ng/ml; 1.46-2.14) and those without plaques (1.89 ng/ml; 1.60-2.21). Both OPG and PTH were independently related to GSM. A significant linear trend for decrease in GSM across quartiles of OPG was found (p=0.003) which remained significant after adjustment for PTH and smoking.

Thus, lower serum OPG levels in subjects with subclinical echogenic carotid plaques and an inverse relation between serum OPG and plaque echogenicity were demonstrated. The findings support the concept that OPG may play an important role in arterial calcification.

4.2 Paper 2

Relation between serum osteoprotegerin and carotid intima media thickness in a general population – The Tromsø Study.

CIMT, measured precisely and noninvasively by B-mode ultrasonography, is a marker of early atherosclerosis. Previous studies have reported conflicting results on the relation between serum OPG concentration and CIMT. The present study was conducted to investigate the relations between OPG, risk factors for cardiovascular diseases and CIMT in a large cross-sectional study including 6516 subjects aged 25-85 years who participated in a population based health survey. CIMT increased significantly across tertiles of OPG after adjustment for traditional cardiovascular risk factors such as age, sex, smoking, total cholesterol, HDL cholesterol, CRP, BMI, systolic blood pressure, cardiovascular disease (CVD) and diabetes mellitus ($p< 0.0001$). There was a significant interaction between age and OPG ($p=0.026$). Increasing OPG concentrations (per SD) reduced the risk of being in the uppermost quartile of CIMT (OR 0.52, 95% CI 0.30-0.88) in subjects < 45 yrs (n=444), whereas subjects ≥ 55 yrs of age (n=4884) had increased risk of being in the uppermost quartile of CIMT (OR 1.19, 95% CI 1.10-1.29) after adjustment for traditional CVD risk factors. Thus, age has differential impact on the association between OPG and CIMT in a general population. The present findings may suggest that increased serum OPG does not promote early atherosclerosis in younger subjects.

4.3 Paper 3

Serum osteoprotegerin, sRANKL and carotid plaque formation and growth in a general population – The Tromsø Study.

Intervention studies in animal models suggest that osteoprotegerin (OPG) functions as an inhibitor or marker of atherosclerosis, whereas one prospective epidemiological study in humans indicated that OPG was an independent risk factor for progression of atherosclerosis. This study was undertaken to explore the association between serum levels of OPG, soluble RANK ligand (sRANKL) and carotid artery plaque formation and plaque growth. The prevalence of carotid plaque and plaque area were assessed by ultrasonographic imaging at baseline and after 7 years follow-up in 2191 men and 2329 women who participated in a population-based study. OPG was significantly associated with atherosclerotic plaque burden and cardiovascular risk factors such as age, body mass index, blood pressure, total cholesterol, HDL cholesterol, HbA1c, fibrinogen at baseline, but not with sRANKL. In subjects without plaque at baseline, OPG predicted plaque formation in crude analysis in both women and men, but not after adjustment for age and other atherosclerotic risk factors. OPG predicted plaque growth in women (+1.8 mm^2, 0.6-3.0) (mean, 95% CI) per 1 SD increase in OPG (p=0.003), whereas no associations were demonstrated in men (0.1 mm^2 (-1.3-1.4), p=0.93). Soluble RANKL did not predict plaque formation or plaque growth.

OPG was an independent predictor of plaque growth in women, but not in men, suggesting sex specific actions of OPG in plaque growth. OPG was not associated with novel plaque formation.

4.4 Paper 4

Serum osteoprotegerin is a predictor for incident cardiovascular disease and mortality in a general population – The Tromsø Study.

Osteoprotegerin (OPG) concentration in serum is associated with the presence and severity of atherosclerosis, and predicts cardiovascular disease and mortality in high-risk populations. The present study was undertaken to investigate the association between serum OPG levels and risk of future myocardial infarction (MI), ischemic stroke (IS) and mortality in a general population. Serum OPG was measured in serum samples from 6265 persons without prior cardiovascular diseases aged 25 to 84 years, who participated in a population health survey, The Tromsø Study, in 1994-95. Incident MI, IS and mortality were registered from the date of inclusion until 31th of December 2005. Cox regression models were used to estimate crude and adjusted hazard ratios (HR; 95% CI) for clinical events. There were 575 MI, 284 IS, and 824 deaths (146 deaths of ischemic heart disease, 78 deaths of stroke, and 600 deaths of other causes) during median 10.6 years of follow-up. Serum OPG concentrations (per SD (1.13 ng/ml) increase in OPG) were associated with increased risk of MI (1.20; 1.11-1.31), IS (1.32; 1.18-1.47), total mortality (1.41; 1.29-1.54), death of ischemic heart disease (1.35; 1.18-1.54), death of stroke (1.44; 1.19-1.75) and death of non-vascular causes (1.31; 1.22-1.41) after adjustment for traditional cardiovascular risk factors. No association was detected between OPG and incident hemorrhagic stroke (HR 1.02; CI 0.73-1.43).

Serum OPG was associated with future risk of MI, IS, total mortality, mortality of ischemic heart disease, stroke and of non-vascular causes independent of traditional cardiovascular risk factors. These findings suggest that serum OPG is a mediator and not merely a marker of cardiovascular diseases, and also plays a role in the pathogenesis of other fatal diseases.

5. General discussion

5.1 Methodological considerations

5.1.1 Measurements of OPG and RANKL

The concentration of total OPG was analyzed by an ELISA assay (R&D Systems, Abingdon, UK) with mouse anti-human OPG as capture antibody. The assay detects both the monomer and dimeric forms of OPG, including OPG bound to its ligands. Biotinylated goat anti-human OPG and streptavidin horseradish peroxydase were used for detection. The OPG assay was performed according to the instructions by the manufacturer. The intra- and interassay coefficients of variation (CV) in our laboratory were 6.5% and 9.3%, respectively.

The concentration of sRANKL was measured by a new, highly sensitive ELISA assay for free sRANKL with a detection limit of 0.02 pmol/L (ampli sRANKL human, Biomedica, Vienna, Austria). The analysis was performed according to the manufacturer's instruction. The intra- and interassay CV for the RANKL assay were 9.3% and 15.0%, respectively. Despite using the most sensitive assay available for measurement of free sRANKL in serum, sRANKL was not detectable in 25.8% of serum samples and below the detection limit of 0.02 pmol/l in 10.4% of samples. In a recently published study by Lieb et al, using the same assay from Biomedica, the concentration of sRANKL was below the detection limit in 25% of the samples [151]. Kiechl et al reported high consistency in absolute levels of serum RANKL and OPG in 3 assessments (1990, 1995, and 2000) indicating long-term stability when stored at -70°C without any thawing-freezing cycle [152]. In contrast, in EDTA plasma others have reported 9.5% and 56.5% decrease in sRANKL compared to baseline when stored at -70 °C for 6 weeks and 6 months, respectively [153]. The OPG plasma concentration was reduced by 5.3% after 6 weeks and 19.7% after 6 months storage at -70 °C [153].

Furthermore, compared to other anticoagulants, EDTA was the preferred anticoagulant in preparation of plasma [153].

Undetectable free sRANKL in a large proportion of samples in our study may indicate that measurement of total RANKL might be favourable. The long storage time of blood samples might also be of importance. Although a reduction in concentration during storage cannot be ruled out, OPG was above the detection limit in all samples. However, it is not likely that this introduced major bias as long as all samples were handled equally.

Blood sampling took place from the morning until the afternoon and participants were not fasting. We have previously shown in young normolipemic males that plasma OPG levels showed a modest, but significant decrease during the day compared to the plasma concentration at 8 am, and that it remained decreased throughout the following 12 h and returned to baseline values the next morning [154]. Others have reported that the circadian rhythm of OPG secretion was characterized by higher daytime concentrations and a nocturnal decrease in post – and premenopausal women and in elderly men [155]. To study the effect of a meal rich in lipids, blood samples were collected in the fasting state and 4 hrs after ingestion of porridge (peak triglyceride concentration in serum appeared 4 hrs after the meal). The standard meal was accompanied by a substantial increase in serum triglycerides from 1.23±0.68 mmol/l in the fasting state to 2.23±1.37 mmol/l in the postprandial state ($p<0.001$) with only a minor decrease in serum OPG levels from 1.45±0.48 ng/ml in the fasting state to 1.32±0.38 ng/ml in the postprandial state, a reduction similar to that observed in young healthy males [154]. Thus, we do not think that non-fasting blood samples and various time points for sampling during the day have introduced severe bias.

5.1.2 Study design, bias and misclassification

Two longitudinal cohort studies, one case-control study and one cross-sectional population-based study are included in this thesis. In cohort studies, the difference in outcome between exposed and nonexposed subjects are studied. Participants often need to be followed for a long time to accumulate sufficient person-time and end-points and thereby they are resource demanding. Bias has been defined as any systematic error in the design, conduct or analysis of a study that results in a mistaken estimate of an exposure's effect on the risk of disease [156]. Cohort studies are vulnerable to selection bias, where the relationship between exposure and disease might differ in subjects participating compared to all that were eligible for the studies [157].

In case-control studies diseased and non-diseased subjects are compared. This design is often used as a first step when searching for a cause of a disease and is very useful in rare diseases. Controls should be selected from the same population, i.e. the source population that gives rise to the study cases. Sometimes it is stressed that cases should be representative for all people with the disease and that controls should be representative for the entire non-diseased population. This can be misleading; a case-control study may be restricted to any type of case that may be of interest as long as it has a sound rationale [158]. A major concern in case-control studies is that cases and controls may differ in characteristics or exposures other than the one that has been targeted for study. One approach to handle this is to match the cases and controls for these characteristics. In our case-control study (paper 1), no significant differences between the groups with regard to cerebrovascular risk factors, included prior cardiovascular disease, strengthen the results despite the low number of participants. Recall bias is a problem in case-control studies where one or more characteristics studied are based on information from the participants. To increase the power of a case-control study with few cases increasing the number of controls up to a ratio of 1 case to 4 controls may be done. A

major limitation with traditional case-control studies is that it is not known whether the disease or condition studied preceded the changes observed in biological characteristics or that they were a result of the disease itself. This problem can be overcome by conducting a nested case-control study, i.e. a case-control study within a cohort where the temporal sequence of exposure and disease can be studied and recall bias is eliminated. This study design is less resource demanding than a cohort study.

Cross-sectional studies can give information about associations. However, it is not possible to establish a temporal relationship and this type of study may only be suggestive of a possible risk factor for a disease. In cohort and nested case-control studies a temporal relationship between exposure and outcome may be found, increasing the possibility of an etiologic relationship. However, observational studies cannot definitively examine whether biomarkers are causally related to a disease.

Information bias can occur when obtained information about the subjects in the study is inadequate, so that the information regarding exposures and/or disease is incorrect. When the gathering of data is inaccurate, subjects may be misclassified and thereby misclassification bias is introduced. Misclassification might be differential or nondifferential. Differential misclassification occurs when the rate of misclassification differs in the different study groups such as in e.g. case control studies where recall bias more often occur in cases than controls. Differential misclassification bias can lead to an apparent association that is false or an apparent lack of association that is false [156]. In nondifferential misclassification there is inaccuracy in the gathering of information in both cases and controls or exposed and unexposed subjects. The effect of this misclassification is usually that the relative risk or odds ratio tends to be diluted, i.e. shifted toward 1.0. Thus, an association is less likely to be

detected [156]. Although a high concordance between plaques in the carotid arteries and other vascular territories have been reported, screening of the left carotid artery as well as other vascular territories in our study (paper 3) would have given a more accurate assessment of plaque burden in each individual, reducing the risk of misclassification. Even though only one carotid artery was screened in our study, a significant association between serum concentration of OPG and plaque area was demonstrated. Many misclassified subjects with a low plaque burden in the screened carotid artery and with a high plaque burden in unscreened arteries arteries would attenuate this association.

Validity

In an epidemiological study the internal validity refers to whether the results are representative (true) for the population under study [158]. Generally, the internal validity may be threatened by selection bias, information bias and confounding. In the Tromsø Study participants were selected by age. Selection bias may be caused by non-attendance. Participants in the study on plaque formation and growth (paper 3) were examined both in Tromsø IV and V. The subjects with follow-up measurements were younger than those lost to follow-up. Moreover, they smoked less, fewer were teetotallers, and they were more physically active and had a lower prevalence of cardiovascular diseases. We believe that it is unlikely that this should invalidate our findings with respect to de novo plaque formation since only subjects without plaques at baseline were included. Whether the findings with regard to OPG and plaque growth would change if persons with more comorbidity were included cannot be definitively ruled out. However, we believe that the internal validity is good due to the high attendance rate. Information bias might influence the quality of data such as smoking habits and self reported diseases. Because diabetes mellitus was self

reported in the Tromsø study, we included HbA1c >6.1% in the definition of diabetes in papers 2-4.

External validity is the degree to which the findings are generalizable to other populations [158]. This can be evaluated by comparing findings between similar studies in different populations and by applying the same models or analyses on other datasets. Due to high net immigration from other parts of Norway, the Tromsø population is relatively young [159]. With regard to the incidence of cardiovascular disease, lifestyle, education, social factors and mortality, the population of Tromsø is similar to other inhabitants in Norway [159].

Confounding and effect modification (interaction)

A confounding factor predicts a disease or outcome, differs between the groups studied and is associated with the exposure under study. The factor's association with disease arises from a causal pathway other than the one under study. A confounding factor must not be affected by the exposure or the disease [158]. Confounding factors may lead to an underestimation or overestimation of the effect of an explanatory variable. Confounding factors may be controlled for by matching in designing of the study or by stratification, or by the use of multivariable statistical methods in analyses of the data [156]. In randomised controlled trials confounding tend to be small in large trials, but might be large in small randomized trials, despite that all potential confounders are expected to be evenly distributed between the groups being compared [160]. However, this is only on average across repetitions of the randomization [158]. In our studies, we have adjusted for known risk factors by the use of multivariable statistical methods. In paper 3, associations between risk factors and change in plaque area was studied. It has been shown that adjustments for baseline values in some situations may induce an overestimation of the relationship between the predictor and changes

over time [161]. Therefore, we presented the changes of the plaque-area in subjects with pre-existing plaques both with and without adjustments for the area at baseline. The relationships did not change significantly. When doing a large number of statistical tests in a data set there is always a chance for detection of false positive associations (type I error). One solution is to be more stringent with "significance" levels, moving to $p<0.001$, rather than $p< 0.05$ [162]. If the test hypothesis is false but is not rejected, the incorrect decision not to reject is called a type II error [163]. The risk of type II errors increase with the number of variables included in the regression models, as degrees of freedom and thus power decrease. Interaction is present when the effect of a risk factor on an outcome is changed by the value of a third variable [164], as in paper 2 where the relation between OPG and CIMT changed across age groups. This is best dealt with by stratifying the sample. Because the value of the third variable changes the effect of the risk on an outcome, interaction is often called effect modification.

Missing values

Subjects with missing values for a covariate in the regression models used (linear regression, logistic regression and proportional hazard model) were not included in the statistical analyses in our studies (complete-subject analysis). It is a valid approach when the missing data are missing completely at random [165]. A drawback is that much recorded data will be discarded. Imputation methods predict and fill in the missing values based on the observed data and the missing-data pattern [165]. A simple method of imputation is to replace missing values with the average value for that variable. This method is likely to reduce the standard deviation (and standard error). In a large sample, few missing values will not be a serious problem. However, if there are many missing values it is potentially dangerous because

smaller standard errors are more likely to lead to significant results that are a product of the data replacement rather than a genuine effect [166].

Causality

To assess causality sir Austin Bradford Hill proposed criteria which was an expansion of rules given by John Stuart Mill (1862) and Hume (1739) (reviewed by Rothman et al [167]). The strength of the statistical associations (relative risk or odds ratio) has been considered important. The stronger the association, the more likely is a causal relationship. However, Rothman et al points out that strong associations are neither necessary nor sufficient for causality, and weakness is neither necessary nor sufficient for absence of causality [167]. Consistency refers to repeated observations of an association in different populations under different circumstances. But as pointed out by Rothman et al [167], lack of consistency does not rule out causality because some effects are produced by their causes only under certain circumstances. Furthermore, a conclusion about inconsistency may be falsely drawn due to different power in the studies compared. Temporality means that a cause must precede the effect in time. As the dose of exposure increases, the risk of disease also increase (biological gradient). Plausibility refers to the scientific plausibility of an association and coherence with the current biologic knowledge. Experimental evidence can refer to laboratory experiments in animals, clinical trails or both. The last point proposed by Hill was analogy. According to Rothman et al [167] "the insight derived from analogy is handicapped by the inventive imagination of scientists who can find analogies everywhere". A set of sufficient criteria for causality does not exist and observational studies cannot definitively examine whether biomarkers are causally related to a disease.

5.2 Discussion of main results

OPG, RANKL, and the cytokine network they are part of, have been proposed to represent the link between the skeletal system and the cardiovascular system because of the frequent coexistence of osteoporosis and cardiovascular disease [47]. In $OPG^{-/-}$ mice severe osteoporosis developed leading to pathological fractures and in two thirds of the animals calcification of the subintima occurred in aorta and renal arteries [62]. These changes could be prevented by transgenic delivery of OPG during gestation. The osteoporosis could be prevented by giving the animals OPG after delivery, however, the vascular calcification could not be reversed [111]. In rats vascular calcification induced by warfarin and vitamin D could to a large extent be prevented by injection of OPG in doses preventing bone loss [168].

Increasing clinical evidence indicates that OPG, RANKL and the cytokine network they are part of, play an important role in cardiovascular disease. Clinically stable symptomatic coronary heart disease (CHD), acute CHD, CHD complicated with heart failure and symptomatic carotid atherosclerosis are associated with increased serum level and/or expression of OPG [110, 127, 169-171]. Serum levels of OPG have also been shown to predict atherosclerotic plaque growth [172], incident cardiovascular disease, and cardiovascular mortality in prospective studies in the general population and among postmenopausal women [151, 172-174].

5.2.1 OPG and cardiovascular risk factors

In the Tromsø cohort we found that serum OPG was significantly associated with several cardiovascular risk factors such as age, BMI (inverse), systolic blood pressure, total cholesterol, HDL cholesterol, HbA1c, hs-CRP (men only), smoking (women only) and fibrinogen after adjustment for age. However, no significant association was found between

OPG and triglycerides. In another study in men (n=522) with suspected coronary artery disease, OPG was positively correlated with serum homocysteine and negatively correlated with triglyceride serum concentrations. OPG was not significantly correlated with body mass index, creatinine, total cholesterol, HDL cholesterol, LDL cholesterol, Apo A1 and Apo B or lipoprotein (a) [170]. In 201 patients with stable chest pain no significant correlation between OPG and BMI was reported and no difference was found in serum OPG levels when stratifying the patients by sex, hypertension, diabetes, hyperlipidemia and current smoking [169]. In a large population based multiethnic study (Dallas Heart Study, n=3386) age, female sex, smoking, hypertension, hypercholesterolemia, CRP, BMI, and diabetes mellitus were independently associated with OPG, whereas no significant correlation between OPG and HDL- and LDL cholesterol was reported [175]. Thus, the relation between OPG and cardiovascular risk factors vary between different studies, and of the traditional cardiovascular risk factors some, but not all are associated with OPG. The interpretation of these findings are unclear, however it might be speculated that if a causal relation between OPG and CVD exists, it might be along a different causal chain than some of the traditional cardiovascular risk factors.

5.2.2 OPG and surrogate markers for early atherosclerosis

It is unclear whether OPG is a marker or mediator for atherosclerosis. Thus, it is relevant to study the relation between OPG and initiation of atherosclerosis. Several studies have demonstrated that increased CIMT is related to cardiovascular risk factor levels [176-179], prevalent cardiovascular disease [180], and atherosclerosis in other parts of the arterial system [181], indicating that CIMT may be regarded as a valid marker of generalized atherosclerosis. Furthermore, CIMT measurement is a more sensitive measure of early atherosclerosis than angiography [132].

In our cross sectional study (n=6516) (paper 2) from a general population, a significant interaction between age and OPG was found. Increasing OPG concentrations reduced the risk of being in the top quartile of CIMT in subjects < 45 years, whereas subjects ≥ 55 years of age had increased risk of being in the top quartile. These age-dependant relations were present in crude- and adjusted models.

Other surrogate markers for early vascular disease are flow-mediated dilatation (FMD) and artery stiffness. Endothelial dysfunction assessed by brachial artery FMD has been associated with cardiovascular events. In a population-based cohort of healthy individuals a single nucleotide polymorphism in the promoter region of the OPG gene was positively associated with IMT and negatively with post-ischemic forearm blood flow [182]. In patients with peripheral artery disease, a negative association between serum OPG level and FMD was reported [183]. However, in multivariate models only metabolic syndrome was associated with FMD [183]. In 70 postmenopausal women with osteoporosis, but without coronary artery disease, serum level of OPG was an independent predictor of artery stiffness measured by pulse wave velocity and augmentation index [184].

The main strengths of our study were the large number of participants and the wide age span. When the dependent variable (CIMT) is measured on a continuous scale, the power to quantify the effect of risk factors, as well as interaction among risk factors, is increased compared with studies in which the end point is defined only by the presence or absence of clinical disease or plaques. Due to low prevalence of atherosclerotic plaques in young subjects, surrogate markers for early atherosclerosis are probably the only feasible method for detecting an interaction between OPG and age in a model studying atherosclerosis as demonstrated in our study.

Two small studies on postmenopausal women and women with previous gestational diabetes found significant positive associations between serum OPG and CIMT [115, 185]. In the Bruneck study (n=915) the positive association between OPG and CIMT in crude analysis fell short of statistical significance in multivariable analyses [172]. The age range was 40-80 years [172], diminishing the possibility to detect an interaction between age and OPG. In our study plaque thickness was included in the measurement of CIMT, whereas in the Bruneck study CIMT was quantified at the far wall of plaque-free sections of the common carotid arteries [172]. This might partly explain why no association was detected in the Bruneck study after adjustment, whereas in our study the positive association between OPG and CIMT in older subjects to some extent reflects the association between OPG and atherosclerotic plaques. However, after exclusion of subjects with plaques in our study, an independent positive association remained in subjects aged ≥ 55 years, indicating that other factors probably are of importance. The switch in the association across age groups was unique for OPG in the multivariable model included traditional cardiovascular risk factors. Howard and co-workers studied the relation between cardiovascular risk factors and subclinical atherosclerosis assessed by ultrasound across age groups [179]. They reported that risk factors associated with atherosclerosis increased across all ages; however, all subjects were older than 45 years [179]. The age-dependent switch in the relation between serum OPG and CIMT also attracts attention to the possible influence of sex hormones and menopausal status. Unfortunately, we do not have accurate information of the menopausal status or serum levels of sex hormones in our cohort. It is unlikely that the menopausal status and serum levels of sex hormones are unrecognized confounders, since the risk estimates are similar in men and women across all age-strata, assuming that women below 45 years are premenopausal and above 55 years are postmenopausal.

Although no firm conclusions can be drawn from a cross sectional study where unrecognized confounders could not be ruled out, it is tempting to suggest that the inverse relation between OPG and CIMT in young age represent a counter regulatory mechanism in order to keep excessive activation of inflammation pathways and other injurious stimuli under control in atherogenesis. Our unexpected finding provide further evidence to the concept that OPG do not promote early atherosclerosis or even inhibits development of atherosclerosis.

5.2.3 OPG and plaque formation and growth.

Consistent with the finding in the Bruneck study [172], we found in our cohort of 4520 subjects (paper 3) followed for up to 7 years, no association between OPG and de novo plaque formation [186]. In subjects with plaques at baseline, a significant association between OPG and growth of plaques was detected in women (n=928), but not in men (n=1100) in our study. Furthermore, we found no significant relations between sRANKL and plaque formation and growth [186]. In the Bruneck study, a significant association between OPG and plaque growth was assessed in 326 subjects with plaques at baseline [172]. In contrast to our study, sex-specific data was not presented [172].

The method for plaque assessment was different between our study and the Bruneck study. In the Bruneck study plaque thickness was measured, and in our study plaque area was calculated. Others have shown that assessment of change in plaque area was superior to change in plaque thickness in detecting changes in plaque burden during 2 years [187]. Whether this is true for longer observation times is uncertain.

Although both the Bruneck study and the Tromsø study are from the general population, a possible difference between the populations was that in the Bruneck study more than 50% of

subjects in tertile 3 of OPG were considered to suffer from a chronic infection [172]. The high number of participants with plaques at baseline in our study reduces the risk of observed associations being the result of statistical errors. The lack of consistency between men and women with regard to the relation between OPG and plaque growth questions the role of serum OPG in growth of plaques. Experimental studies in mice indicate that OPG is not involved in initiation and growth of plaques [114, 188, 189].

5.2.4 OPG and plaque echogenicity

Previously, data from the Tromsø Study has shown an association between low bone mineral density and presence of echogenic carotid plaques [54]. In our case-control study (paper 1), serum OPG was lower in subjects with echogenic plaques compared to subjects with echolucent plaques. No difference in OPG was found between controls and subjects with echolucent plaques. In subjects with plaques, OPG levels decreased linearly with increasing echogenicity assessed by GSM [190]. Plaques that appear echolucent are lipid-rich, whereas echogenic plaques have higher content of dense fibrous tissue and calcification [191].
No significant differences between the groups in our study with regard to cerebrovascular risk factors, included prior cardiovascular disease, strengthen the results despite the low number of participants. Interestingly, the difference between OPG serum levels in subjects with echogenic plaques and controls increased after exclusion of subjects with prior cardiovascular disease. To the best of our knowledge, this was the first study to show an association between serum OPG and plaque morphology in humans.

Most studies have shown increased serum OPG in subjects with atherosclerosis and in subjects with heart failure, probably due to continuous inflammatory stimulation. However, in a case-control study in young survivors of uncomplicated myocardial infarction (no heart

failure or relapse of angina/myocardial infarction) serum OPG was equal to age and sex matched controls 1 to 4 years after the infarction [116]. A possible explanation might be that the acute inflammatory response had subsided years after the acute event. In the present study (paper 1), the participants were recruited from a population study and included on the basis of their plaque status and not clinical disease. This may partly explain that there was no difference in OPG serum level between controls and subjects with echolucent plaques, and even significantly lower OPG levels in subjects with echogenic plaques. These findings support the notion that the increased serum level of OPG observed in clinical disease could be a result of chronic inflammation.

Animal studies indicate that OPG influences vascular calcification and plaque morphology. Both osteoporosis and subintimal vascular calcification ocurred in $OPG^{-/-}$ mice [62], and vascular calcification induced by warfarin treatment or high doses of vitamin D was reduced in rats treated with OPG [168]. Increased vascular calcification and plaque size appeared in double knockout mice (Apo $E^{-/-}$ and $OPG^{-/-}$) compared to Apo $E^{-/-}$ $OPG^{+/+}$ mice, indicating that OPG acted as an inhibitor of vascular calcification and of plaque growth [114]. In Apo $E^{-/-}$ mice with intact endogenous OPG production, treatment with OPG did not influence atherosclerotic lesion size, but promoted smooth muscle cell accumulation, collagen fiber formation and development of fibrous caps in the atherosclerotic plaques [188]. OPG treatment did not affect markers of inflammation [188]. Serum OPG increased within few weeks in $ldlr^{-/-}$ mice fed a diet promoting atherosclerosis [189]. Administration of recombinant OPG did not influence atherosclerotic plaque size, but reduced vascular calcification [189]. Thus, lack of OPG seems to promote vascular calcification, whereas treatment with OPG promoted fibrous cap formation and reduced calcification in animals with intact endogenous OPG production. Less expression of OPG has been demonstrated in aortic

stenosis with calcified valves in humans than in normal controls [192]. RANKL was not expressed in controls, but in calcified valves [192] indicating that the OPG/RANKL system seems to be involved in the regulation of calcification also in the human vascular system.

Recently, a strong negative association between serum OPG and GSM ($r=-0.575$, $p<0.001$) was reported in subjects with prior cerebrovascular disease [193]. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) are used to prevent atherosclerotic vascular events and death through lowering of LDL cholesterol levels [194, 195]. Statins seem to be able to prevent ischemic coronary artery disease (CAD), heart failure and left ventricular dysfunction [196, 197]. Therefore, it has been hypothesized that statins have favorable pleiotropic effects including improvement of endothelial function and reduction of inflammation beyond their cholesterol-reducing effects [196, 197]. In humans, use of statins has been reported to be associated with increased bone mineral density [198]. Atorvastatin increased OPG mRNA levels and protein secretion three fold in human osteoblasts, expression of differentiation markers, alkaline phosphatase and osteocalcin increased, and the inhibitory effect of glucocorticoids on OPG production was abrogated [199]. This may contribute to the beneficial effects of statins on the skeleton [199]. In human endothelial cells and SMCs, atorvastatin reduced TNF-alpha induced OPG expression [93]. In subjects with hypercholesterolemia and type 2 diabetes, treatment with low doses of pravastatin (5-10 mg/per day) increased serum OPG significantly (6-7%) after 3 months and it remained elevated after six months [200]. In two other studies in type 2 diabetic patients with microalbuminuria and nephropathy, respectively, simvastatin treatment reduced OPG whereas lovastatin treatment increased OPG serum level [201, 202]. In a double blinded study in 27 patients with stable CAD patients received either simvastatin 40 mg/d or fenofibrate 160 mg/d. The OPG serum concentration increased significantly after one month of treatment in

both groups, by 26.8% and 33.3%, respectively, and the effect remained stable after additional two months of treatment [203]. In subjects (n=140) with moderate carotid stenosis atorvastatin reduced serum hsCRP and OPG levels from baseline to follow-up at 1 year. Aggressive lipid lowering therapy reduced the concentrations more than moderate doses of atorvastatin. The GSM score increased, reflecting increased plaque echogenicity [204].

Thus, the effects of statins on serum OPG diverge in different populations and between statins. Whether the effect of statins on plaque echogenicity is mediated by the OPG/RANKL axis and/or other mediators is not known. However, the consistent inverse relation between OPG and echogenicity in subjects with prior cerebrovascular disease [193], in the intervention study [204] and in our study strengthen the notion that OPG and plaque echogenicity are related also in humans as demonstrated in experimental animal studies.

5.2.5 OPG and future cardiovascular disease and mortality

Limited data are available on the impact of OPG to predict risk of incident myocardial infarction, stroke and mortality in the general population. In our study (paper 4) OPG predicted risk of myocardial infarction and ischemic stroke. In tertile 3 versus tertile 1 of OPG the relative risk of myocardial infarction and ischemic stroke increased 1.4 fold and 2.0 fold, respectively, demonstrating a dose response relationship. In the Bruneck study OPG was reported to be associated with incident cardiovascular disease (CVD) and cardiovascular mortality, respectively [172]. However, CVD (number of events=128) was a composite of transient ischemic attacks, ischemic stroke, myocardial infarction, peripheral artery disease, revascularization procedures, and vascular deaths (number of deaths=56). Thus, vascular deaths represented more than 40% of incident cardiovascular disease, and a significant association between OPG and vascular deaths alone was reported [172]. In the same

population, no association was reported between serum level of sRANKL and carotid- and femoral atherosclerosis, whereas a significant association between sRANKL and cardiovascular events was found [152].

In a nested prospective case-control study (within the EPIC-Norfolk study) 951 apparently healthy individuals, who developed a coronary event during 6 years of follow-up, were matched by age and sex by 1705 healthy controls. Baseline serum OPG was associated with future coronary events, in contrast to baseline sRANKL concentration [173]. In the Offspring cohort of the Framingham Heart Study (n=3250), OPG serum levels, but not sRANKL, were associated with cardiovascular disease (fatal and non-fatal myocardial infarction, coronary insufficiency, heart failure and stroke) and all-cause mortality during a mean follow-up of 4.6 years [205]. The association with CVD failed to reach statistical significance when OPG was categorized in quartiles [151]. In 897 patients with acute coronary syndrome followed for median 89 months, baseline OPG serum concentration was significantly associated with mortality and hospitalization for heart failure, but not for stroke and recurrent myocardial infarction after adjustment for other risk factors [206]. In patients with suspected stable angina pectoris undergoing elective coronary angiography (n=1025), strong associations between OPG concentrations and all-cause mortaility, CVD mortality and incidence of myocardial infarction in univariable analyses were reported [207]. However, after adjustment for cardiovascular risk factors no significant associations were reported, except for the subgroup of patients with OPG serum concentration above the 90^{th} percentile [207].

In patients with large vessel disease stroke, OPG were significantly higher compared to patients with small vessel disease and controls [208]. In a case-control study in

postmenopausal women (n=490), OPG was measured at baseline and subjects were followed for 12 years [124]. 241 incident strokes occurred whereas 247 controls were randomly selected from the cohort. Associations between serum OPG and risk of fatal ischemic stroke, cardiovascular mortality and all-cause mortality, but not with nonfatal ischemic strokes were reported [124]. In our prospective cohort study (paper 4) OPG showed a significant interaction with sex for ischemic stroke as endpoint, the risk estimate for women was higher than in men. The discordance between the case-control study in postmenopausal women and our study might be explained by different study populations and study design. In a nested case-control study (median length of follow-up 3.1 years) within a large unselected cohort from the general population, 254 cases with verified incident acute ischemic stroke and 254 age- and sex-matched controls were studied [209]. The adjusted odds ratio for ischemic stroke was 0.87 (95% CI 0.46-1.63) comparing participants in the highest quartile of OPG concentrations with those in the lowest quartile. The authors suggested that this could indicate a different pathogenic process in stroke development from that in ischemic heart disease, where OPG has been shown to be a significant predictor. The result of our study does not support this notion.

In our study significant associations between OPG and total mortality and death of myocardial infarction and ischemic stroke were detected, respectively. We also found a significant association between OPG and death of non-vascular causes. To our knowledge, this has not been previously reported. In the Bruneck study, subjects with cardiovascular disease prior to baseline was included in most analyses, and no association between OPG and death of non-vascular causes (number of events=100) was found [172]. However, after exclusion of subjects in the Bruneck study who had experienced cardiovascular disease before study baseline (n=65) the risk (hazard ratio (95% confidence interval)) of death after

adjustment for other risk factors was in OPG tertile 2; 1.1 (0.6-2.1) and in tertile 3; 1.6 (0.9-2.9) versus tertile 1 [172]. In our study the risk of death in tertile 2 was 1.2 (0.9-1.5) and in tertile 3 1.6 (1.3-2.1) versus tertile 1. Thus, the risk estimates are practically identical in the two population based studies. The difference in statistical significance between the two studies is most likely due to differences in statistical power.

5.2.6 OPG/RANKL/RANK - marker or causal factor for cardiovascular disease?

The relation between OPG and vascular disease could be a causal relationship. Alternatively, OPG might be a marker of atherosclerosis or OPG and atherosclerosis could share a common etiologic factor. Whether studies in cell cultures reflect the in vivo situation and to what degree the results of studies in animals are transferable to humans, are uncertain. Browner et al [124] hypothesized that increased serum OPG levels in humans could be a response to, rather than a cause of atherosclerosis or vascular calcification, perhaps in an attempt to regulate those processes. Another explanation could be that the higher OPG levels are a result of decreased clearance of OPG, perhaps because of increased binding of RANKL [124].

An inherent limitation using serum concentration to study the relation to disease is to what extent serum concentrations reflect what is going on within the tissues. Different effects of the OPG/RANKL system on the various stages of atherosclerosis such as plaque formation, plaque modelling and calcification are plausible.

Our epidemiological studies suggest that OPG is not a main player in atherosclerotic plaque formation or growth of plaques (paper 3). However, we found that serum OPG predicted myocardial infarction and ischemic stroke during 12 years of follow up (paper 4) independent of traditional risk factors. With each SD higher level of OPG the future risk of cardiovascular diseases and mortality increased 20-44% after multivariable adjustment. In comparison, the

risk of myocardial infarction in our study with each SD higher level of total cholesterol was 30% (unpublished data). In a recently published meta-analysis the increased risk of coronary heart disease with each SD higher levels of log CRP was 44 % after adjustment for conventional risk factors and 23% after inclusion of fibrinogen in the multivariable model [210]. In a large population based study in apparently healthy women followed for a mean of eight years the relative risk of first cardiovascular events according to quintiles of LDL cholesterol compared to the lowest quintile was 0.9, 1.1, 1.3 and 1.5, respectively [211]. The relative risk in our population for first myocardial infarction was in comparison 1.0, 1.1, 1.1 and 1.5 compared to the lowest quintile of OPG. A causal relationship between OPG and cardiovascular disease is supported by the clear temporal sequence. Furthermore, serum OPG at baseline discriminated between thrombotic and hemorrhagic stroke, possibly indicating that OPG is not merely an unspecific marker for poor health and chronic inflammation. However, the number of hemorrhagic events was low so interpretation should be done with caution. The strength of associations found for established causal risk factors and for OPG cannot be used to conclude that there is a causal relationship or that a causal relationship does not exist. Although several studies have shown an association between OPG and cardiovascular disease and mortality, mechanisms are uncertain.

Homocysteine is another biomarker where extensive epidemiological data support that homocysteine might be a causal risk factor for cardiovascular disease (reviewed in [212]). In a nested case-control study in the Tromsø population a 4 µmol/L (1SD 4.7 µmol/L and 3.7 µmol/L in cases and controls, respectively) increment in serum homocysteine was associated with 32% increased risk of coronary heart disease after adjustment [213]. However, intervention trials with B-vitamins lowering plasma homocysteine have not shown a clear beneficial effect in patients with vascular disease [214-217].

Treatment with recombinant OPG or the monoclonal antibody denosumab to RANKL, have shown beneficial effects in the skeletal system. Large trials, such as the denosumab trial for the prevention of fractures in postmenopausal women, might also as a "side effect" contribute to increased knowledge about the relation between the OPG/RANKL system and the cardiovascular system.

Since the OPG/RANKL system most likely is not causally related to plaque formation and growth, an alternative role in the pathogenesis of cardiovascular disease should be searched for. Besides an effect on plaque modeling and stability, it might be speculated that the OPG/RANKL system could be involved in thrombosis (see section 1.7).

The utility of serum markers as risk predictors may not depend on their pathogenic importance, but rather on their ability to reflect important pathogenic processes [173]. An experimental study in mice suggested that RANKL, rather than RANKL inhibiton was responsible for exacerbation of vascular disease. Human RANKL knock-in mice had implanted prednisolone pellets to induce osteoporosis. These mice were responsive to denosumab in contrast to wild type mice [218]. Treatment with denosumab reduced aortic calcium deposition of prednisolone-treated huRANKL-knock-in mice by up to 50%. TRAIL might also be of importance for atherosclerosis. An experimental study in apo E-/- mice with diabetes mellitus showed that recombinant human TRAIL significantly attenuated the development of atherosclerotic plaques, induced apoptosis of infiltrating macrophages and increased the vascular smooth muscle cell content, suggesting that OPG could contribute to cardiovascular risk by inhibiting a possible anti-atherosclerotic activity of circulating TRAIL

[219]. Reduced level of soluble TRAIL in patients with acute myocardial infarction and stable coronary disease compared to controls has recently been reported [220].

Conclusions

In the case control study (paper 1) OPG and carotid plaque echogenicity measured by GSM showed an inverse relationship, i.e. the OPG serum level was significantly lower in subjects with more echogenic (fibrotic and calcified) plaques than in subjects with echolucent (lipid rich) plaques and controls. This finding is consistent with animal studies where lack of OPG was associated with calcification and treatment with OPG decreased calcification.

In our cross sectional population based study (paper 2) we found that the relation between serum OPG and carotid intima media thickness (CIMT) changed across age groups. In young subjects (25- <45 years), higher OPG levels reduced the risk of being in the uppermost quartile of CIMT, whereas subjects ≥ 55 years of age showed a reversed association. Of all the established risk factors for cardiovascular disease included in the multivariable model, the switch in the association across age groups was unique for OPG.

In the population based prospective study (paper 3), no association was found between serum level of OPG at baseline and de novo carotid plaque formation during seven years of follow up. A significant association between OPG and plaque growth was demonstrated in women, but not in men. This inconsistency challenges the concept that OPG is related to plaque growth. Together, paper 2 and 3 suggest that OPG is not involved in the initiation of atherosclerosis. The negative association between OPG and CIMT (paper 2) may indicate that increased serum OPG could be favourable in younger subjects.

Serum OPG at baseline was independently associated with incident myocardial infarction and ischemic stroke, but not hemorrhagic stroke during 12 years of follow-up (paper 4). OPG predicted total mortality, deaths of myocardial infarction and ischemic stroke, respectively. Furthermore, an association between OPG and death of non-vascular causes was detected.

These findings may indicate that OPG is a mediator in cardiovascular disease and may also play a role in the pathogenesis of other fatal diseases.

Further implications

Our studies do not support a role for OPG in initiation of atherosclerosis and question the role of OPG in plaque growth. OPG predicts myocardial infarction and ischemic stroke, diseases that most often are caused by plaque rupture with subsequent thromboembolic complications. OPG has been reported to bind to trombospondin-1 and vWF. A possible role for OPG in the regulation of thrombus formation has been proposed. Thus, both basal mechanistic studies and population based studies on the relation between OPG and venous thrombosis are needed. Intervention trials are important for gaining more knowledge about the relation between risk factors and disease. Intervention trials focusing on reducing OPG in humans will probably not be performed due to the pleiotropic effects of OPG and risk of serious side effects.

OPG, RANKL and possible effects of medication

The TRAIL/OPG/RANKL system affects the cardiovascular system, the immune system and apoptosis. OPG seems to downregulate the immune response by decreasing dentritic cell survival [70]. In vitro studies indicate that OPG may act as a survival factor for tumour cells from both solid tumours [74, 78] and haematological malignancies [79]. Denosumab is a human monoclonal antibody with a high affinity and specificity for RANKL, and thereby inhibits the differentiation, activity, and survival of osteoclasts. A clinical trial in women with osteoporosis showed a reduction in the risk of vertebral, nonvertebral, and hip fractures [90]. Although denosumab is specific for RANKL and does not block TRAIL, it might be speculated that possibly shifting the balance between the cytokines in the TRAIL/OPG/RANKL system could lead to side effects that could be harmful or beneficial. If the effect of OPG/RANKL system on the cardiovascular system is harmful and that effect is mainly mediated by RANKL, prevention of binding of RANKL to RANK could be associated with a beneficial effect. If binding of denosumab to RANKL leads to enhanced

activity of OPG, it might be speculated that increased risk of infection or cancer could occur. During 36 months of treatment with denosumab in postmenopausal women (n=7868), no significant increase in the risks of cancer, infection or cardiovascular disease were observed [90]. The overall incidence of new malignancies was 4.3% in the placebo and 4.8% in the denosumab groups. According to Amgen's home page there were no significant impact of denosumab on new malignancies in the breast (0.7% placebo vs. 0.9% denosumab), reproductive (0.2% placebo vs. 0.5% denosumab), and gastrointestinal systems (0.6% placebo vs. 0.9% denosumab) [221]. In men with prostate cancer treated with denosumab every 6 months for three years, no increase in new primary cancers occurred in the treatment group compared with the placebo group [222]. In the Bruneck study, no significant associations were reported between sRANKL, OPG and the incidence of cancer (n=146) and deaths from cancer (n=81) [223].

The observed association between OPG and non-vascular mortality in our study (paper 4) needs further exploration. The relation between OPG and cancer needs to be addressed in larger studies with long follow-up time.

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Paper 1

Paper 2

Paper 3

Paper 4

Appendix A

Letter of invitation

Questionnaires from the fourth Tromsø Study

Du er innbudt til den store helseundersøkelsen i Tromsø kommune 1994 - 95

Vi når fram til alle

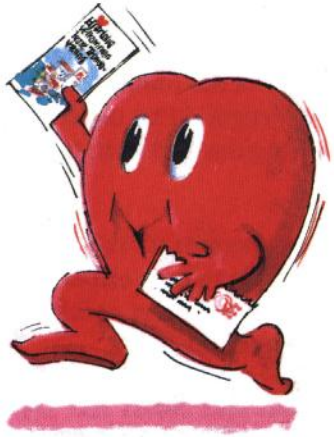
Vi begynner i de ytre distriktene i kommunen. Her vil undersøkelsen pågå i skolehus og andre lokaler - se opplysningene i innbydelsen som følger dette brevet.
Fra slutten av oktober 1994 til sommeren 1995 vil undersøkelsen foregå i Mellomveien 50 (Elisabeth-senteret; den gamle kvinneklinikken). Vi ser helst at du møter på stedet som er oppført i innbydelsesbrevet.

Hvorfor har du fått tilbudet ?

Fordi vi tilbyr undersøkelsen til alle som er født i 1969 eller tidligere.

Hva er formålet ?

Undersøkelsen er i første rekke rettet mot hjerte-karsykdom, men er også viktig for å få ny viten om andre alvorlige kroniske sykdommer (bl.a. kreft).
Denne gangen vil en i tillegg se spesielt på smertetilstander i muskler og skjelett, blant annet fibromyalgi. Derfor vil noen høsten 1995 bli invitert til en spesialundersøkelse.
Store hjerte-karundersøkelser ble gjort i Tromsø i 1974, 1979-80 og 1986-87. Det var stort frammøte, og det ble funnet en rekke tilfeller av hjerte-karsykdom - som nå får behandling.
Undersøkelsene har også gitt oss viktig kunnskap for å bekjempe disse sykdommene. Den kunnskap vi har fått gjennom de tidligere undersøkelsene, har gjort Universitetet i Tromsø til et av de fremste forskningsmiljøer i verden på hjerte-karsykdommer. Også denne gangen tar vi sikte på å finne personer som har hjerte-karsykdom uten å vite det. Vi vil også gjerne nå dem som har særlig høy risiko, slik at de kan få tilbud om forebygging og andre tiltak som kan hindre at sykdom utvikler seg. Hjerte-karsykdom er fortsatt et av våre største helseproblemer.



Ikke bare for din egen skyld.....

Undersøkelsen har ikke bare betydning for deg personlig. Det er også viktig at resultatene blir brukt i medisinsk forskning, bl.a. ved at vi sammenholder dem med framtidig forekomst av sykdom. Dermed lærer vi mer om hvordan hjerte-karsykdom, kreft og andre folkesykdommer oppstår og hvordan de kan forebygges. Ved å møte fram er du med i kampen mot disse sykdommene.

Undersøkelsen omfatter

- **Måling av høyde og vekt**
- **Måling av blodtrykk**
- **Blodprøve.** I denne måler vi innholdet av fettstoffer (bl.a. kolesterol), kalk og et leverenzym. Resultatet av disse målingene sendes din lege om du ønsker det. Resultatet av andre prøver blir bare brukt til medisinsk forskning. Prøven blir frosset ned, slik at det senere kan måles andre stoffer om det blir nødvendig for utforskning av sykdom. Før slike målinger blir gjort, blir studien forelagt den forskningsetiske komité for Nord-Norge.
- **EKG** er en undersøkelse som registrerer hjertets aktivitet. Den gjøres på en forenklet måte, og registreringene blir bare brukt til forskning.

- **Spørreskjema**
- **Spesialundersøkelse.** Alle født mellom 1920-1939, og et utvalg av de øvrige, blir tilbudt en mer omfattende undersøkelse gratis. Hva undersøkelsen omfatter varierer noe, men gir en bedre beskrivelse av hjertet, hovedpulsårens funksjon, åreforkalkning, og tendens til beinskjørhet. Du får time til undersøkelsen ved frammøte.

Spørreskjema

Dette finner du på baksiden av det brevet du har fått. Vennligst fyll ut skjemaet på forhånd og ta det med til undersøkelsen. Dersom enkelte spørsmål er vanskelige å fylle ut, kan du få hjelp når du møter fram.

Om samtykke

Opplysningene om deg blir behandlet strengt fortrolig. De oppbevares og brukes etter regler gitt av Datatilsynet og den forskningsetiske komité for Nord-Norge. For at opplysningene skal brukes i medisinsk forskning, må du samtykke til det. Samtykke er også nødvendig for at din lege skal få resultat av de målinger som gjøres (og som du selv får tilsendt resultat av) og svar du gir på spørreskjemaet som ligger ved dette brevet. Vi ber derfor at du ved frammøte samtykker i:

- at melding om dine resultat sendes til din faste lege, og inngår i din journal hos legen.
- at blodprøven kan brukes til analyser som ledd i medisinsk forskning. Hensikten med slike analyser er å forstå årsak til sykdom.
- at dine resultater kan brukes til medisinsk forskning, ved å sammenholde opplysningene med andre helse- og sykdomsregister (f.eks. kreftregister og dødsårsaksregister) og opplysninger fra de tidligere helseundersøkelsene i Tromsø. Før opplysningene analyseres, blir navn og person-nummer fjernet. Selv om du gir samtykke, kan du senere reservere deg mot bruk av dine resultat.

Etterundersøkelse

Noen av dem som blir undersøkt, blir senere innkalt til egen lege for nærmere kontroll. Trenger du behandling, får du tilbud om det.

Hva koster undersøkelsen ?

Det er nødvendig med en egenandel ved undersøkelsen. Den er beskjeden i forhold til de totale kostnadene. Beløpets størrelse vil du finne i brevet du nå har mottatt. Spesialundersøkelsen er gratis. Trenger du ny undersøkelse hos egen lege eller ved Regionsykehuset, betaler du vanlig egenandel.

Antrekk

Av hensyn til blodtrykkmålingen ber vi om at du tar på plagg uten ermer eller med korte ermer som ikke strammer. Det er ikke nødvendig å ta av seg på overkroppen.

Steder som får besøk av helseundersøkelsen

- Kaldfjord
- Tromvik
- Lakselvbukt
- Sjursnes
- Breivikeidet
- Fagernes
- Skittenelv
- Ersfjordbotn
- Straumsbukta
- Brensholmen
- Vikran
- Trondjord
- Sjøtun
- Tromsø sentrum

Vel møtt!

Hjertelig hilsen

- Kommunehelsetjenesten
- Fagområdet medisin, Universitetet i Tromsø

Statens helseundersøkelser

Repro & Grise A/S 380 86 955

Hjertelig velkommen, kjære Tromsøværing

Innbydelse til

HELSEUNDERSØKELSEN

Fødselsdato	Personnr.	Kommune	Kretsnr.

Velkommen til helseundersøkelsen i Tromsø!

Helseundersøkelsen kommer nå til Tromsø. Tid og sted for frammøte finner du nedenfor. Du finner også en orientering om undersøkelsen i den vedlagte brosjyren.

Vi ber deg fylle ut spørreskjemaet på baksiden og ta det med til undersøkelsen.

Undersøkelsen blir mest verdifull om frammøtet blir så fullstendig som mulig. Vi håper derfor at du har mulighet til å komme. Møt selv om du kjenner deg frisk, om du er under legebehandling, eller om du har fått målt kolesterol og blodtrykk i den senere tid.

Vennlig hilsen
Kommunehelsetjenesten
Fagområdet medisin, Universitetet i Tromsø
Statens helseundersøkelser

EGEN HELSE

Hvordan er helsen din nå? *Sett bare ett kryss.*

Dårlig	12	1
Ikke helt god		2
God		3
Svært god		4

Har du, eller har du hatt:

	JA	NEI	Alder første gang
Hjerteinfarkt 13			år
Angina pectoris (hjertekrampe) 16			år
Hjerneslag/hjerneblødning 19			år
Astma 22			år
Diabetes (sukkersyke) 25			år

Bruker du medisin mot høyt blodtrykk?

Nå	28	1
Før, men ikke nå		2
Aldri brukt		3

Har du i løpet av det siste året vært plaget med smerter og/eller stivhet i muskler og ledd som har vart i minst 3 måneder sammenhengende? 29 — JA / NEI

Har du de siste to ukene følt deg:

	Nei	Litt	En god del	Svært mye
Nervøs og urolig? 30				
Plaget av angst? 31				
Trygg og rolig? 32				
Irritabel? 33				
Glad og optimistisk? 34				
Nedfor/deprimert? 35				
Ensom? 36				
	1	2	3	4

RØYKING

Røykte noen av de voksne hjemme da du vokste opp? 37 — JA / NEI

Bor du, eller har du bodd, sammen med noen dagligrøykere etter at du fylte 20 år? 38 — JA / NEI

Hvis "JA", hvor mange år tilsammen? 39 — Antall år

Hvor lenge er du vanligvis daglig tilstede i røykfylt rom? 41 — Antall timer

Sett 0 hvis du ikke oppholder deg i røykfylt rom.

Røyker du selv:

	JA	NEI
Sigaretter daglig? 43		
Sigarer/sigarillos daglig? 44		
Pipe daglig? 45		

Hvis du har røykt daglig tidligere, hvor lenge er det siden du sluttet? 46 — Antall år

Hvis du røyker daglig nå eller har røykt tidligere:

Hvor mange sigaretter røyker eller røykte du vanligvis daglig? 48 — Antall sigaretter

Hvor gammel var du da du begynte å røyke daglig? 52 — Alder år

Hvor mange år tilsammen har du røykt daglig? 54 — Antall år

MOSJON

Hvordan har din fysiske aktivitet i fritiden vært det siste året? *Tenk deg et ukentlig gjennomsnitt for året.*
Arbeidsvei regnes som fritid.

	Timer pr. uke: Ingen	Under 1	1-2	3 og mer
Lett aktivitet *(ikke svett/andpusten)* 56				
Hard fysisk aktivitet *(svett/andpusten)* 57				
	1	2	3	4

KAFFE

Hvor mange kopper kaffe drikker du daglig?
Sett 0 hvis du ikke drikker kaffe daglig.

Kokekaffe	58	Antall kopper
Annen kaffe	60	Antall kopper

ALKOHOL

Er du total avholdsmann/-kvinne? 62 — JA / NEI

Hvor mange ganger i måneden drikker du vanligvis alkohol? *Regn ikke med lettøl.*
Sett 0 hvis mindre enn 1 gang i mnd. 63 — Antall ganger

Hvor mange glass øl, vin eller brennevin drikker du vanligvis i løpet av to uker? 65
Regn ikke med lettøl.
Sett 0 hvis du ikke drikker alkohol.

Øl	Vin	Brennevin
glass	glass	glass

FETT

Hva slags margarin eller smør bruker du vanligvis på brødet? *Sett ett kryss.*

Bruker ikke smør/margarin	71	1
Meierismør		2
Hard margarin		3
Bløt (soft) margarin		4
Smør/margarin blanding		5
Lettmargarin		6

UTDANNING/ARBEID

Hvilken utdanning er den høyeste du har fullført?

Grunnskole, 7-10 år, framhaldsskole, folkehøgskole	72	1
Realskole, middelskole, yrkesskole, 1-2-årig videregående skole		2
Artium, øk.gymnas, allmennfaglig retning i videregående skole		3
Høgskole/universitet, mindre enn 4 år		4
Høgskole/universitet, 4 år eller mer		5

Hva slags arbeidssituasjon har du nå?

Lønnet arbeid	73	
Heltids husarbeid	74	
Utdanning, militærtjeneste	75	
Arbeidsledig, permittert	76	

Hvor mange timer lønnet arbeid har du i uka? 77 — Antall timer

Mottar du nå noen av følgende ytelser?

Syketrygd (sykmeldt)	79	
Attføring	80	
Uførepensjon	81	
Alderspensjon	82	
Sosialstøtte	83	
Arbeidsløshetstrygd	84	

SYKDOM I FAMILIEN

Har en eller flere av foreldre eller søsken hatt hjerteinfarkt (sår på hjertet) eller angina pectoris (hjertekrampe)? 85 — JA / NEI / VET IKKE

Helseundersøkelsen i Tromsø

Hovedformålet med Tromsøundersøkelsene er å skaffe ny kunnskap om hjerte-karsykdommer for å kunne forebygge dem. I tillegg skal undersøkelsen øke kunnskapen om kreftsykdommer og andre alminnelige plager som f.eks. allergier, smerter i muskulatur og nervøse lidelser. Vi ber deg derfor svare på noen spørsmål om forhold som kan ha betydning for risikoen for disse og andre sykdommer.

Skjemaet er en del av Helseundersøkelsen som er godkjent av Datatilsynet og av Regional komite for medisinsk forskningsetikk. Svarene brukes bare til forskning og behandles strengt fortrolig. Opplysningene kan senere bli sammenholdt med informasjon fra andre offentlige helseregistre etter de regler som Datatilsynet og Regional komite for medisinsk forskningsetikk gir.

Hvis du er i tvil om hva du skal svare, sett kryss i den ruten som du synes passer best.

Det utfylte skjema sendes i vedlagte svarkonvolutt. Portoen er betalt.

På forhånd takk for hjelpen!

Med vennlig hilsen

Fagområdet medisin
Universitetet i Tromsø **Statens helseundersøkelser**

Hvis du ikke ønsker å besvare spørreskjemaet, sett kryss i ruten under og returner skjemaet. Da slipper du purring.

Jeg ønsker ikke å besvare spørreskjemaet ..... 17 ❑

Dag Mnd År

Dato for utfylling av skjema: ..... 18 ...../...../.....

OPPVEKST

I hvilken kommune bodde du da du fylte 1 år?

..... 24 - 28

Hvis du ikke bodde i Norge, oppgi land i stedet for kommune.

Hvordan var de økonomiske forhold i familien under din oppvekst?

- Meget gode ..... 29 ❑
- Gode ..... ❑
- Vanskelige ..... ❑
- Meget vanskelige ..... ❑

Hvor mange av de første 3 årene av ditt liv
- bodde du i by? ..... 30 _____ år
- hadde dere katt eller hund i hjemmet? ..... 31 _____ år

Hvor mange av de første 15 årene av ditt liv
- bodde du i by? ..... 32 _____ år
- hadde dere katt eller hund i hjemmet? ..... 34 _____ år

BOLIG

Hvem bor du sammen med?

Sett ett kryss for hvert spørsmål og angi antall.		Ja	Nei	Antall
Ektefelle/samboer	36	❑	❑	
Andre personer over 18 år	37	❑	❑	_____
Personer under 18 år	40	❑	❑	_____

Hvor mange av barna har plass i barnehage? ..... 43 _____

Hvilken type bolig bor du i?
- Enebolig/villa ..... 45 ❑ 1
- Gårdsbruk ..... ❑ 2
- Blokk/terrasseleilighet ..... ❑ 3
- Rekkehus/2-4 mannsbolig ..... ❑ 4
- Annen bolig ..... ❑ 5

Hvor stor er din boenhet? ..... 46 _____ m^2

I omtrent hvilket år ble boligen bygget? ..... 49 _____

		Ja	Nei
Er boligen isolert etter 1970?	53	❑	❑
Bor du i underetasje/kjeller?	54	❑	❑
Hvis "Ja", er gulvbelegget lagt på betong?	55	❑	❑

Hvordan er boligen hovedsakelig oppvarmet?
- Elektrisk oppvarming ..... 56 ❑
- Vedfyring ..... ❑
- Sentralvarmeanlegg oppvarmet med:
  - Parafin ..... ❑
  - Elektrisitet ..... ❑

		Ja	Nei
Er det heldekkende tepper i stua?	60	❑	❑
Er det katt i boligen?	61	❑	❑
Er det hund i boligen?	62	❑	❑

ARBEID

Hvis du er i lønnet eller ulønnet arbeid, hvordan vil du beskrive ditt arbeid?
- For det meste stillesittende arbeid? ..... 63 ❑ 1
  (f.eks. skrivebordsarbeid, montering)
- Arbeid som krever at du går mye? ..... ❑ 2
  (f.eks. ekspeditørarb., lett industriarb., undervisning)
- Arbeid hvor du går og løfter mye? ..... ❑ 3
  (f.eks. postbud, pleier, bygningsarbeid)
- Tungt kroppsarbeid? ..... ❑ 4
  (f.eks. skogsarb., tungt jordbruksarb., tungt bygn.arb.)

Kan du selv bestemme hvordan arbeidet ditt skal legges opp?
- Nei, ikke i det hele tatt ..... 64 ❑ 1
- I liten grad ..... ❑ 2
- Ja, i stor grad ..... ❑ 3
- Ja, det bestemmer jeg selv ..... ❑ 4

		Ja	Nei
Har du skiftarbeid, nattarbeid eller går vakter?	65	❑	❑

Har du noen av følgende yrker (heltid eller deltid)?

Sett ett kryss for hvert spørsmål.		Ja	Nei
Sjåfør	66	❑	❑
Bonde/gårdbruker		❑	❑
Fisker		❑	❑

EGNE SYKDOMMER

Har du noen gang hatt:
Sett ett kryss for hvert spørsmål. Oppgi alderen ved hendelsen.
*Hvis det har skjedd flere ganger, hvor gammel var du **siste** gang?*

	Ja	Nei	Alder
Lårhalsbrudd 69	❑	❑	_____
Brudd ved håndledd/underarm 72	❑	❑	_____
Nakkesleng (whiplash) 75	❑	❑	_____
Skade som førte til sykehusinnleggelse 78	❑	❑	_____
Sår på magesekken 81	❑	❑	_____
Sår på tolvfingertarmen 84	❑	❑	_____
Magesår-operasjon 87	❑	❑	_____
Operasjon på halsen 90	❑	❑	_____

Har du eller har du hatt:
Sett ett kryss for hvert spørsmål.

	Ja	Nei
Kreftsykdom 93	❑	❑
Epilepsi (fallesyke)	❑	❑
Migrene	❑	❑
Kronisk bronkitt	❑	❑
Psoriasis	❑	❑
Benskjørhet (osteoporose) 98	❑	❑
Fibromyalgi/fibrositt/kronisk smertesyndrom	❑	❑
Psykiske plager som du har søkt hjelp for	❑	❑
Stoffskiftesykdom (skjoldbruskkjertel)	❑	❑
Sykdom i leveren	❑	❑
Nyrestein 103	❑	❑
Blindtarmsoperasjon	❑	❑
Allergi og overfølsomhet		
Atopisk eksem (f.eks. barneeksem)	❑	❑
Håndeksem	❑	❑
Høysnue	❑	❑
Matvareallergi 108	❑	❑
Annen overfølsomhet (ikke allergi)	❑	❑

Hvor mange ganger har du hatt forkjølelse, influensa, "ræksjuka" og lignende siste halvår? ..110 ____ ganger

	Ja	Nei
Har du hatt dette siste 14 dager? 112	❑	❑

SYKDOM I FAMILIEN

Kryss av for de slektningene som har eller har hatt noen av sykdommene:
Kryss av for "Ingen" hvis ingen av slektningene har hatt sykdommen.

	Mor	Far	Bror	Søster	Barn	Ingen
Hjerneslag eller hjerneblødning 113	❑	❑	❑	❑	❑	❑
Hjerteinfarkt før 60 års alder 119	❑	❑	❑	❑	❑	❑
Kreftsykdom 125	❑	❑	❑	❑	❑	❑
Astma 131	❑	❑	❑	❑	❑	❑
Mage/tolvfingertarm-sår 137	❑	❑	❑	❑	❑	❑
Benskjørhet (osteoporose) 143	❑	❑	❑	❑	❑	❑
Psykiske plager 149	❑	❑	❑	❑	❑	❑
Allergi 155	❑	❑	❑	❑	❑	❑
Diabetes (sukkersyke) 161	❑	❑	❑	❑	❑	❑
– alder da de fikk diabetes 167	____	____	____	____	____	____

SYMPTOMER

	Ja	Nei
Hoster du omtrent daglig i perioder av året? 177	❑	❑
Hvis "Ja":		
Er hosten vanligvis ledsaget av oppspytt? 178	❑	❑
Har du hatt slik hoste så lenge som i en 3 måneders periode i begge de to siste år? 179	❑	❑
Har du hatt episoder med piping i brystet? 180	❑	❑
Hvis "Ja", har dette oppstått:		
Sett ett kryss for hvert spørsmål.		
Om natten 181	❑	❑
Ved luftveisinfeksjoner	❑	❑
Ved fysiske anstrengelser	❑	❑
Ved sterk kulde	❑	❑
Har du merket anfall med plutselig endring i pulsen eller hjerterytmen siste år? 185	❑	❑

Hvor ofte er du plaget av søvnløshet?

Aldri, eller noen få ganger i året 186	❑ 1
1-2 ganger i måneden	❑ 2
Omtrent en gang i uken	❑ 3
Mer enn en gang i uken	❑ 4

Hvis du er plaget av søvnløshet i perioder, når på året er du mest plaget?

Ingen spesiell tid 187	❑ 1
Særlig i mørketiden	❑ 2
Særlig i midnattsoltiden	❑ 3
Særlig vår og høst	❑ 4

	Ja	Nei
Har du det siste året vært plaget av søvnløshet slik at det har gått ut over arbeidsevnen? 188	❑	❑

Hvor ofte er du plaget av hodepine?

Sjelden eller aldri 189	❑ 1
En eller flere ganger i måneden	❑ 2
En eller flere ganger i uken	❑ 3
Daglig	❑ 4

Hender det at tanken på å få alvorlig sykdom bekymrer deg?

Ikke i det hele tatt 190	❑ 1
Bare i liten grad	❑ 2
En del	❑ 3
Ganske mye	❑ 4

BRUK AV HELSEVESENET

Hvor mange ganger har du siste året, på grunn av egen helse eller sykdom, vært:
*Sett **0** hvis du **ikke** har hatt slik kontakt.*

	Antall ganger siste år
Hos vanlig lege/legevakt 191	_____
Hos psykolog eller psykiater	_____
Hos annen legespesialist utenfor sykehus	_____
På poliklinikk 197	_____
Innlagt i sykehus	_____
Hos bedriftslege	_____
Hos fysioterapeut 203	_____
Hos kiropraktor	_____
Hos akupunktør	_____
Hos tannlege 209	_____
Hos naturmedisiner (homøopat, soneterapeut o.l.)	_____
Hos håndspålegger, synsk eller "leser"	_____

LEGEMIDLER OG KOSTTILSKUDD

Har du det siste året periodevis brukt noen av de følgende midler daglig eller nesten daglig? Angi hvor mange måneder du brukte dem.
*Sett **0** hvis du **ikke** har brukt midlene.*

Legemidler
- Smertestillende ....215 _____ mnd.
- Sovemedisin .... _____ mnd.
- Beroligende midler .... _____ mnd.
- Medisin mot depresjon ....221 _____ mnd.
- Allergimedisin .... _____ mnd.
- Astmamedisin .... _____ mnd.

Kosttilskudd
- Jerntabletter ....227 _____ mnd.
- Kalktabletter eller benmel .... _____ mnd.
- Vitamin D-tilskudd .... _____ mnd.
- Andre vitamintilskudd ....233 _____ mnd.
- Tran eller fiskeoljekapsler .... _____ mnd.

Har du de siste 14 dager brukt følgende legemidler eller kosttilskudd?
*Sett **ett** kryss for **hvert** spørsmål.*

	Ja	Nei
Legemidler		
Smertestillende medisin 237	❑	❑
Febersenkende medisin	❑	❑
Migrenemedisin	❑	❑
Eksemsalve	❑	❑
Hjertemedisin (ikke blodtrykksmedisin)	❑	❑
Kolesterolsenkende medisin 242	❑	❑
Sovemedisin	❑	❑
Beroligende medisin	❑	❑
Medisin mot depresjon	❑	❑
Annen nervemedisin	❑	❑
Syrenøytraliserende midler 247	❑	❑
Magesårsmedisin	❑	❑
Insulin	❑	❑
Tabletter mot diabetes (sukkersyke)	❑	❑
Tabletter mot lavt stoffskifte (thyroxin)	❑	❑
Kortisontabletter 252	❑	❑
Annen medisin	❑	❑
Kosttilskudd		
Jerntabletter	❑	❑
Kalktabletter eller benmel	❑	❑
Vitamin D-tilskudd	❑	❑
Andre vitamintilskudd 257	❑	❑
Tran eller fiskeoljekapsler	❑	❑

VENNER

Hvor mange gode venner har du som du kan snakke fortrolig med og gi deg hjelp når du trenger det? ....259 ____ gode venner
Tell ikke med de du bor sammen med, men ta med andre slektninger!

Hvor mange av disse gode vennene har du kontakt med minst en gang i måneden? ....261 ____

	Ja	Nei
Føler du at du har nok gode venner? 263	❑	❑

Hvor ofte tar du vanligvis del i foreningsvirksomhet som f.eks. syklubb, idrettslag, politiske lag, religiøse eller andre foreninger?
- Aldri, eller noen få ganger i året ....264 ❑ 1
- 1-2 ganger i måneden .... ❑ 2
- Omtrent en gang i uken .... ❑ 3
- Mer enn en gang i uken .... ❑ 4

KOSTVANER

Hvis du bruker smør eller margarin på brødet, hvor mange skiver rekker en liten porsjonspakning vanligvis til? Vi tenker på slik porsjonspakning som du får på fly, på kafé o.l. (10-12 gram).

Den rekker til omtrent ....265 _____ skiver

Hva slags fett blir vanligvis brukt til **matlaging** (ikke på brødet) i din husholdning?
- Meierismør ....266 ❑
- Hard margarin .... ❑
- Bløt (Soft) margarin .... ❑
- Smør/margarin blanding .... ❑
- Oljer ....270 ❑

Hva slags type brød (kjøpt eller hjemmebakt) spiser du vanligvis?
Sett ett eller to kryss!

	Loff	Fint brød	Kneip-brød	Grov-brød	Knekke-brød
Brødtypen ligner mest på:	❑ 271	❑	❑	❑	❑ 275

Hvor mye (i **antall** glass, kopper, poteter eller brødskiver) spiser eller drikker du vanligvis **daglig** av følgende matvarer?
*Kryss av for **alle** matvarene.*

	0	Færre enn 1	1-2	3-4	5-6	Mer enn 6
Helmelk (søt eller sur) (glass) 276	❑	❑	❑	❑	❑	❑
Lettmelk (søt eller sur) (glass)	❑	❑	❑	❑	❑	❑
Skummet melk (søt eller sur) (glass)	❑	❑	❑	❑	❑	❑
Te (kopper)	❑	❑	❑	❑	❑	❑
Appelsinjuice (glass)	❑	❑	❑	❑	❑	❑
Poteter 281	❑	❑	❑	❑	❑	❑
Brødskiver totalt (inkl. knekkebrød)	❑	❑	❑	❑	❑	❑
Brødskiver med						
– fiskepålegg (f.eks. makrell i tomat)	❑	❑	❑	❑	❑	❑
– magert kjøttpålegg (f.eks. skinke)	❑	❑	❑	❑	❑	❑
– fetere kjøttpålegg (f.eks. salami)	❑	❑	❑	❑	❑	❑
– gulost 286	❑	❑	❑	❑	❑	❑
– brunost	❑	❑	❑	❑	❑	❑
– kaviar	❑	❑	❑	❑	❑	❑
– syltetøy og annet søtt pålegg	❑ 1	❑ 2	❑ 3	❑ 4	❑ 5	❑ 6

Hvor mange **ganger i uka** spiser du vanligvis følgende matvarer?
*Kryss av for **alle** matvarene.*

	Aldri	Færre enn 1	1	2-3	4-5	Omtrent daglig
Yoghurt 290	❑	❑	❑	❑	❑	❑
Kokt eller stekt egg	❑	❑	❑	❑	❑	❑
Frokostblanding/havregryn o.l.	❑	❑	❑	❑	❑	❑
Middag med						
– rent kjøtt	❑	❑	❑	❑	❑	❑
– pølser/kjøttpudding/-kaker	❑	❑	❑	❑	❑	❑
– feit fisk (f.eks. laks/uer) 295	❑	❑	❑	❑	❑	❑
– mager fisk (f.eks. torsk)	❑	❑	❑	❑	❑	❑
– fiskeboller/-pudding/-kaker	❑	❑	❑	❑	❑	❑
– grønnsaker	❑	❑	❑	❑	❑	❑
Majones, remulade o.l.	❑	❑	❑	❑	❑	❑
Gulrøtter 300	❑	❑	❑	❑	❑	❑
Blomkål/kål/brokkoli	❑	❑	❑	❑	❑	❑
Epler/pærer	❑	❑	❑	❑	❑	❑
Appelsiner, mandariner o.l.	❑	❑	❑	❑	❑	❑
Sukkerholdige leskedrikker	❑	❑	❑	❑	❑	❑
Sukkerfrie («Light») leskedrikker	❑	❑	❑	❑	❑	❑
Sjokolade	❑	❑	❑	❑	❑	❑
Vafler, kaker o.l. 307	❑ 1	❑ 2	❑ 3	❑ 4	❑ 5	❑ 6

ALKOHOL

Hvor ofte pleier du å drikke	øl?	vin?	brennevin?
Aldri, eller noen få ganger i året	❑ 308	❑	❑ 1
1-2 ganger i måneden	❑	❑	❑ 2
Omtrent 1 gang i uken	❑	❑	❑ 3
2-3 ganger i uken	❑	❑	❑ 4
Omtrent hver dag	❑	❑	❑ 5 310

Omtrent hvor ofte har du i løpet av siste år drukket alkohol tilsvarende minst 5 halvflasker øl, en helflaske vin eller 1/4 flaske brennevin?

- Ikke siste år ..... 311 ❑ 1
- Noen få ganger ..... ❑ 2
- 1 - 2 ganger per måned ..... ❑ 3
- 1 - 2 ganger i uken ..... ❑ 4
- 3 eller flere ganger i uken ..... ❑ 5

I omtrent hvor mange år har ditt alkoholforbruk vært slik du har svart i spørsmålene over? ..... 312 _____ år

SLANKING

Omtrent hvor mange ganger har du bevisst prøvd å slanke deg? Sett **0** hvis ingen forsøk.

- – før 20 år ..... 314 _____ ganger
- – senere ..... 316 _____ ganger

Hvis du har slanket deg, omtrent hvor mange kilo har du på det meste gått ned i vekt?

- – før 20 år ..... 318 _____ kg
- – senere ..... 320 _____ kg

Hvilken vekt ville du være tilfreds med (din "trivselsvekt")? ..... 322 _____ kg

UFRIVILLIG URINLEKKASJE

Hvor ofte har du ufrivillig urinlekkasje?

- Aldri ..... 325 ❑ 1
- Ikke mer enn en gang i måneden ..... ❑ 2
- To eller flere ganger i måneden ..... ❑ 3
- Ukentlig eller oftere ..... ❑ 4

Dine kommentarer:

BESVARES BARE AV KVINNER

MENSTRUASJON

Hvor gammel var du da du fikk menstruasjon første gang? ..... 326 _____ år

Hvis du ikke lenger har menstruasjon, hvor gammel var du da den sluttet? ..... 328 _____ år

Når du ser bort fra svangerskap og barselsperiode, har du noen gang vært blødningsfri i minst 6 måneder? 330	Ja ❑	Nei ❑

Hvis "Ja", hvor mange ganger? ..... 331 _____ ganger

Hvis du fremdeles har menstruasjon eller er gravid:

Hvilken dato startet din siste menstruasjon? ..... 333 dag/ mnd/ år ___/___/___

Bruker du vanligvis smertestillende legemidler for å dempe menstruasjonsplager? 339	Ja ❑	Nei ❑

SVANGERSKAP

Hvor mange barn har du født? ..... 340 _____ barn

Er du gravid nå? 342	Ja ❑	Nei ❑	Usikker ❑

Har du i forbindelse med svangerskap hatt for høyt blodtrykk og/eller eggehvite (protein) i urinen? 343	Ja ❑	Nei ❑

Hvis "Ja", i hvilket svangerskap?

	Svangerskap Første	Senere
For høyt blodtrykk 344	❑	❑
Eggehvite i urinen 346	❑	❑

Hvis du har født, fyll ut for hvert barn barnets fødselsår og omtrent antall måneder du ammet barnet.

Barn:	Fødselsår:	Antall måneder med amming:
1	348 _____	_____
2	_____	_____
3	356 _____	_____
4	_____	_____
5	364 _____	_____
6	_____	_____

PREVENSJON OG ØSTROGEN

Bruker du, eller har du brukt:	Nå	Før	Aldri
P-pille (også minipille) 372	❑	❑	❑
Hormonspiral	❑	❑	❑
Østrogen (tabletter eller plaster) 374	❑	❑	❑
Østrogen (krem eller stikkpiller)	❑ 1	❑ 2	❑ 3

Hvis du bruker p-pille, hormonspiral eller østrogen; hvilket merke bruker du nå?

376 .....

Hvis du bruker eller har brukt p-pille:

Alder da du begynte med P-piller? ..... 380 _____ år

Hvor mange år har du tilsammen brukt P-piller? ..... 382 _____ år

Dersom du har født, hvor mange år brukte du P-piller før første fødsel? ..... 384 _____ år

Hvis du har sluttet å bruke P-piller:

Alder da du sluttet? ..... 386 _____ år

Takk for hjelpen! Husk å postlegge skjemaet idag!

Helseundersøkelsen i Tromsø

Helseundersøkelsen i Tromsø

for dem som er 70 år og eldre.

Hovedformålet med Tromsøundersøkelsene er å skaffe ny kunnskap om hjerte-karsykdommer for å kunne forebygge dem. De skal også øke kunnskapen om kreftsykdommer og alminnelige plager som f.eks. allergier, smerter i muskulatur og nervøse lidelser. Endelig skal de gi kunnskap om hvorledes den eldste delen av befolkningen har det. Vi ber deg derfor svare på spørsmålene nedenfor.

Skjemaet er en del av Helseundersøkelsen som er godkjent av Datatilsynet og av Regional komite for medisinsk forskningsetikk. Svarene brukes bare til forskning og behandles strengt fortrolig. Opplysningene kan senere bli sammenholdt med informasjon fra andre offentlige helseregistre etter de regler som Datatilsynet og Regional komite for medisinsk forskningsetikk gir.

Hvis du er i tvil om hva du skal svare, sett kryss i den ruten som du synes passer best.

Det utfylte skjema sendes i vedlagte svarkonvolutt. Portoen er betalt.

På forhånd takk for hjelpen!

Med vennlig hilsen

Fagområdet medisin
Universitetet i Tromsø

Statens helseundersøkelser

Hvis du ikke ønsker å besvare spørreskjemaet, sett kryss i ruten under og returner skjemaet. Da slipper du purring.

Jeg ønsker ikke å besvare spørreskjemaet ..... 17 ❑

Dato for utfylling av skjema: ..... 18 Dag ...../ Mnd ...../ År .....

OPPVEKST

I hvilken kommune bodde du da du fylte 1 år?

..... 24 -28

Hvis du ikke bodde i Norge, oppgi land i stedet for kommune.

Hvordan var de økonomiske forhold i familien under din oppvekst?

- Meget gode ..... 29 ❑ 1
- Gode ..... ❑ 2
- Vanskelige ..... ❑ 3
- Meget vanskelige ..... ❑ 4

Hvor gamle ble dine foreldre?

- Mor ble ..... 30 _____ år
- Far ble ..... 32 _____ år

BOLIG

Hvem bor du sammen med?

Sett ett kryss for hvert spørsmål og angi antall.	Ja	Nei	Antall
Ektefelle/samboer 34	❑	❑	
Andre personer over 18 år 35	❑	❑	_____
Personer under 18 år 38	❑	❑	_____

Hvilken type bolig bor du i?

- Enebolig/villa ..... 41 ❑ 1
- Gårdsbruk ..... ❑ 2
- Blokk/terrasseleilighet ..... ❑ 3
- Rekkehus/2-4 mannsbolig ..... ❑ 4
- Annen bolig ..... ❑ 5

Hvor lenge har du bodd i boligen du bor i nå? ..... 42 _____ år

	Ja	Nei
Er boligen tilpasset til dine behov? 44	❑	❑
Hvis "Nei", er det problemer med:		
Plassen i boligen 45	❑	❑
Ujevn, for høy eller for lav temperatur 46	❑	❑
Trapper 47	❑	❑
Toalett 48	❑	❑
Bad/dusj 49	❑	❑
Vedlikehold 50	❑	❑
Annet (spesifiser) 51	❑	❑
Ønsker du å flytte til en eldrebolig? 52	❑	❑

TIDLIGERE ARBEID OG ØKONOMI

Hvordan vil du beskrive det arbeidet du hadde de siste 5-10 årene før du ble pensjonist?

- For det meste stillesittende arbeid? ..... 53 ❑ 1
  (f.eks. skrivebordsarbeid, montering)
- Arbeid som krever at du går mye? ..... ❑ 2
  (f.eks. ekspeditørarbeid, husmor, undervisning)
- Arbeid hvor du går og løfter mye? ..... ❑ 3
  (f.eks. postbud, pleier, bygningsarbeid)
- Tungt kroppsarbeid? ..... ❑ 4
  (f.eks. skogsarb., tungt jordbruksarb., tungt bygn.arb.)

Har du hatt noen av følgende yrker (heltid eller deltid)?

Sett ett kryss for hvert spørsmål.	Ja	Nei
Sjåfør 54	❑	❑
Bonde/gårdbruker 55	❑	❑
Fisker 56	❑	❑

Hvor gammel var du da du ble pensjonert? ..... 57 _____ år

Hva slags pensjon har du?

- Minstepensjon ..... 59 ❑
- Tilleggspensjon ..... 60 ❑

Hvordan er din økonomi nå?

- Meget god ..... 61 ❑ 1
- God ..... ❑ 2
- Vanskelig ..... ❑ 3
- Meget vanskelig ..... ❑ 4

HELSE OG SYKDOM

Er helsen din blitt forandret det siste året?

- Ja, dårligere ..... 62 ❑ 1
- Nei, uforandret ..... ❑ 2
- Ja, bedre ..... ❑ 3

Hvordan synes du at helsen din er nå i forhold til andre på samme alder?

- Mye dårligere ..... 63 ❑ 1
- Litt dårligere ..... ❑ 2
- Omtrent lik ..... ❑ 3
- Litt bedre ..... ❑ 4
- Mye bedre ..... ❑ 5

EGNE SYKDOMMER

Har du noen gang hatt:

Sett ett kryss for hvert spørsmål. Oppgi alderen ved hendelsen. Hvis det har skjedd flere ganger, hvor gammel var du siste gang?

	Ja	Nei	Alder
Lårhalsbrudd 64	❑	❑	_____
Brudd ved håndledd/underarm 67	❑	❑	_____
Nakkesleng (whiplash) 70	❑	❑	_____
Skade som førte til sykehusinnleggelse 73	❑	❑	_____
Sår på magesekken 76	❑	❑	_____
Sår på tolvfingertarmen 79	❑	❑	_____
Magesår-operasjon 82	❑	❑	_____
Operasjon på halsen 85	❑	❑	_____

Har du eller har du hatt:

Sett ett kryss for hvert spørsmål.	Ja	Nei
Kreftsykdom 88	❑	❑
Epilepsi (fallesyke)	❑	❑
Migrene	❑	❑
Parkinsons sykdom	❑	❑
Kronisk bronkitt	❑	❑
Psoriasis 93	❑	❑
Benskjørhet (osteoporose)	❑	❑
Fibromyalgi/fibrositt/kronisk smertesyndrom	❑	❑
Psykiske plager som du har søkt hjelp for	❑	❑
Stoffskiftesykdom (skjoldbruskkjertel)	❑	❑
Sykdom i leveren 98	❑	❑
Gjentatt, ufrivillig urinlekkasje	❑	❑
Grønn stær	❑	❑
Grå stær	❑	❑
Slitasjegikt (artrose)	❑	❑
Leddgikt 103	❑	❑
Nyrestein	❑	❑
Blindtarmsoperasjon	❑	❑
Allergi og overfølsomhet		
Atopisk eksem (f.eks. barneeksem)	❑	❑
Håndeksem	❑	❑
Høysnue 108	❑	❑
Matvareallergi	❑	❑
Annen overfølsomhet (ikke allergi)	❑	❑

Hvor mange ganger har du hatt forkjølelse, influensa, "ræksjuka" og lignende siste halvår? 111 ____ ganger

	Ja	Nei
Har du hatt dette de siste 14 dager? 113	❑	❑

SYKDOM I FAMILIEN

Kryss av for de slektningene som har eller har hatt noen av sykdommene:

Kryss av for "Ingen" hvis ingen av slektningene har hatt sykdommen.

	Mor	Far	Bror	Søster	Barn	Ingen
Hjerneslag eller hjerneblødning 114	❑	❑	❑	❑	❑	❑
Hjerteinfarkt før 60 års alder 120	❑	❑	❑	❑	❑	❑
Kreftsykdom 126	❑	❑	❑	❑	❑	❑
Høyt blodtrykk 132	❑	❑	❑	❑	❑	❑
Astma 138	❑	❑	❑	❑	❑	❑
Benskjørhet (osteoporose) 144	❑	❑	❑	❑	❑	❑
Slitasjegikt (artrose) 150	❑	❑	❑	❑	❑	❑
Psykiske plager 156	❑	❑	❑	❑	❑	❑
Alderdomssløvhet 162	❑	❑	❑	❑	❑	❑
Diabetes (sukkersyke) 168	❑	❑	❑	❑	❑	❑
– alder da de fikk diabetes 174	__	__	__	__	__	

SYMPTOMER

	Ja	Nei
Hoster du omtrent daglig i perioder av året? 184	❑	❑
Hvis "Ja":		
Er hosten vanligvis ledsaget av oppspytt? 185	❑	❑
Har du hatt slik hoste så lenge som i en 3 måneders periode i begge de to siste år? 186	❑	❑
Har du hatt episoder med piping i brystet? 187	❑	❑
Hvis "Ja", har dette oppstått:		
Sett ett kryss for hvert spørsmål.		
Om natten 188	❑	❑
Ved luftveisinfeksjoner	❑	❑
Ved fysiske anstrengelser	❑	❑
Ved sterk kulde 191	❑	❑
Har du merket anfall med plutselig endring i pulsen eller hjerterytmen siste år? 192	❑	❑
Har du gått ned i vekt siste året? 193	❑	❑
Hvis "Ja":		
Hvor mange kilo? 194	_____ kg	

Hvor ofte er du plaget av søvnløshet?

- Aldri, eller noen få ganger i året ..... 196 ❑ 1
- 1-2 ganger i måneden ..... ❑ 2
- Omtrent en gang i uken ..... ❑ 3
- Mer enn en gang i uken ..... ❑ 4

Hvis du er plaget av søvnløshet i perioder, når på året er du mest plaget?

- Ingen spesiell tid ..... 197 ❑ 1
- Særlig i mørketiden ..... ❑ 2
- Særlig i midnattsoltiden ..... ❑ 3
- Særlig vår og høst ..... ❑ 4

	Ja	Nei
Pleier du å ta en lur på dagen? 198	❑	❑
Føler du at du vanligvis får nok søvn?	❑	❑

Er du plaget av:	Nei	Litt	I stor grad
Svimmelhet 200	❑	❑	❑
Dårlig hukommelse	❑	❑	❑
Kraftløshet	❑	❑	❑
Forstoppelse 203	❑	❑	❑

Hender det at tanken på å få alvorlig sykdom bekymrer deg?

- Ikke i det hele tatt ..... 204 ❑
- Bare i liten grad ..... ❑
- En del ..... ❑
- Ganske mye ..... ❑

LEGEMLIGE FUNKSJONER

Klarer du selv disse gjøremålene i det daglige uten hjelp fra andre?	Ja	Med noe hjelp	Nei
Gå innendørs i samme etasje 205	❑	❑	❑
Gå i trapper	❑	❑	❑
Gå utendørs	❑	❑	❑
Gå ca. 500 meter	❑	❑	❑
Gå på toalettet	❑	❑	❑
Vaske deg på kroppen 210	❑	❑	❑
Bade eller dusje	❑	❑	❑
Kle på og av deg	❑	❑	❑
Legge deg og stå opp	❑	❑	❑
Spise selv	❑	❑	❑
Lage varm mat 215	❑	❑	❑
Gjøre lett husarbeid (f.eks. oppvask)	❑	❑	❑
Gjøre tyngre husarbeid (f.eks. gulvvask)	❑	❑	❑
Gjøre innkjøp	❑	❑	❑
Ta bussen	❑	❑	❑

	Ja	Vanskelig	Nei
Kan du høre vanlig tale (evt. med høreapparat)? 220	❑	❑	❑
Kan du lese (evt. med briller)? 221	❑	❑	❑

Er du avhengig av noen av disse hjelpemidlene?

	Ja	Nei
Stokk 222	❑	❑
Krykke	❑	❑
Gåstol (rullator)	❑	❑
Rullestol	❑	❑
Høreapparat	❑	❑
Trygghetsalarm 227	❑	❑

BRUK AV HELSEVESENET

Hvor mange ganger har du siste året, på grunn av egen helse eller sykdom, vært: *Sett 0 hvis du ikke har hatt slik kontakt.*	Antall ganger siste år
Hos vanlig lege/legevakt 228	_____
Hos psykolog eller psykiater	_____
Hos annen legespesialist utenfor sykehus	_____
På poliklinikk 234	_____
Innlagt i sykehus	_____
Hos fysioterapeut	_____
Hos kiropraktor 240	_____
Hos akupunktør	_____
Hos tannlege	_____
Hos fotterapeut 246	_____
Hos naturmedisiner (homøopat, soneterapeut o.l.)	_____
Hos håndspålegger, synsk eller "leser"	_____

Har du hjemmehjelp?	Ja	Nei
Privat 252	❑	❑
Kommunal	❑	❑

Har du hjemmesykepleie? ..... ❑ ❑

Er du fornøyd med helse- og hjemmetjenesten i kommunen?	Ja	Nei	Vet ikke
Prinsippet med fast lege 255	❑	❑	❑
Hjemmesykepleien	❑	❑	❑
Hjemmehjelpen	❑	❑	❑

Er du trygg på at du kan få hjelp av helse- og hjemmetjenesten hvis du trenger det?

- Trygg ..... 258 ❑ 1
- Ikke trygg ..... ❑ 2
- Svært utrygg ..... ❑ 3
- Vet ikke ..... ❑ 4

LEGEMIDLER OG KOSTTILSKUDD

Har du det siste året periodevis brukt noen av de følgende midler daglig eller nesten daglig? Angi hvor mange måneder du brukte dem.
Sett 0 hvis du ikke har brukt midlene.

Legemidler

- Smertestillende ..... 259 _____ mnd.
- Sovemedisin ..... _____ mnd.
- Beroligende midler ..... _____ mnd.
- Medisin mot depresjon ..... 265 _____ mnd.
- Allergimedisin ..... _____ mnd.
- Astmamedisin ..... _____ mnd.
- Hjertemedisin (ikke blodtrykksmedisin) ..... 271 _____ mnd.
- Insulin ..... _____ mnd.
- Tabletter mot diabetes (sukkersyke) ..... _____ mnd.
- Tabletter mot lavt stoffskifte (thyroxin) ..... 277 _____ mnd.
- Kortisontabletter ..... _____ mnd.
- Midler mot forstoppelse ..... _____ mnd.

Kosttilskudd

- Jerntabletter ..... 283 _____ mnd.
- Vitamin D-tilskudd ..... _____ mnd.
- Andre vitamintilskudd ..... _____ mnd.
- Kalktabletter eller benmel ..... 289 _____ mnd.
- Tran eller fiskeoljekapsler ..... _____ mnd.

FAMILIE OG VENNER

Har du nær familie som kan gi deg hjelp og støtte når du trenger det? 293	Ja	Nei
	❑	❑

Hvis "Ja": Hvem kan gi deg hjelp?

- Ektefelle/samboer ..... 294 ❑
- Barn ..... ❑
- Andre ..... ❑

Hvor mange gode venner har du som du kan snakke fortrolig med og gi deg hjelp når du trenger det? ..297 ____ **gode venner**
Tell ikke med dem du bor sammen med, men ta med andre slektninger!

	Ja	Nei
Føler du at du har nok gode venner? 299	❑	❑

Føler du at du hører med i et fellesskap (gruppe av mennesker) som stoler på hverandre og føler forpliktelse overfor hverandre (f.eks. i politisk parti, religiøs gruppe, slekt, naboskap, arbeidsplass eller organisasjon)?

- Sterk tilhørighet ..... 300 ❑ 1
- Noe tilhørighet ..... ❑ 2
- Usikkert ..... ❑ 3
- Liten eller ingen tilhørighet ..... ❑ 4

Hvor ofte tar du vanligvis del i foreningsvirksomhet som f.eks. syklubb, idrettslag, politiske lag, religiøse eller andre foreninger?

- Aldri, eller noen få ganger i året ..... 301 ❑ 1
- 1-2 ganger i måneden ..... ❑ 2
- Omtrent en gang i uken ..... ❑ 3
- Mer enn en gang i uken ..... ❑ 4

KOSTVANER

Hvor mange måltider spiser du vanligvis daglig (middag og brødmåltid)? ..... 302 _____ Antall

Hvor mange ganger i uken spiser du varm middag? ..... 304 _____

Hva slags type brød (kjøpt eller hjemmebakt) spiser du vanligvis?
Sett ett eller to kryss.

	Loff	Fint brød	Kneip-brød	Grov-brød	Knekke-brød
Brødtypen ligner mest på:	❑ 306	❑	❑	❑	❑ 310

Hva slags fett blir til vanligvis brukt til *matlaging* (ikke på brødet) i din husholdning?

- Meierismør ..... 311 ❑
- Hard margarin ..... ❑
- Bløt (Soft) margarin ..... ❑
- Smør/margarin blanding ..... ❑
- Oljer ..... 315 ❑

Hvor mye (i antall glass, poteter eller brødskiver) spiser/drikker du vanligvis daglig av følgende matvarer?
Kryss av for alle matvarene.

	Ingen	Mindre enn 1	1-2	3 og mer
Melk alle sorter (glass) 316	❑	❑	❑	❑
Appelsinjuice (glass)	❑	❑	❑	❑
Poteter	❑	❑	❑	❑
Brødskiver totalt (inkl. knekkebrød) ...	❑	❑	❑	❑
Brødskiver med				
– fiskepålegg (f.eks. makrell i tomat)	❑	❑	❑	❑
– gulost	❑	❑	❑	❑
– kaviar 322	❑ 1	❑ 2	❑ 3	❑ 4

Hvor mange ganger i uka spiser du vanligvis følgende matvarer?
Kryss av for alle matvarene.

	Aldri	Sjeldnere enn 1	1	2 og mer
Yoghurt 323	❑	❑	❑	❑
Kokt eller stekt egg	❑	❑	❑	❑
Frokostblanding/havregryn o.l.	❑	❑	❑	❑
Middag med				
– rent kjøtt	❑	❑	❑	❑
– feit fisk (f.eks. laks/uer)	❑	❑	❑	❑
– mager fisk (f.eks. torsk) 328	❑	❑	❑	❑
– grønnsaker (rå eller kokte)	❑	❑	❑	❑
Gulrøtter (rå eller kokte)	❑	❑	❑	❑
Blomkål/kål/brokkoli	❑	❑	❑	❑
Epler/pærer	❑	❑	❑	❑
Appelsiner, mandariner o.l. 333	❑ 1	❑ 2	❑ 3	❑ 4

TRIVSEL

Hvordan trives du med å bli gammel - alt i alt?

- Godt ..... 334 ❑ 1
- Ganske bra ..... ❑ 2
- Opp og ned ..... ❑ 3
- Dårlig ..... ❑ 4

Hvordan ser du på livet fremover?

- Lyst ..... 335 ❑ 1
- Ikke så verst ..... ❑ 2
- Nokså bekymret ..... ❑ 3
- Mørkt ..... ❑ 4

BESVARES BARE AV KVINNER

MENSTRUASJON

Hvor gammel var du da du fikk menstruasjon første gang? ..... 336 _____ år

Hvor gammel var du da menstruasjonen sluttet? ..... 338 _____ år

SVANGERSKAP

Hvor mange barn har du født? ..... 340 _____ barn

Hvis du har født, fyll ut for hvert barn barnets fødselsår og omtrent antall måneder du ammet barnet.
Hvis du har født mer enn 6 barn, noter fødselsår og antall måneder med amming for dem nederst på siden.

Barn:	Fødselsår:	Antall måneder med amming:
1	342 _____	_____
2	346 _____	_____
3	_____	_____
4	_____	_____
5	358 _____	_____
6	_____	_____

Har du i forbindelse med svangerskap hatt for høyt blodtrykk og/eller eggehvite (protein) i urinen? ..... 366 Ja ❑ Nei ❑

Hvis "Ja", i hvilket svangerskap?

	Svangerskap Første	Senere
For høyt blodtrykk 367	❑	❑
Eggehvite i urinen 369	❑	❑

ØSTROGEN-MEDISIN

Bruker du, eller har du brukt, østrogen-medisin?

	Nå	Før	Aldri
Tabletter eller plaster 371	❑	❑	❑
Krem eller stikkpiller 372	❑	❑	❑

Hvis du bruker østrogen, hvilket merke bruker du nå?

..... 373

Dine kommentarer:

Takk for hjelpen! Husk å postlegge skjemaet idag!
Helseundersøkelsen i Tromsø

Appendix B

Protocol for ultrasound measurements

Protocol for digitizing images (”grabbing”)

Protocol for standardization of digitized plaque images

PROCEDURES FOR MEASUREMENTS OF INTIMA-MEDIA THICKNESS AND RECORDING AND MEASUREMENTS OF PLAQUE OF THE RIGHT CAROTID ARTERY. THE TROMSØ STUDY 1994/1995

by
Oddmund Joakimsen

1. The Acuson ultrasound instrument is switched on.

2. A videocassette is inserted in the videorecorder.

3. Check that the videotape has been wound to the right position, do not overwrite previous recordings.

4. Cassettes are marked with serial numbers, uneven numbers for Acuson I, even numbers for Acuson II.

5. The initials and the identity numbers of the participant and the sonographer number (Jon=1, Eva=2, Oddmund=3) are written on each ultrasound image recorded. Labels with the ID-number of the participants are attached to the registration form, in which all ultrasound data obtained from the participants are filled (plaque localization, size, number per artery, "missing measures" codings, etc).

6. A RES-field, appropriately adjusted to a maximum width of the screen and a depth of a little more than 2 cm of the B-mode image, is positioned on the screen for obtaining images from the carotid artery of optimal quality.

7. The subject is examined in a supine position with the head slightly rotated to the left. ECG-pads are attached to both arms and the right leg (or abdomen) (lead I), and the right carotid is insonated by a 5-7 MHz ultrasound transducer.

8. The examination starts with identification of crossectional B-mode images of the carotid artery, and, if necessary for identifying purposes, in combination with colour-Doppler and/or pulsed wave Doppler 5 MHz. The examination starts caudally in the neck, normally just above the clavicle, then moving the probe upstream with simultaneous rotation movements to search for plaques also at the circumference of the vessel. Thus, the carotid artery is searched from the proximal part of the common carotid artery (CCA), upstream to the bifurcation (BIF), and as far up in the internal carotid artery (ICA) as technically possible. A plaque is defined as a presumed atherosclerotic lesion of the intima layer of the vessel wall presenting as a focal protrusion of more than 50% of the intima-media thickness (IMT) of the surrounding vessel wall, often with deviating echogenicity compared to other part of the wall of the artery. Whether a plaque is present or not is a decision taken by the sonographer during the examination. Live, crossectional imaging of the whole carotid artery is recorded on the videotape.

9. A ultrasound examination sequence is then performed in the triplex modus (i.e., combination of pulsed wave Doppler, colour Doppler, and B-mode examination) from just above the clavicle and as high upstream above BIF as possible. The objective of this part of the examination is to look for stenotic areas along the artery. However, if plaques later during the B-mode scanning procedures are found suspicious of a hemodynamic significant stenosis, a new triplex examination is performed to reevaluate the flow conditions. A live triplex sequence of the relevant part of the carotid artery is recorded on the videotape if a stenosis is suspected.

10. B-mode longitudinal ultrasound scanning of the carotid artery is then performed. To get an optimal topographic reference, the examination is starting as proximally as possible in CCA. The probe is then moving upstream with simultaneous rotating movements to look for plaques in all segments, both in the near and the far wall.

    If a plaque is found, a frozen image of the vessel wall with the plaque presented as distinctly as possible and after guidelines according to elementary ultrasound principles such as vertical propagation of the ultrasound beam, presentation of the plaque in the full diameter of the vessel and not in chord, not cutting the plaque skewly causing a falsely too large thickness of the plaque. To ensure the quality of plaque registration, some technical points may be of help: The plaque should be "attached" at its both ends to the typical double-lined intima-media structures visible on the B-mode image, and these

double-lined structures should best be visible both in the near and the far wall at the same time. When the echogenicity obtained is as high as possible(as bright as possible), this is an indication that the ultrasound waves have cut the plaque optimally.

The presentation of the plaque causing the largest thickness of the plaque is chosen for recording of a frozen image on the videotape. An electronic caliper is put on the top of the plaque (at the interface between the surface of the plaque and the vessel lumen) and another caliper in the presumed transition zone between the media and the adventitia layer. The distance between the calipers is the thickness of the plaque, and that value is put on the registration form in the appropriate box. The B-mode image of the plaque is identified correctly by marking on the display what has been found, and where: PLAQUE ICA FW (a plaque in the far wall of the internal carotid artery), PLAQUE BULB NW (a plaque in the near wall of the bifurcation), etc. A short recording of approximately 5 sec. is videotaped. If more than one plaque is present at a site (e.g., in the far wall of ICA), the largest is chosen and recorded.

After identifying and recording of plaques, imaging procedures to get optimal measures of IMT from CCA and BIF are performed. Optimal images are available when distinct double contours of the vessel wall typical for the intima-media complex can be seen. It is important that the longitudinal axis of the insonated vessel wall is perpendicular to the ultrasound beam direction. To avoid falsely too thick intima-media layer, the IMT should be measured in the full diameter of the artery and not in a chord. When satisfactory images are achieved, R-wave triggered IMT-registrations are recorded on a cineloop containing more than 20 images. Afterwards, the images stored in the cineloop are scrutinized and 3 of most representative images, and each at least 10 images apart, are selected for recording on the videotape.

Regarding IMT measurements in the BIF, the start of the BIF is first identified and then marked with an arrow. This is the point where the parallell walls of CCA are starting to diverge. If the probe throughout the recording process in the cineloop has changed position, the placeing of the arrow marker must be adjusted accordingly. It is important to underline that it is the sonographer who places the marker and not the off-line reader of the IMT measurements. The arrow-setting has to be as precise as possible, particularly when a plaque is located in the borderzone between BIF and CCA to avoid over- or underestimating of IMT.

The target site for IMT measurements of BIF is the 1 cm area from the start of the BIF and upstream, distally. If only a part of this distance is measurable, a recording

may, however, be performed on this shorter distance if the live sequence shows that this part of the vessel wall is representative of the rest of the 1 cm area. This shorter, measurable distance is marked with an electronic star. The 3 chosen images are marked BULB1, BULB2, and BULB3 and recorded on the videotape. If no measurable image is possible to obtain, an image from BIF still is recorded and marked MB, i.e., "missing bulb". If only one or two images from the cineloop is considered measurable, these are recorded and MB for one ore two images also recorded. IMT measurements from the near wall IMT in BIF were not recorded.

11. After examination of the BIF, B-mode scanning of CCA is performed, starting at the bifurcation and downstream as far as possible. Registration and measurements of plaque are done in the same way as mentioned above. The images with plaques are marked PLAQUE CCA FW and PLAQUE CCA NW, videorecording is performed of both the live sequence and of the frozen, marked images. Three optimal images for measuring IMT are chosen from the cineloop, from the arrow mark indicating the transition between BIF and CCA and 1 cm distally as described above. The images are marked CCA1, CCA2, and CCA3. Non-measurable images of IMT are also handled as described previously: an image of the CCA vessel wall is frozen and marked MC. All measurements on the far wall refer to the so-called "leading edge" principle (or "upper demarcation line" principle). These structures are not being different in thickness when the emitted power (mW/cm^2) or of the ultrasound instrument's gainsetting are changed (nor are biologic different conditions of subjects examined).

    Near wall measurements, however, are performed on "far edge" principles which means that IMT to some degree may be dependent on some of the technical conditions mentioned above (e.g., gainsetting). Standardized examination conditions therefore are particularly important for near wall measurements. It is, however, not possible, in technical terms, to obtain such ideal conditions because individually instrument adjusting alternatives always are more or less involved in processing optimal B-mode images. However, setting of functions such as emitted power of ultrasound, preprocession, postprocession, gainsetting, etc, should be standardized as much as possible. Biologic inter-individual differences (obesity, position of the neck arteries, short or long necks, etc.) causing need of some different adjustments, however, are not possible to standardize. If the visibility of IMT and plaques is not optimal, the gainsetting (both the general and the segmental) should first be adjusted to improve the quality of the image.

The gain should all the time be set high enough to identify soft, echolucent plaques but not too high to conceal small plaques due to "ultrasound noise" Only as an exception, adjustments of the other functions should be done.

After examination:

12. Do not remove the cassette from the videorecorder before end of the day, or when the cassette is full.

13. Check that the registration form is completed appropriately. In the "Remarks" box, coding for reasons for missing of measurable images should be done:

    MB 1= missing images from BIF due to obesity
    MB 2= missing images from BIF due to a steep angle between CCA and BIF
    MB 3= missing images from BIF due to technically difficult examinations (e.g., short neck)
    MB 4= missing images from BIF due to previous surgery or radiation
    MB 5=other reasons

    In the same way, missing coding for CCA and ICA is performed: MC 1, MC 2, etc.

14. A referral form to Department of Neurology, University Hospital, Tromsø is completed when a suspected carotid stenosis or occlusion are found. Two criteria for defining a stenosis is used. Either a velocity increase across an atherosclerotic plaque in BIF of 0.1 m/sec or more or 0.2 m/sec or more in ICA, compared to the reference velocity distally in ICA; or a plaque thickness that constitutes 35% or more of the lumen diameter at the plaque site. The velocities should be manually angle-corrected for the angle at which Doppler-beams are emitted into the vessel. Occlusion is suspected when the open lumen of the artery is not visible on B-mode or if there is a visible occluding plaque in the artery, and there is no detectable flow in the artery by pulsed Doppler or by colour-Doppler. The referral threshold should be low to avoid false negative stenosis cases. The person who is referred, should be given a written and verbal information of the findings and clinical implications before leaving the room.

Protokoll for "Grabbing"

Digitalisering av plakk opptak fra SVHS-kassett

- PC + skjerm, samt Panasonic 7560 + skjerm slåes på. Videoskjermen er ferdig forhåndsinnstilt på PAL.

- Videokassetten settes i. Spol fram til aktuelle plakk-opptak. Sjekk hele tiden mot skjemaet over alle plakkopptak (Excel-ark) for å forsikre seg om at man får digitalisert alle opptakene. Fin-innstill inntil framet man har på videoskjermen er stillestående, uten 'snø'.

- Start programmet **Matrox Intellicam** fra PC'ens skrivebord.

- Dersom det er ***første 'grab'*** fra kassetten skal GSM for bakgrunnsfargen i opptaket testes med følgende underprosedyre (1-5), ellers fortsett på neste punkt:

    1. Grab et bilde (se under prosedyren for GRAB! under).
    2. Save bildet under katalogen C:\My documents\Plaque\Test som test.tif.
    3. Last bildet inn i Adobe Photoshop 3.0.
    4. Trykk på Bilde**m**odus – **G**råtoner, og deretter '**OK**' på minimenyen 'Ta bort fargeinformasjonen?'
    5. Med firkantpekeren, avgrens et 'svart' område utenfor delen av skjermen ved Doppler-bildet, og trykk B**i**lde – **H**istogram. Middelverdien skal være mellom 1-3. Dersom den er <1 eller >3, stilles knappen 'BLACK LEVEL' på Panasonic 7650's 'TBC CONTROL' hhv litt med/mot klokka, og prosedyren gjentas inntil middelverdien er mellom 1-3 (men ikke 0, da kan 'BLACK LEVEL' være stilt for lavt!). Dette gjentas ved oppstart, og hver gang man skifter kassett, for å sikre seg om at bakgrunns-svart *er* svart.

GRAB!

- Trykk **Ctrl + M**, eller trykk på **kamera-ikonet** under menylinja (nr 6 fra venstre) for å grabbe bildet.Dette kan gjøres gjentatte ganger inntil man har et mest mulig rent bilde. Første gang man 'grabber' etter oppstart av Matrox Intellicam, får man opp undermenyen 'Digitizer Configuration Format' – Velg 'PAL' i boksen og trykk '**OK**'.

- Lagre bildet ved å trykke **Alt+F – A**, eller **F**ile – Save **A**s, (men ***ikke* Ctrl+S**, eller **Alt+F – S**, da lagres nemlig over sist lagret fil!) og skriv da inn filnavnet som er oppgitt i skjemaet. *OBS – dobbelsjekk for evt. skrivefeil.* Filen lagres i katalogen som samsvarer med tapenummeret, (Tape 01 osv…) under hhv Tr4 eller Tr5. (For å forenkle rutinen med filnavn, kan navnet kopieres fra Excel, og limes inn i filnavn-rubrikken i Intellicam, for deretter å trykke <Enter> for å lagre…men dobbelsjekk for skrivefeil likevel!!). Skriv inn 'grabe' – dato (format: ddmmåå, for eksempel 011102, 150103) i Excel-arket som en 'kvittering'.

- Spol så fram til neste plakk-opptak, fin-innstill og gjenta prosedyren…11000 ganger!!

Einar Fosse 2002

"Grabbing"-protocol

(Digitizing plaque images from SVHS-cassette)

- PC + monitor, and Panasonic 7560 video recorder + monitor are switched on. The video screen is preset to PAL.

- The videocassette is inserted in the video recorder. Wind on to the plaque image of interest. Check continuously the plaque registration form (Excel-sheet) to ensure that no plaque images are missed. The frame on the video screen should be smoothly adjusted until it is stationary, without any "snow".

- Start **Matrox Intellicam** on the PC desktop.

- If this is the ***first 'grab'*** from the videocassette, the GSM-value for the background colour of the recorded image should be calibrated according to the following procedure (1-5). If not, proceed to the next step.

    1. Grab an image (see the procedure for GRAB!).
    2. Save the image in the catalogue C:\My documents\Plaque\Test as test.tif.
    3. Export the image to Adobe Photoshop 3.0.
    4. Press the Image **m**ode – **G**reyscale, and then '**OK**' on the mini-menu 'Discard colour information?'
    5. Delimit a 'black' area outside the B-mode picture on the screen with the squared tool function, and press **I**mage – **H**istogram. The mean value should be between 1-3. If it is <1 or >3, the 'BLACK LEVEL'-button on the Panasonic 7650's 'TBC CONTROL' is turned a little clockwise/counter-clockwise respectively, and the procedure is repeated until the mean value is between 1-3 (but not 0, then the 'BLACK LEVEL' is too low!). This procedure should be repeated at every start-up, and each time a new videocassette is inserted, to ensure that background-black really *is* black.

GRAB!

- Press **Ctrl + M**, or press the **camera-icon** in the menu (nr 6 from left) to grab the image. Repeat until you have an optimal image. Every time Matrox Intellicam is started, the 'Digitizer Configuration Format' menu will appear on the screen – Choose 'PAL' in the box and press 'OK'.

- Save the image by pressing **Alt+F – A**, or **F**ile – Save **A**s, (but ***not*** **Ctrl+S**, or **Alt+F – S**, then the previous image will be erased!) and use the file name from the plaque registration form. *PS – check for writing error.* The file is saved in the catalogue corresponding to the tape number (Tape 01 etc…) under Tr4 or Tr5 respectively. (To simplify the file name routine, the file can be copied from Excel, and pasted in the file name column in Intellicam, and thereafter press <Enter> to save…but still check for writing error!!). Fill in 'grab' – date (format: ddmmyy, f. ex 011102, 150103) on the Excel sheet as a 'receipt'.

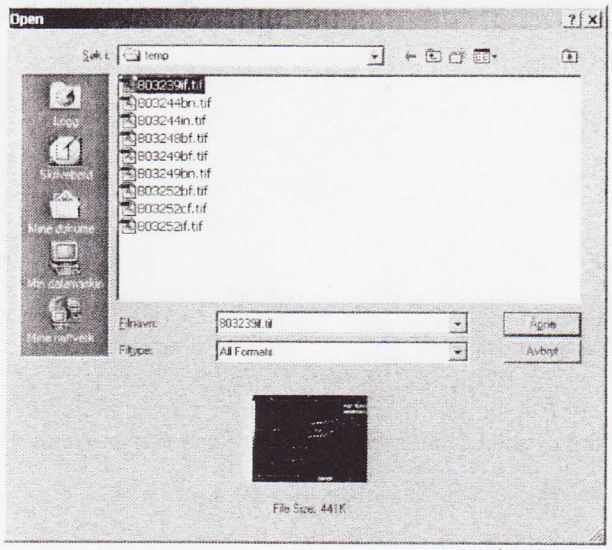
- Wind on to the next plaque recording, adjust smoothly and repeat the procedure…11000 times!

Stein Harald Johnsen 2005

Protokoll for plakk-standardisering

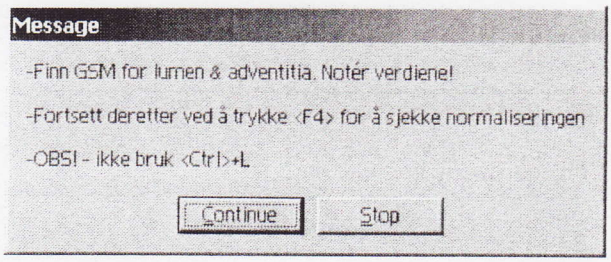
Programvare: Adobe Photoshop v7.0.1.

Trinn 1: Åpne bildefil:



Trykk **<F2>**, uthev filen som skal brukes, og trykk **<ENTER>**.

(Følgende skjer automatisk: Bildet åpnes i Photoshop, gjøres om til gråtoner, og lagres, deretter gjøres gråtone 0 pixler om til gråtone 1 pixler, og bildet forstørres til 200%)



Følgende meny kommer opp:

Trykk **<ENTER>**

Trinn 2: Finn GSM for lumen & adventitia:

LUMEN: avtegn enten med 'Lasso Tool' eller 'Rectangular Marquee Tool' det området i lumen som:

- er mørkest/fri for støy
- fortrinnsvis ligger i nærheten av plakket
- Ved fargeopptak, må et område uten farge velges.

Trykk **<Alt+I, H>** for å få frem histogram. Notér median-verdien, og så trykk **<Esc>** for å få bort histogrammet.

ADVENTITIA: avtegn enten med 'Lasso Tool' eller 'RectangularMarquee Tool' et område av adventitia som

- ligger mest mulig horisontalt/vinkelrett til proben
- er i nærheten av plakket (evt under plakket dersom dette er lysest og det ikke er skygge fra plakket)
- er lysest/har høyest gråtone
- området som velges skal være ca 0,25cm i bredde, og taes fra den indre (lumen-nære) halvdelen av adventitia, og vil da utgjøre minst 150 pixler.

Trykk **<Alt+I, H>** for å få frem histogram. Notér median-verdien, og så trykk **<Esc>** for å få bort histogrammet.

VIKTIG: *ikke gjør endringer på bildet (med* **<Ctrl+L>***) i dette trinnet!*

Trinn 3: Foreta 'prøve-standardisering':

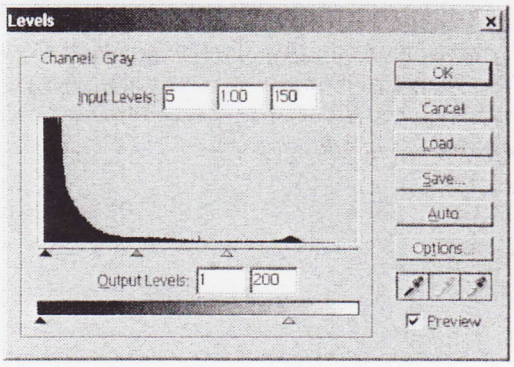
Denne del-rutinen er for å teste hvorvidt GSM-verdiene fra lumen og adventitia gir 'riktige' GSM-verdier etter normalisering. Lumen-GSM skal ligge mellom 1-5, adventitia mellom 190-200. ***OBS: alle plakk skal normaliseres, også de med GSM verdier innenfor de definerte områdene før normalisering!*** Har bildet i utgangspunktet GSM-verdier som er nære opp til de definerte områdene, kan Trinn 3 hoppes over, forsett med Trinn 4.

Trykk **<F4>**

(Følgende skjer automatisk: Et duplikat av bildet lages med navn 'Prøve', bildet forstørres til 200%)

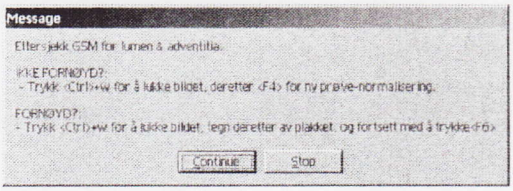
Menyen for innføring av 'input'-verdier kommer opp:

Før *kun* inn *input* verdiene (lumen i venstre boks, adventitia i høyre boks. Midtre boks skal ikke endres – *må* være 1.0)



Trykk **<Enter>** eller ***OK***

Følgende meny kommer opp:



Trykk **<Enter>** eller ***Continue***, ettersjekk deretter GSM for lumen og adventitia.

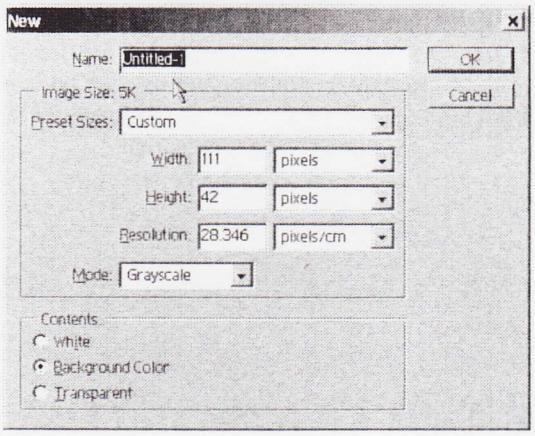
- ***Ønsker du nytt forsøk?***
  - Trykk **<Ctrl+w>** (*viktig!!*) for å fjerne prøvebildet
  - ta nye GSM målinger på utgangs-bildet
  - gjenta prosedyren ved å trykke **<F4>**.

- ***Ønsker du ikke nytt forsøk?***
  - Før inn verdiene for hhv lumen- og adventitia-GSM brukt som grunnlag for standardiseringen.
  - Gå videre til Trinn 4.

Trinn 4: Avtegning av plakk, lagring av ikke-standardisert & standardisert fil:

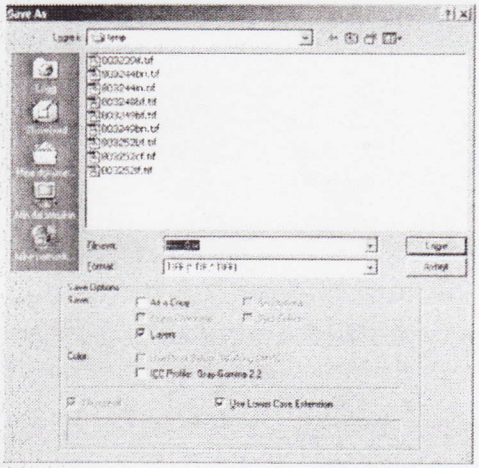
Trykk **<Ctrl+w>** *(viktig!!)* for å fjerne prøvebildet, avtegn plakket med 'Lasso Tool' (se kommentarer til slutt), og trykk deretter **<F6>**.

Følgende meny kommer opp:

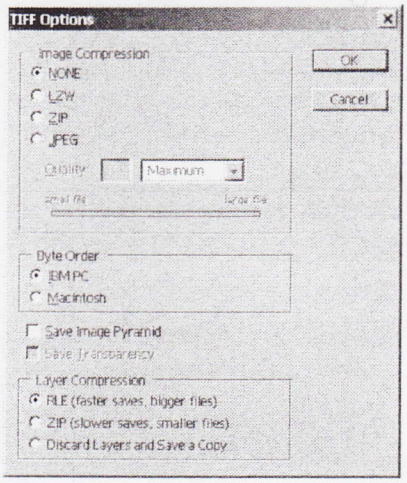


Bare trykk **<Enter>** eller ***OK***.
Ikke kopiér/lim fra Excel-filen her!

Men på neste meny kan **PlakkFilNavn_0** hentes fra Excel-filen, og limes inn i feltet **Name** (hvor det default står 'Untitled-1.tif').



Trykk deretter <Enter> eller OK på neste meny.



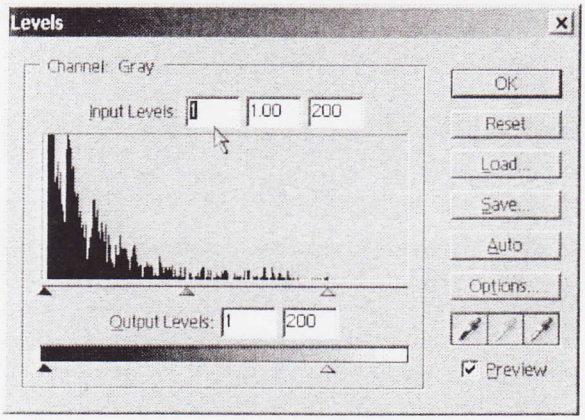
(Og dermed er det ikke-standardiserte plakk-bildet lagret! I neste steg føres input-verdier for standardisering.)

Menyen for innføring av 'input'-verdier kommer opp:

Før kun inn input verdiene *(lumen i venstre boks, adventitia i høyre boks. Midtre boks skal ikke endres – må være 1.00. Outputverdiene skal ikke endres)*

OBS: alle plakk skal standardiseres, også de med GSM verdier innenfor de definerte områdene før normalisering!

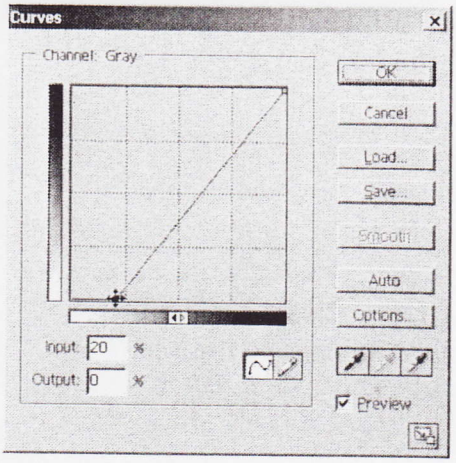
Trykk **<Enter>** eller ***OK***.



(Følgende skjer automatisk: det standardiserte plakk-bildet lagres med endret filnavn, og Photoshop gjøres klar for ny omgang)

FERDIG!

Kommentarer til Trinn 4: Avtegning av plakk:



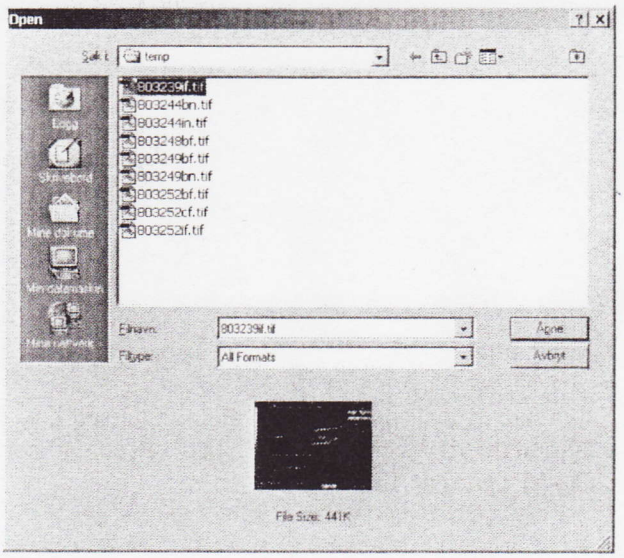
Tips: dersom plakket er vanskelig å visualisere, kan man foreta en temporær grå-skala forskyvning ved å bruke 'Curves'-funksjonen (**<Ctrl+m>**). Set pekeren over nedre venstre ende av linje, hold venstre museknapp nede, og dra punktet horisontalt til høyre. Plakk-bildet oppdateres automatisk. Husk å trykke **<Esc>** eller ***Cancel***, ikke ***OK***, for å avsluttet Curves-funksjonen!

Dersom plakket delvis ligge i akustisk skygge, skal kun de synlige deler av plakket avmerkes. Er plakket 'delt', kan 2 eller flere områder merkes med 'Lasso Tool'avtegnes som vanlig ved å holde venstre museknapp nede mens markøren merkes rundt plakk-delen. For avtegning av neste plakk, holdes <shift>-tasten nede og neste området avtegnes med venstre museknapp. Er mindre en 50% av plakket 'synlig', ekskluderes plakket.

Protocol - standardization of plaque

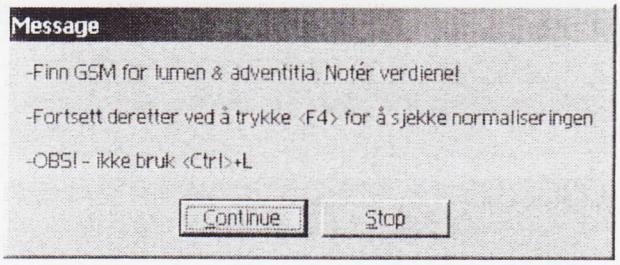
Software: Adobe Photoshop v7.0.1.

Step 1: Open image file:



Press **<F2>**, italicize the file of interest, and press **<ENTER>**.

(The following occurs automatically: The image is opened in Photoshop, transformed to gray levels, and saved. Then gray level 0 pixels are transformed to gray level 1 pixels, and the image is blown up 200%)



The following menu appears:

Press **<ENTER>**

Step 2: Define lumen-GSM & adventitia-GSM:

LUMEN: Using the 'Lasso Tool' or 'Rectangular Marquee Tool' function, outline the lumen area which:

- Is darkest/without noise
- Is located near the plaque
- If the recording has been coloured , an area without colour must be chosen.

Press **<Alt+I, H>** for histogram function. Mark the median-value, and then press **<Esc>** to erase the histogram.

ADVENTITIA: Using the 'Lasso Tool' or 'Rectangular Marquee Tool' function, outline a part of the adventitia which is

- as horizontal as possible/perpendicular to the probe
- located near the plaque (possibly under the plaque if this is the brightest part and without plaque shadow)
- brightest/ highest gray level
- the chosen area should at least have a width of 0,25cm, and represent the innermost (close to lumen) half of the adventitia, and will then consist of at least 150 pixels.

Press **<Alt+I, H>** to have the histogram. Mark the median-value, and then press **<Esc>** to erase the histogram.

IMPORTANT: *Do not make any changes to the image (by* **<Ctrl+L>***) at this step!*

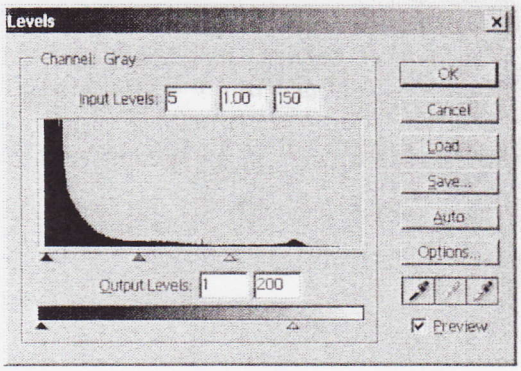
Step 3: Do a 'test-standardization':

This part is for testing whether the GSM-values from lumen and adventitia give 'the right' GSM-values after standardization. The lumen-GSM should be between 1-5, adventitia between 190-200. ***PS: All plaques should be standardized, including those that have GSM values within the predefined limits before standardization!*** If the image has GSM-values close up to the predefined limits, then Step 3 may be skipped, proceed to Step 4.

Press **<F4>**

(The following occurs automatically: A duplicate of the image is created with the title 'Prøve', the image is blown up 200%)

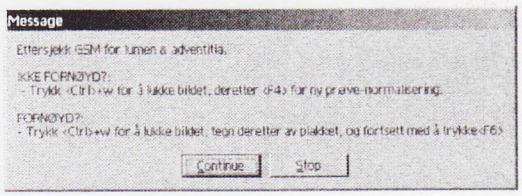
The menu for inserting 'input'-values appears:



Fill in *input* values only (lumen values in the left box, adventitia values in the right box. The box in between should not be changed – *must* be 1.0)

Press **<Enter>** or ***OK***

The following menu appears:



Press **<Enter>** or ***Continue***, then check the GSM values for lumen and adventitia.

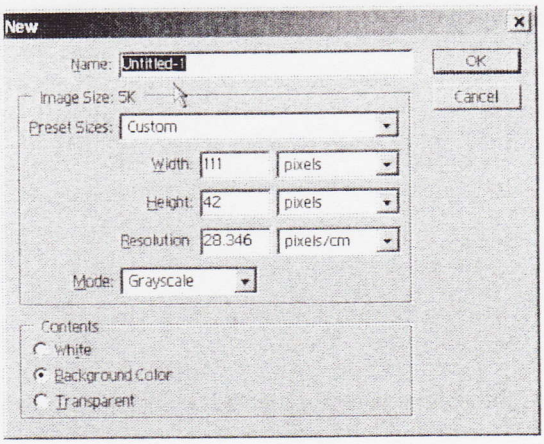
- ***Do you want a new trial?***
  - Press **<Ctrl+w>** (*important!!*) to erase the test image
  - Take new GSM measurements from the original image
  - Repeat the procedure by pressing **<F4>**.

- ***Do you not want a new trial?***
  - Fill in the values for lumen- and adventitia-GSM that were used in the test standardization.
  - Proceed to Step 4.

Step 4: Outlining of the plaque, saving the non-standardized & standardized file:

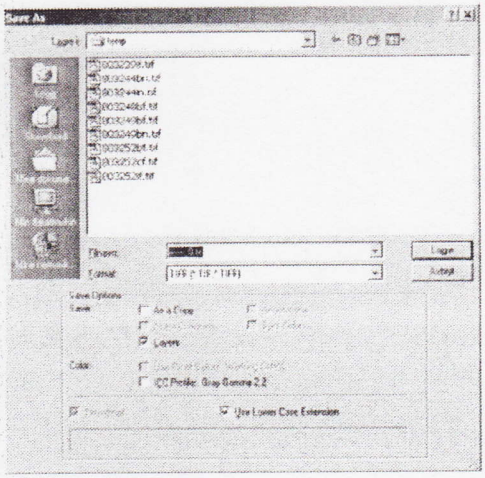
Press **<Ctrl+w>** (*important!!*) to erase the test image, outline the plaque using the 'Lasso Tool' (see comments in the end), and then press **<F6>**.

The following menu appears:

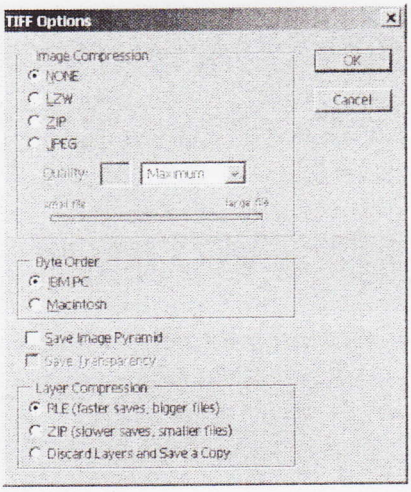


Press **<Enter>** or ***OK***.
Do not copy/paste from the Excel-file at this step!

On the following menu **PlaqueFileName_0** is copied from the Excel-file, and then pasted in the box **Name** (by default titled 'Untitled-1.tif').

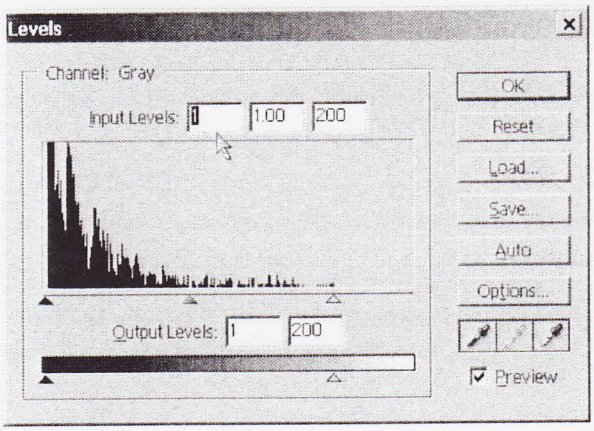


Then press <Enter> or OK on the next menu.



(And then the non-standardized plaque image is saved! In the next step the input-values for standardization are filled in.)

The menu for inserting 'input'-values appears:



Fill in input values only *(lumen in the left box, adventitia in the right box. The box in between must not be changed – should be 1.00. The output values should not be changed)*

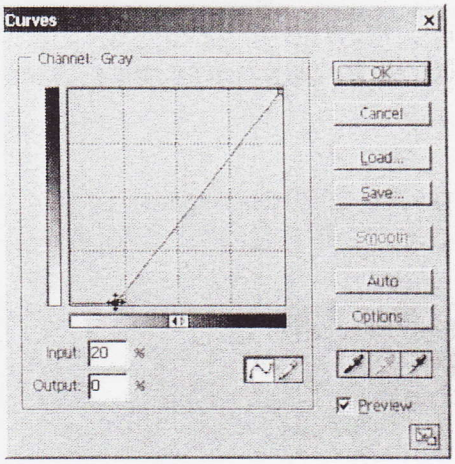
PS: all plaques should be standardized, including those with GSM values within the predefined limits before standardization!

Press **<Enter>** or ***OK***.

(The following occurs automatically: the standardized plaque image is saved with a new file name, and Photoshop makes ready for a new turn)

FINISHED!

Comments to Step 4: Outlining of the plaque:



Tip: If the plaque is difficult to visualize, a temporary gray-scale shift can be performed using the 'Curves'-function (**<Ctrl+m>**). Place the pointer over the left part of the bottom line, press the left mouse button, and draw the point horizontally to the right. The plaque image is automatically updated. Remember to press **<Esc>** or ***Cancel***, <u>not</u> ***OK***, to close the Curves-function!

If the plaque is partly located in an acoustic shadow, only the visible parts of the plaque should be outlined. If the plaque is divided by the shadow, two or more parts of the plaque can be outlined with the 'Lasso Tool' as usual by pressing the left mouse button while the plaque is being outlined. When the next part of the plaque is to be outlined, press the <shift>-key and then the plaque area is outlined by using the left mouse button. If less than 50% of the plaque is visible, the plaque has to be excluded from analysis.

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