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# Infection, inflammation and atherosclerosis

*Dag S. Halvorsen*

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**Institute of Community Medicine,
University of Tromsø**



**Department of Microbiology,
University Hospital of North Norway**

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by

**Dag S. Halvorsen**

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**Department of Microbiology, University Hospital of North Norway**

**Institute of Community Medicine, University of Tromsø**

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*Chlamydia pneumoniae* IgA- and IgG antibodies in young survivors of myocardial infarction. A comparison of antibody detection by a microimmunofluorescence test and an enzyme immunoassay. D. S. Halvorsen, T. Børvik, I. Njølstad, T. J. Gutteberg, L. H. Vorland, J.-B. Hansen. *Journal of Internal Medicine.* 2002;251:142-147.

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No detectable *Chlamydia pneumoniae* and cytomegalovirus DNA in leukocytes in subjects with echolucent and echogenic carotid artery plaques. Dag S. Halvorsen, Joachim Karlsen, Ann-Trude W. Notø, Ellisiv B. Mathiesen, Inger Njølstad, Tore J. Gutteberg, Lars H. Vorland, John-Bjarne Hansen. *International Journal of Cardiology.* 2007;117:388-394.

**3**

The association between white blood cells, fibrinogen and C-reactive protein, and carotid atherosclerosis is sex dependent. The Tromsø Study. Dag S. Halvorsen, Stein H. Johnsen, Ellisiv B. Mathiesen, Inger Njølstad. Submitted.

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Persistent inflammatory response does not predict mortality in long-term ischemic stroke survivors. Dag S. Halvorsen, Torgeir Engstad, Jan Brox, Bjarne Østerud, Inger Njølstad. Submitted.

**Abbrevations**

| | |
|---|---|
| BLAST | Basic Local Allignment Sequence Tool |
| CHD | Coronary heart disease |
| CRP | C-reactive protein |
| CV | Coeffisient of variation |
| CVD | Cardiovascular disease |
| CMV | Cytomegalovirus |
| *C. pneumoniae* | *Chlamydia pneumoniae* |
| DNA | Deoxyribonucleic acid |
| EB | Elementary body |
| EIA | Enzyme immuno assay |
| GSM | Gray scale median |
| HR | Hazard ratio |
| HRT | Hormone replacement therapy |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IMT | Intima-media thickness |
| LPS | Lipopolysaccharide |
| MIF | Microimmunofluorescence |
| OR | Odds ratio |
| PCR | Polymerase chain reaction |
| PBMC | Peripheral blood mononuclear cells |
| RB | Reticulate body |
| TNF | Tumor necrosis factor |
| WBC | White blood cell count |

**Introduction**

Myocardial infarction and stroke are leading causes of death and disability in the Western world, and substantial resources are spent on treatment, care and rehabilitation. A marked reduction in cardiovascular mortality has been observed in both genders in Norway during the 1980s and 1990s (Statistics Norway), probably due to both improved prevention and treatment of the acute ischemic event. In spite of this, cardiovascular diseases (CVD) accounted for nearly 40 % of the total mortality in 2004 (Statistics Norway). Atherosclerosis is an age-related disorder, and as the population ages, the burden of CVD will increase. A better understanding of the pathogenesis and mechanisms behind atherosclerosis is needed to improve preventive and therapeutic strategies.

Endothelial dysfunction with progressive lipid accumulation in the vessel wall and formation of an atheromatous plaque is the hallmark of atherosclerosis, and an important cause of cardiovascular events. Atherosclerosis is considered a dynamic inflammatory process rather than a passive process of lipid accumulation [1]. Recent research indicates that plaque pathology is more essential than the stenotic flow-reduction for the risk of acute ischemic events [2-4].

Some authors claim that a significant number of patients with coronary heart disease (CHD) lack conventional risk factors (cigarette smoking, diabetes, hyperlipidemia, and hypertension) [5,6]. This claim implies that other risk factors may play a pivotal role in atherosclerosis, and the search for non-traditional risk factors, such as infectious and/or inflammatory causes, has been extensive during the last decade.

**Epidemiology of coronary heart disease and stroke**

Autopsy studies confirm that early atherosclerotic changes are localised to the coronary arteries and the aorta in young people [7]. Atherosclerosis is a global vascular phenomenon, and a strong relation between carotid, coronary, aortic and peripheral vascular atherosclerosis has been reported [8].

The prevalence and the incidence of CVD increase with age in both genders. Women develop atherosclerotic disease later in life than men, and the difference has been attributed to the specific effects of sex steroids on lipid metabolism, the coagulation and fibrinolytic system [9]. The CVD protection during women's reproductive years might empirically be expected to disappear after the menopause, but this is not the case [10]. Obviously, the absolute number of women suffering CVD continues to rise due to an aging female population. In population-based studies, the prevalence of CHD was similar in both genders,

whereas myocardial infarction appeared more often in men than in women in all age groups [11,12]. Even among the oldest (> 70 years), myocardial infarction appeared twice as often in men than women [11,12]. Women seem to have a worse prognosis after myocardial infarction than men, and the increased risk of death may partly be explained by older age, and the presence of more comorbidities [13].

Stroke (all cause) is the third leading cause of death in Norway after CHD and malignancy (Statistics Norway), and ischemic strokes, affecting large and middle sized arteries, account for 80-85 % of all strokes [14]. In contrast to myocardial infarction, the stroke incidence rates are similar in men and women [14-16]. In a population based study from UK, non-fatal cerebrovascular events were the most frequent acute manifestation of vascular disease, with half of all events in people aged > 75 years [16]. Stroke outcome is worse in women, partly explained by a higher proportion of hemorhhagic strokes [17] and presence of comorbidities [13].

**Atherothrombosis – an inflammatory disorder**
The atherosclerotic and thrombotic process underlying CVD appear somewhat interdependent and may be integrated under the term atherothrombosis [18]. Atherothrombosis is a multifactorial, multistep disorder involving low grade inflammation at every stage, from initiation to progression, culminating with plaque rupture and thrombus formation [19]. The atherosclerotic lesion is an asymmetric focal thickening of the intima layer of the vessel wall, consisting of immunocompetent cells, endothelial and smooth muscle cells, connective-tissue elements, lipids and debris [19].

The earliest event in atherogenesis is endothelial cell dysfunction [18], caused by noxious stimuli from smoking, hypertension, dyslipidemia, diabetes mellitus or other unknown factors. Inflamed endothelium expresses leukocyte adhesion molecules, which attract and attach leukocytes. Following adherence to the endothelium, chemoattractant cytokines, such as monocyte chemoattractant protein-1, mediate transmigration of inflammatory cells into the subendothelial space [19].

A T-cell infiltrate is always present within the lesion [1], and $T_{h-1}$ cells produce interferon-$\gamma$ and TNF, which activate macrophages, endothelial and smooth muscle cells, leading to enhanced and sustained inflammation [19]. Secretion of macrophage colony stimulating factor differentiates monocytes into macrophages, and promotes phagocytosis of apoptotic cell fragments and oxidized LDL particles [20]. Transformation of lipid loaded

macrophages into foam cells is the hallmark of the fatty streak lesions, and as the plaque matures, the lipid core grows due to disorded apoptosis [19,21].

If fatty streak formation progresses, smooth muscle cells migrate into the intima, proliferate and form the fibrous cap, which is crucial for the plaque stability. Thinning of the cap is often seen at the shoulder regions of the plaque, in areas with ongoing inflammation and neovascularity [22], which promote recruitment of more immunocompetent cells. Foam cells release a broad range of inflammatory cytokines and matrix-degrading proteinases, which affects plaque instability and promote cap breakdown [2,19]. Once coagulation factors in the blood gain access to the lipid core, thrombus formation ensues. Low-grade inflammation by itself is associated with hyper-coagulability [23], as inflammatory mediators increase platelet count and reactivity, consume coagulation inhibitors, expose tissue factor and generate thrombin [23].

**Atherosclerotic plaque morphology**
Postmortem studies have shown presence of plaques in many vascular locations, presence of immunocompetent cells in the affected arteries, and appearance of multiple disrupted plaques in patients with acute coronary syndromes, suggesting the systemic nature of atherosclerotic disease [3,24]. Histopathological studies of plaques prone to rupture show unique structural characteristics; a large lipid pool mixed with numerous inflammatory cells covered with a thin fibrous cap with ongoing inflammation and neovascularity at the shoulder regions [2,18]. Younger individuals tend to have more plaques containing inflammatory cells than older individuals, and men more often than women [25,26].

Inflammatory regulation seems essential for plaque stability [1,19], and thrombus formation is dependent on plaque morphology, as the lipid core contains tissue factor, which is strongly thrombogenic [19,20]. Inflammatory plaques are also named "high risk", "vulnerable" or "thrombosis-prone" plaques. The concept of atherosclerosis as a systemic atherotrombotic inflammatory process is gaining support [18,27], and recently published papers discuss how to identify "vulnerable patients" with vulnerable plaques [3].

**Inflammatory markers**
**White blood cell count (WBC)**
Leukocytosis or elevated WBC reflects the normal bone marrow response to infectious or inflammatory stimuli. The maturation of WBC in the bone marrow, and their release into the circulation are tightly regulated by colony-stimulating factors, interleukins, complement

factors and hormones [28,29]. A two- to three fold increase in circulating leukocytes, including immature cells, can be achieved within few hours [30]. The circulating pool of neutrophils contains one pool of circulating cells, and one pool lining the blood vessel walls, the latter easily mobilized by stress hormones [28,29]. From a clinical point of view, leukocytosis is defined as WBC > 11 x $10^9$ /l.

**Interleukin-6 (IL-6)**

IL-6 expression and secretion is mainly induced by IL-1 and TNF-α, and IL-6 is secreted by a number of different cells including activated monocytes, macrophages, lymphocytes, endothelial cells, fibroblasts and smooth muscle cells [31,32]. Adipocytes secrete IL-6, and as much as one third of total circulating IL-6 may originate from adipose tissue [31]. IL-6 is a powerful inducer of the hepatic acute phase response [32], and it is postulated that IL-6 is the main circulating mediator linking local pathology and systemic inflammation [32].

**C-reactive protein (CRP)**

CRP, named for its capacity to react with the pneumococcal capsular C-polysaccharide, is a very sensitive, unspecific systemic marker of inflammation and tissue damage [33]. Small amounts are produced by cells inside the atherosclerotic lesion [34], but hepatic synthesis, tightly regulated by IL-6, is the main determinant of the CRP concentration [33]. Hepatic synthesis starts rapidly, and the serum concentration peaks around 48 hours with a half-life of 19 hours [30,33]. The CRP structure affects stability and function; circulating CRP has a pentameric structure, whereas monomeric CRP (mCRP) promotes higher proinflammatory activity and is located within atheromatous lesions [35]. From a clinical point of view, CRP serum values > 10 mg/l are considered pathological. American Heart Association and Center for Disease Control state that individuals with CRP values > 3 mg/l are at increased risk of CHD [36].

**Fibrinogen**

Fibrinogen is a soluble glycoprotein produced by hepatocytes, mainly regulated by IL-6 with a circulating half-life of 100 hours [37]. It is an acute phase protein and peak concentration is reached at 96 hours [30]. Fibrinogen plays a pivotal role in the coagulation cascade as the substrate for thrombin [38], and the plasma levels range from 1.5 to 4.5 g/l, exceeding the minimum concentration of 0.5 g/l needed for haemostasis maintenance [37]. Fibrinogen is a

major determinant of plasma and whole blood viscosity, and red blood cell aggragation, the pathophysiological mechanisms behind the erythrocyte sedimentation rate [38].

**Infectious agents as triggers of atherosclerosis**

Given their association with inflammation, infectious agents have been explored as initiators and promoters of atherosclerosis, and most studies have focused on *C. pneumoniae* and cytomegalovirus (CMV).

*C. pneumoniae* causes respiratory tract infections, usually mild or asymptomatic. The organism accounts for 1-10 % of community acquired pneumonias, and 1-5 % of upper respiratory tract infections [39,40]. Sixty to 80 % of the elderly population has IgG antibodies [39,41]. *C. pneumoniae* epidemics occur at intervals of 4 to 5 years [42], infections are more frequent in winter, and are often reported in closed communities such as military camps [43].

*C. pneumoniae* is a gram-negative bacteria harbouring lipopolysaccharide (LPS) in the outer membrane. It is an obligate intracellular bacterium capable of chronic and persistent infection with a unique developmental cycle, involving two different morphological forms. The infectious elementary body (EB) is internalised by the host cell, remains within a host-derived vacuole and differentiates into a larger, metabolically active reticulate body (RB) [41]. After 10-12 rounds of RB multiplication, the RBs differentiate to EBs, which are released from the host cell to initiate another cycle. The organism may also evolve into a "persistent body", an intracellular, metabolically inactive, nonreplicating but viable form that may contribute to chronic inflammation [41,44].

Whether (asymptomatic) nasopharyngeal *C. pneumoniae* carriage contributes to circulatory *C. pneumoniae* antigens is questioned. Presence of *C. pneumoniae* without serological response has been reported in young children [45], and a high prevalence of pharyngeal *C. pneumoniae* carriage was reported in CHD patients (36 %) and in controls (22 %) in 1998, with similar antibody status [46]. To my knowledge, this observation in CHD patients has not been confirmed by other groups.

CMV is a prevalent viral pathogen and 60-90 % of adults have IgG antibodies [47]. Most people contract CMV infection from close contact with individuals secreting the virus in their body fluids, during childhood or adolescence. Primary infection is usually mild or asymptomatic, but severe infections involving the liver, lungs and the central nervous system have been reported [48]. After primary infection, CMV remains latent in endothelial cells, epithelial cells, fibroblasts and different parenchyma cells [47]. Reactivation and viral shedding occur when the host is immunosuppressed, and generalized CMV endothelitis is a

major threat to all solid organ transplant recipients [49]. The clinical relevance of CMV reactivity in immunocompetent elderly and/or CVD patients is unclear [47].

**How to diagnose a CMV or *C. pneumoniae* infection**

Analyses of acute phase reactants, pathogen detection in a relevant clinical specimen, and antibody detection in paired sera, is the optimal diagnostic approach in acute infections. Diagnosing a chronic or persistent infection is more complex, as the inflammatory response is modest and less specific when organisms are hiding intracellularly. Culture or detection of pathogen DNA from infected tissue, and to a lesser extent, serological analysis is part of the diagnostic workup. Detection of an intracellular pathogen indicates that the individual once has been infected, but uncertainty still remains whether the organism is associated with the clinical manifestations.

The incubation period for a CMV infection is 3-8 weeks, and IgM and IgG antibodies are detectable 1-2 and 2-4 weeks after disease onset. IgG antibodies persists lifelong and is a marker of a previous primary infection or latent infection [50]. During CMV reactivation, less than half of subjects will present IgM antibodies, and circulating CMV DNA is a more reliable marker of viral replication and reactivity [50]. Culture and antigen staining is not part of routine diagnostics any more, and detection of CMV DNA in blood and urine, with PCR methodology, is available in all centres treating organ transplant recipients [49].

The incubation period for a *C. pneumoniae* infection is 2-4 weeks, and IgM and IgG antibodies are detectable 2-3 and 4-8 weeks after disease onset, dependent on test format and antigens [43,51-54]. IgM antibodies will gradually decline and become undetectable, whereas IgG antibodies persist, often lifelong, as a marker of previous infection. During reinfection, IgM may be absent or low, and IgG appears within 1-2 weeks, whereas data on IgA reactivity is sparse [42,51,54]. Culture of *C. pneumoniae* is difficult and not part of routine diagnostics [51,54]. Detection of *C. pneumoniae* DNA in nasopharyngeal samples, with PCR methodology, is a relevant approach in acute respiratory tract infections [51]. Circulating *C. pneumoniae* DNA has been reported in acute pneumonia, but is not a suitable marker [55].

**The evidence linking *C. pneumoniae* and CMV with atherosclerosis**
**Sero-epidemiological studies**

In 1988, Saikku and colleagues reported that patients with CHD had significantly higher levels of *C. pneumoniae* IgA and IgG antibodies as compared to healthy controls [56]. Numerous cross-sectional and case-control studies have been published, and the vast majority

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report a positive association between *C. pneumoniae* antibodies and atherosclerotic disease (mainly IgA, and to lesser extent IgG antibodies) [57].

Prospective studies minimize the relationship between *C. pneumoniae* IgA and IgG antibodies and atherosclerotic disease in the general population [57-61], and in individuals with pre-existing CVD [62,63], when adjusted for conventional risk factors, as male sex, smoking and increased lipid levels have been associated with *C. pneumoniae* IgG seropositivity [64,65]. In a meta-analysis, the overall OR was 1.1 (95% CI 0.8-1.4) when studying the relationship between *C. pneumoniae* IgG antibodies and CVD events [66]. Wald et al reported no association between IgA (OR 1.09, 95% CI, 0.82-1.43) and IgG antibodies (OR 1.26, 95% CI, 0.95-1.68) and cardiovascular mortality [67].

Most cross-sectional studies report a positive association between CMV IgG antibodies and measures of atherosclerotic disease [68]. Exposure to CMV was associated with a slight excess risk of subsequent cardiovascular events (HR 1.24, 95 % CI; 1.01-1.53) among subjects with previous CVD [69], whereas CMV IgG seropositivity was not associated with risk of ischemic events in hypertensive men in another study [70]. In one study, CMV seropositive individuals were more likely to develop post-angioplasty restenosis than seronegative subjects [71], but CMV reactivation was not demonstrated after angioplasty in another study [72].

### *In vitro* studies

*C. pneumoniae* can infect and survive in lung epithelium cells, resident macrophages, circulating monocytes, vascular endothelium and arterial smooth muscle cells [73]. *C. pneumoniae* infected human endothelial cells increases the expression of adhesion molecules and promotes transendothelial migration of phagocytes [74]. Infected macrophages ingest excess lipoprotein to form foam cells [75], and enhance the inflammatory action of oxidized LDL, leading to endothelial cell necrosis rather than apoptosis [76]. Infected macrophages may also enhance the production of matrix-degrading proteinases affecting the cap stability [77]. Further, infected macrophages secrete proinflammatory cytokines, such as TNFα, IL-1β, IL-6, and induce procoagulant activity [73].

Interaction of CMV immediate early gene products and the tumor suppressor gene p53, promotes proliferation of smooth muscle cells and inhibition of apoptosis [78]. CMV infected endothelial cells exhibit enhanced adhesion molecule and scavenger receptor expression, increased cytokine production and procoagulant activity [68].

**Animal studies**

In mice and rabbits, fed a normal and a cholesterolenriched diet, *C. pneumoniae* was detected in atheromas after experimentally induced infections [73,79]. Gieffers et al showed that monocytes transmit *C. pneumoniae* from the lungs to the vascular wall [80], and treatment with azithromycin modifies *C. pneumoniae* induced atherosclerotic lesions in rabbits [81]. In a porcine model, acute *C. pneumoniae* infection induced endothelial dysfunction with pro-coagulant reactivity [82]. In mice fed a cholesterol enriched diet, CMV induced endothelial injury with accumulation of lipids and accelerated atherosclerotic lesion formation [68].

**Histopathological and morphological studies**

*C. pneumoniae* organisms were detected by electron microscopy in atherosclerotic lesions in the early 1990s [83]. Preparations revealed variable and degenerative organisms, localised in smooth muscle cells, foam cells and within extracellular debris. Tissues with minimal lesions were as often positive as complex lesions, questioning the specificity of the procedures [84]. Several investigators have used immunohistochemistry (IHC) with genus and species-specific monoclonal antibodies to detect *C. pneumoniae*, but IHC fluorescence reading is subjective and hindered by unspecific background staining, and positive IHC samples could not be confirmed by PCR or immunoblotting [57,85].

**Culture of *C. pneumoniae* from vascular tissue**

To culture *C. pneumoniae* is difficult [57], and few research groups have provided evidence of viable *C. pneumoniae* in atheromas [86,87]. Detection of *C. pneumoniae* EBs and/or cell inclusions is difficult due to the unspecific reactions caused by the inoculated material, and cell cultures are at risk of contamination with *Mycoplasma* species and environmental chlamydia like organisms [88,89].

**Detection of pathogen DNA in atheromas**

*C. pneumoniae* DNA has been detected in atheromas from coronary [90] and carotid arteries [91,92], and from the aorta [93] with PCR methodology. Absence of *C. pneumoniae* DNA in specimens from the same locations has also been published [94-96], and the inconsistency could be due to low concentrations and patchy distributions [97]. Presence of CMV in atheromas has been confirmed by some groups [68, 98], but not by others [99,100].

**Detection of pathogen DNA in peripheral blood mononuclear cells (PBMC)**

Presence of pathogen DNA in atheromas is reported to correlate with DNA extracted from PBMCs, and circulating *C. pneumoniae* DNA was proposed to be a feasible marker of *C. pneumoniae* infected plaques [91,92,101,102]. However, positivity rates of circulating *C. pneumoniae* DNA range from 0 to 86 % in CVD patients [91,103,104]. Yetkin et al reported increased *C. pneumoniae* DNA prevalence after coronary angioplasty [105], whereas no increase was reported by another group [106]. Scepticism to PBMC as marker of vascular infection arose when it was reported that 46 % of healthy blood donors had *C. pneumoniae* DNA detected in their PBMCs [107]. Few reports describe presence of CMV DNA in PBMCs among immunocompetent CVD patients, and the prevalence ranged from from 0 to 17 % [106,108].

**Antibiotic intervention trials**

Early reports showed a benefit of short courses of azithromycin and roxithromycin in CHD patients [109,110]. The promising results have not been confirmed in larger trials with longer treatment duration [111,112], and a recent meta-analysis including six randomized azithromycin trials, conclude that azithromycin had no significant benefit versus placebo on a composite cardiovascular endpoint in CHD patients (OR 0.93, 95% CI, 0.84-1.03) [113].

Few studies used *C. pneumoniae* seropositivity as inclusion criteria, and to my knowledge, no studies selected individuals on basis of circulatory *C. pneumoniae* DNA. Analysing data from patients with and without evidence of *C. pneumoniae* infection may reduce the ability to demonstrate a benefit of antibiotics on clinical end points in individuals with a past infection. Worth to mention, tetracyclines and macrolides have anti-inflammatory effects, which might influence the study results [113].

Despite the early optimism, large scale antibiotic treatment in CVD patients has major public health implications, and as a medical microbiologist I am concerned that increased antibiotic consumption will increase antibiotic resistance [114].

**Inflammatory markers and atherosclerosis**

The inflammatory markers are produced directly by cells inside the atheromatous plaque(s) or indirectly by the liver [31,33,34,37]. Inflammation is a non-specific event, and the inflammatory process in other tissues/organs may confound the relation between low grade inflammation and atherosclerosis. CRP, fibrinogen and WBC are the most commonly studied

markers in epidemiological studies due to commercially available large scale automated assays, and to lesser extent IL-6 due to absence of such assays.

Inflammation is positively associated with most of the conventional cardiovascular risk factors, and the levels of inflammatory markers increase in a dose dependent manner with the number of risk factors in both genders [115-117]. I have summarized the current opinions on the associations between inflammatory markers and cardiovascular risk factors in Table 1.

Table 1.

| | CRP | Fibrinogen | WBC | IL-6 |
|---|---|---|---|---|
| Age | ↑ | ↑ | ↑ | ↑ |
| Male gender | ↑ | ↓ | | |
| Female gender | ↑ | ↑ | | |
| Cigarette Smoking | ↑ | ↑ | ↑ | ↑ |
| Hypertension | ↑ | ↑ | ↑ | ↑ |
| High LDL-cholesterol | (↑) | ↑ | | |
| Low HDL-cholesterol | ↑ | ↑ | | ↑ |
| Diabetes mellitus | ↑ | ↑ | | |
| Metabolic syndrome | ↑ | ↑ | | |
| Obesity | ↑ | ↑ | | ↑ |
| Alcohol consumption | (↓) | ↓ | | (↓) |
| Endurance exercise | ↓ | ↓ | ↓ | ↓ |
| Hormone (replacement) therapy | ↑ | ↓ | | |
| Chronic infections | ↑ | ↑ | ↑ | ↑ |
| Chronic inflammation | ↑ | ↑ | ↑ | ↑ |

↑ positive association, ↓ negative association, ( ) less pronounced association
References; 31, 32, 36-38, 115-119

**The evidence linking inflammatory markers with atherosclerosis**

The levels of inflammatory markers correlate with acute myocardial and brain infarction volume, and may serve as prognostic markers, as patients with increased levels of CRP on admission or at discharge are associated with a worse outcome [119-121].

Cross-sectional associations between inflammatory markers and measures of carotid [122-124] and coronary atherosclerosis [115,125] have been reported. In prospective population-based studies, WBC [126,127], fibrinogen [128,129] and CRP [36,130,131] predict first ever myocardial infarction and/or stroke. A recent meta-analysis including four studies and 8 years follow-up, indicates that the risk of stroke in healthy individuals with highest quartile of CRP concentrations increased by nearly 70 % as compared to those with the lowest quartile (OR 1.68, 95% CI, 1.40-2.01) [132]. Combining 22 studies, Danesh et al reported a nearly 60 % increase in risk of CHD in healthy subjects with CRP levels in the upper third compared with the lower third (OR 1.58, 95% CI, 1.48-1.68) [133]. The gender

specific effects of inflammatory markers are not clear, and few studies present data stratified by gender [134].

Few epidemiological studies on secondary CVD prevention have included measurement(s) of inflammatory markes, and their relevance is unclear. WBC [135,136], fibrinogen [137,138] and CRP [138,139] are reported to predict recurrent events in subjects with pre-existing CVD. The effect of inflammatory markers is attenuated after controlling for traditional risk factors in the primary and secondary prevention settings [121,133,140,141].

## "CRP lowering drugs" and cardiovascular disease

Clinical statin trials showed that statins lower median CRP levels by 15-30 % largely independent of the lipid reduction [142], and the benefit achieved with statin therapy seems to depend on reductions in both cholesterol and inflammation [143].

Therapy with acetylsalicylate (ASA) reduces the risk of myocardial infarction, stroke and vascular death in high-risk individuals [144]. ASA impairs platelet activation and aggregation, but there is uncertainty whether ASA reduces CRP levels [142].

Populations who consume diets rich in omega-3 fatty acids (FA) experience less inflammatory disorders and low CVD mortality. Cross-sectional associations between a high intake of omega-3 FA and lower CRP levels has been reported, but prospective studies offer inconclusive results on the CRP lowering effect of omega-3 FA [145].

## Aims of thesis

To compare two serological assays detecting *Chlamydia pneumoniae* IgA- and IgG antibodies in young survivors of myocardial infarction. Although highly subjective and non-standardized, the microimmunofluorescence (MIF) test has been considered the serological gold standard for years, and we wanted to compare the MIF test with an enzyme immunoassay (EIA), based on the same antigen preparation from the same manufacturer. (Study 1).

To investigate whether *Chlamydia pneumoniae* DNA or cytomegalovirus DNA is present in peripheral blood mononuclear cells in subjects with echolucent or echogenic carotid artery plaques. Modern real-time PCR technology was applied for this study. (Study 2).

To describe the association between inflammatory markers and measures of carotid atherosclerosis in men and women in the Tromsø Study (2001). WBC, fibrinogen and CRP

were measured in 5341 participants, and the relationship with carotid plaque area and plaque morphology was studied. (Study 3).

To investigate the predictive ability of inflammatory markers (WBC, fibrinogen, IL-6 and CRP) and *Chlamydia pneumoniae* IgA- and IgG antibodies as mortality risk predictors in long-term ischemic stroke survivors and stroke-free subjects in an 8-year follow-up study. (Study 4).

## Materials and Methods
### Study populations
In study 1, all patients (both genders, age 40-60 years) hospitalized because of first ever acute myocardial infarction or chest pain with negative exercise-ECG at the University Hospital of North Norway (1993 to 1995), were identified from computer records. Patients met the internationally accepted International classification of diseases – ninth revision (ICD-9) criteria (code 410 and 786.5). Age and sex matched controls to patients with myocardial infarction were randomly drawn from the fourth Tromsø Study (1994). Patients suffering from hypercholesterolemia, malignant hypertension, diabetes mellitus, coagulation disorders, manifest chronic inflammation, renal failure, liver disease, thyroid and malignant disorders were excluded. After a screening visit, the final study population consisted of 61 patients hospitalized with myocardial infarction, and 51 patients with chest pain and negative exercise-ECG, and 61 age and sex-matched controls.

In study 2, participants were recruited from the fifth Tromsø Study (2001), which included ultrasound examination of the right carotid artery. Subjects aged 56-80 years were eligible for the plaque group if they had $\geq 1$ plaque (plaque thickness of $\geq 2.5$ mm) in the carotid bifurcation or internal carotid artery, and plaque morphology was classified as echolucent or echogenic. Persons of the same age without carotid plaques served as controls. We excluded persons with hypercholesterolemia, malignant hypertension, coagulation disorders, manifest chronic inflammation, renal failure, liver disease, thyroid and malignant disorders, and use of lipid-lowering drugs or anticoagulants. After a screening visit with bilateral ultrasound examination of the carotids, the final study population consisted of 29 persons with echolucent plaques, 28 with echogenic plaques and 38 controls without carotid pathology.

In study 3, participants were selected from the fifth Tromsø Study (2001). A total of 8130 subjects attended the survey, and ultrasound examination of the right carotid artery was

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performed in 5454 persons. A plaque was defined as a localised protrusion of the vessel wall into the lumen (> 50% compared with the adjacent intima-media thickness), and was recorded from the near and the far walls of the common carotid, the bifurcation and the internal carotid arteries. A total of 5341 persons (2982 women and 2359 men) were enrolled in the study, as 83 subjects did not consent to medical research, and 30 subjects were excluded due to technical and data management difficulties (Figure 1).

Figure 1. Flow chart of the study population in Study 3.

| | |
|---|---|
| Subjects attending the Tromsø Study (2001) | n=8130 |
| Ultrasound examination of the right carotid artery | n=5454 |
| | 83 individuals did not consent to medical research |
| Study population | |
| Women | n=2998 |
| Men | n=2373 |
| | 16 men and 14 women were excluded due to technical and data management difficulties |
| Final Study population | |
| Women | n=2982 |
| Men | n=2359 |
| Subjects with carotid plaques | |
| Women | n=1613 |
| Men | n=1592 |

In study 4, participants were recruited from the fourth Tromsø Study (1994). In 1997, 269 individuals with a self-reported stroke and 262 individuals with no self-reported stroke were re-examined. A history of stroke was verified in 221 individuals, and our final study population comprised 187 individuals with a previous ischemic stroke, as we excluded 20 subjects with subarachnoid and 14 subjects with intracerebral haemorrhage. The final control group consisted of 243 population-based individuals without a history of stroke or transient ischemic attack (TIA) (Figure 2). The number of stroke free subjects was larger, as they were drawn on basis of a tentative larger and more heterogenic stroke-cohort than the final study sample of long-term ischemic stroke survivors.

Figure 2. Flow chart of the study population in Study 4.

Subjects attending the
Tromsø Study (1994)                  n=27159

No self-reported stroke              n=26741      n=418 Subjects with self-reported stroke

Invited to re-examination            n=361        n=362

Re-examined in 1997
(defined as baseline)                n=262        n=269

No stroke                            n=243        n=221 Subjects with a verified stroke

                                                  20 subjects with subarachnoidal and
                                                  14 subjects with intracerebral
                                                  haemorrhage were excluded
Study Sample
Subjects with no stroke              n=243        n=187 Verified ischemic stroke

Survival after
8 years follow-up                    n=201        n=118


**Antibody detection**

*C. pneumoniae* IgA- and IgG antibodies were analysed by a MIF test and an EIA test from Labsystems OY (Helsinki, Finland) in Study 1. LPS depleted EBs serve as antigen, and according to the manufacturer, the antigen is identical in both tests. The EBs are fixed on glass surfaces in the MIF test, whilst the EBs are attached to plastic wells in the EIA test. For the IgA analyses, all sera were incubated overnight at 4 °C with rheumatoid factor-adsorbent to remove IgG antibodies.

In the MIF test, sera were incubated on slide for 30 (IgG) and 60 (IgA) minutes, and after washing, fluorescein isothiocyanate conjugated goat antihuman IgA or IgG was added for another 30 minutes. The slides were washed and dried before immunofluorescence reading by one experienced staff member. IgA samples were examined at dilutions 1:16, 1:32 and 1:64, whereas all IgG samples were screened at 1:32 and positive samples were further examined at dilutions 1:64, 1:128, 1:256 and 1:512 [151].

In the EIA test, sera were diluted 1:100, and 10 µl samples were inserted into the antigen coated plastic wells. After 1 hour incubation, plates were washed, and horseradish peroxidase conjugated sheep antihuman IgA or IgG was added for another hour. After washing, tetramethylbenzine substrate was added for 30 minutes, before adding a stop-

solution, containing $H_2SO_4$. The reaction was read photometrically at 450 nm, and the end product is proportional to the concentration of *C. pneumoniae* antibodies, expressed as enzyme immunounits (EIU). According to the manufacturer, the EIA kit is scaled and calibrated in such a way that the EIU corresponds to the inverted titre of the MIF test. Based on the results in paper 1, the *C. pneumoniae* EIA test was applied in Studies 2 and 4.

CMV IgG antibodies were analyzed by a microparticle enzyme immunoassay technique from Abbott Laboratories (Illinois, USA) in Study 2. The reaction well contains microparticles coated with CMV, and present antibodies bind to the antigen coated microparticles forming antigen-antibody complexes. Assay diluent is added, the reagent is transferred to a matrix cell, and the antigen-antibody complex binds irreversibly to the glass surface of the matrix cell. Alkaline phophatase conjugated anti human IgG antibody is added, and after washing, 4-methylumbelliferyl phosphate substrate is added and the reaction is read photometrically. All serological assays were performed at the Department of Microbiology, University Hospital of North Norway.

**Polymerase chain reaction (PCR)**

PCR is used in clinical and research laboratories to identify and detect genes and DNA sequences, and for the rapid diagnosis of infectious diseases, as PCR enables to produce millions of copies of a specific DNA sequence within hours. The basic principles are described in this section, and a more detailed discussion of the applied real-time PCR methodology (Study 2) is described on page 35.

A PCR vial contains all the necessary components for DNA amplification; DNA template that contains the region of the DNA fragment to be amplified, the primers (short single-stranded DNA fragments) which are complementary to the DNA regions at the 5' and 3' ends, the DNA polymerase (enzyme), large quantities of the four nucleotides, and buffer solution containing necessary cations. The vial is inserted into a thermal cycler, a pre-programmed and automated machine that heats and cools the reaction tubes to achieve precise temperatures required for each step of the PCR reaction; denaturation, annealing and extension, which are repeated for 30 to 40 cycles.

We applied real-time PCR technology, targeting two different sequences of the genome for both *C. pneumoniae* and CMV, and details concerning targets, primer/probe sequences, amplicon length and GenBank accession numbers are given in Paper 2. Prior to the first cycle, the PCR reaction was heated to 50 °C (2 minutes) for activation of uracil-N-glycosylase, and thereafter to 95 °C (10 minutes) to activate the hot start enzyme, and to

ensure that the DNA template and primers are denatured. Cycling began with one step at 95 °C (15 seconds), followed by annealing-elongation for 1 minute at 60 °C. The temperature was lowered so that the primers could attach to the single stranded DNA template, followed by elongation during which the DNA polymerase copies the DNA template, starting at the 3' end of the primers annealed to both strands. PCR product visualization was done with different fluorescent dyes (SYBR green and FAM) in the real-time PCRs [146].

**Ultrasound examination of the carotid artery**

Ultrasound imaging allows assessment of carotid atherosclerosis and plaque composition, and has been validated against histology [147]. Plaques that appear echolucent have a thin fibrous cap overlaying a lipid core with numerous macrophages, and are recognized as inflammatory plaques. Echogenic or non-inflammatory plaques contain more fibrous tissue, and less lipids and inflammatory cells [148]. Computer assisted analysis of gray scale content and calculation of gray scale median (GSM) was used to assess echogenicity. In Study 2, one experienced examiner performed the ultrasound examinations, and in Study 3, 3 sonographers performed the ultrasound analyses.

High-resolution ultrasonography (Acusan Xp10 128, ART upgraded, with a 7.5 MHz linear-array transducer, aperture size 38 mm) of both carotid arteries (Study 2) and of the right carotid artery (Study 3) was performed as previously described [149,150]. The sonographers were blinded to laboratory data and questionnaires. For each plaque a still image was recorded with the transducer parallel to vessel wall and vertical to the point of maximum plaque thickness. All recordings were done on a Panasonic 7650 video player with super VHS tape. Still images were digitalized offline using a PC with the Matrox Meteor II frame grabber card and Matrox Intelliscan v2.07 software, at a resolution of 768x576 pixels. In subjects with more than one plaque, the areas of plaques were summarized to give the total plaque area and a maximum of six plaques were recorded.

The histogram function in Adobe Photoshop was used to generate GSM values. The plaque GSM was standardized using the GSM of the lumen and media-adventitia as reference structures, and the GSM of the total plaque area was given as a weighted mean of the GSM value of each single plaque [150]. Median GSM was used to define an echolucent and an echogenic group in Study 2, whereas GSM values were grouped into quartiles in Study 3.

## Cardiovascular risk factors and blood measurements

Participants were asked to report previous myocardial infarction and stroke, present angina pectoris and diabetes mellitus (yes/no), use of blood-pressure and lipid-lowering drugs, and acetylsalicylate (yes/no) and current smoking (yes/no). CHD was defined as prevalent angina pectoris or previous myocardial infarction. Cardiovascular disease (CVD) was defined as CHD and/or previous stroke.

Height, weight, blood pressure and lipids (fasting in Studies 1 and 2, non-fasting in Studies 3 and 4) were measured in participants in all papers presented. WBC with leukocyte fractions is presented in Study 2, whereas total WBC is presented in Studies 3 and 4. Fibrinogen was assayed with a clotting method in a STA-Compact analyzer (Diagnostica Stago, Asnieres, France); the detection limit was 0.6 g/l and the coefficient of variation (CV) was 4%. CRP was measured by a particle-enhanced immunoturbidimetric assay from Roche Diagnostics (Mannheim, Germany), with detection limit 0.12 mg/l (Study 3) and 0.175 mg/l (Study 4) and the CV was 1.4% and 4.0%, respectively. IL-6 was analyzed with an ELISA (Quantikine Immunoassay, R&D Systems, Abingdon, UK) with detection limit 0.70μg/l and CV 4.2 % (Study 4).

All laboratory measurements were performed at the Department of Clinical Chemistry, University Hospital North Norway, except for the IL-6 analyses, which were done in the research laboratory of Professor Bjarne Østerud, Institute of Medical Biology, University of Tromsø.

## Data management and statistics

The computer programme EpiInfo (CDC, Atlanta, GA, USA) (Study 1) and the SAS software package (SAS Institute, Cary, NC, USA) (Studies 2-4) were used for data management and analyses. A p-value < 0.05 was considered significant.

In Study 1, matched analyses between patients with myocardial infarction and controls were done with McNemar's test (categorical data) and paired t-test (continuous data). Sensitivity and specificity were calculated according to Galen et al [151], and interrater agreement was estimated by kappa (K) and weighted kappa (Kw) according to Altman et al [152]. The interrater agreement was estimated as poor (<0.20), fair (0.21-0.40), moderate (0.41-0.60), good (0.61-0.80) or very good (0.81-1.00) [152]. The agreement was calculated for two different cut-off levels; between negative and weak positive and between weak positive and strong positive samples. Between group seroprevalence differences were assessed by the $\chi^2$ test in Study 2.

In Study 3, we used student's t-test, $\chi^2$ test and analysis of covariance (ANCOVA) to test differences between groups. CRP was log transformed to correct for skewed distributions. We performed sex stratified analyses due to interactions between sex and log CRP (p=0.003) and sex and WBC (p=0.04), when modelling plaque area and plaque morphology, respectively.

When subjects without carotid plaque(s) (total plaque area = 0 $mm^2$) were included in the analyses, the distribution of plaque area was skewed and did not satisfy the model criteria for linear and for ordinal logistic regression analyses. Therefore, in a separate analysis we compared the levels of inflammatory markers in subjects with and without plaques. Within the plaque group, the relationship between inflammatory markers and total plaquea area and plaque echogenicity were tested in regression analyses.

We used multiple regression analysis to test the independent association between plaque area and inflammatory markers. Plaque area was used as dependent variable, with inflammatory markers as continuous independent variables. We controlled for age, current smoking, total cholesterol and HDL cholesterol, systolic blood pressure, diabetes mellitus, CVD and treatment with lipid lowering drugs, known to be associated with atherosclerosis [119]. Linear trend for plaque area and plaque morphology (GSM) across quartiles of the inflammatory markers were tested by linear regression.

The independent relations between plaque morphology (GSM) and inflammatory markers were tested in multiple logistic regression analysis (cumulative ordinal logit model), where quartiles of GSM were used as dependent variable. The model calculates the OR for being in a higher quartile of the dependent variable. The score test for the proportional odds assumption was > 0.05 in all logistic regression models for both genders. In additional logistic regression analyses, GSM was dichotomized, where GSM quartile 1 was defined as echolucent plaque and quartiles 2 to 4 as echogenic plaque, and we controlled for all covariates mentioned above.

In Study 4, CRP and IL-6 was log transformed to correct for skewed distributions. We used student's t-test, $\chi^2$ test and ANCOVA to test differences between groups of subjects with and without ischemic stroke, controlling for age, sex, smoking (yes/no), comorbidities (diabetes and/or previous myocardial infarction) and medication with statins or ASA.

The effect of stroke on mortality was examined with stroke status (yes/no) as an explanatory variable in Cox proportional hazards models, and the effect of inflammatory markers or presence of *C. pneumoniae* antibodies on mortality were examined in similar models in stroke free subjects and stroke survivors separately. The inflammatory markers

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were analysed both as continuous variables and grouped into tertiles. *C. pneumoniae* antibodies were analysed as a dichotomous variable with the commonly used cut-off values for IgA- (≥ 16) and IgG (≥ 32) antibodies. Due to a high correlation between CRP and fibrinogen (Pearsons, r=0.66), these variables were not jointly included in the regression models.

**Summary of main results**

**Study 1**

Presence of IgA (cut-off=16) antibodies was significantly higher in CHD patients compared with controls for both assays (p=0.02 by the MIF and p=0.05 by the EIA test)(Table 2). The presence of IgG (cut-off=32) antibodies was significantly higher among patients (p=0.05) when analysed by the MIF-test, but not with the EIA-test (p=0.16). The strength of agreement between the assays was good for both IgA- (Kw=0.67) and IgG (Kw=0.79) analyses. Our data indicate better agreement for IgA cut-off=16 (K=0.74) than cut-off=32 (K=0.56), and for IgG cut-off=64 (K=0.83) than cut-off=32 (K=0.73). The EIA-analysis revealed less difference between groups and concealed the statistical associations described with the MIF-test.

We conclude that the choice of serological method is of importance when evaluating a possible relationship between *C. pneumoniae* and CHD. Dependent on assay and cut-off level, there is an increased *C. pneumoniae* IgA- and IgG seroprevalence in young survivors of myocardial infarction compared to controls. Despite the subjective interpretation of MIF-titres, the strength of agreement between the EIA and MIF tests was good for both antibody classes.

Table 2. Prevalence (%) of *Chlamydia pneumoniae* IgA- and IgG antibodies analysed with a MIF test and an EIA test, according to commonly used cut-off levels.

| | Patients with myocardial infarction (n=61) | | Controls (n=61) | | p-value[a] | |
|---|---|---|---|---|---|---|
| Cut-off levels | MIF | EIA | MIF | EIA | MIF | EIA |
| IgA (cut-off ≥16) | 45.9 | 47.5 | 26.2 | 29.5 | 0.02 | 0.05 |
| IgA (cut-off ≥32) | 24.6 | 21.3 | 16.4 | 9.8 | 0.33 | 0.12 |
| IgG (cut-off ≥32) | 86.9 | 86.9 | 72.1 | 75.4 | 0.05 | 0.16 |
| IgG (cut-off ≥64) | 73.8 | 75.4 | 49.2 | 52.4 | 0.009 | 0.02 |

[a]Matched analyses (McNemar's test) between patients with myocardial infarction and controls

**Study 2**

*C. pneumoniae* DNA and CMV DNA was not detected in a single PBMC sample from subjects with carotid atherosclerosis, regardless of plaque echogenicity, nor in controls without carotid pathololgy. *C. pneumoniae* and CMV IgG seropositivity was frequent in all groups, confirming previous exposure. Our results indicate that persistent *C. pneumoniae* or CMV infection is not a common phenomenon in subjects with carotid atherosclerosis.

**Study 3**

Women and men with carotid plaque(s) had significantly elevated levels of WBC and fibrinogen, but not CRP, as compared to subjects without plaques. All inflammatory markers were significantly associated with plaque area in men in multivariable adjusted models. WBC was significantly associated with plaque echogenicity in women in multivariable adjusted models (OR 1.12, 95% CI, 1.01-1.25), but not in men. Fibrinogen was significantly associated with plaque echogenicity in age-adjusted models in men only (OR 1.11, 95% CI, 1.02-1.22), but not in multivariable adjusted models (OR 1.10, 95% CI, 0.99-1.21). Although our study had no clinical end points, the results indicate sex dependent associations between inflammatory markers and measures of carotid atherosclerosis. CRP did not discriminate echolucent from echogenic carotid plaques in either gender. Our data highlights the importance of sex-specific analyses when studying the relationship between inflammatory markers and carotid atherosclerosis.

**Study 4**

Stroke survivors had significantly elevated levels of WBC, fibrinogen, IL-6 and CRP at baseline, as compared to stroke-free subjects, after adjustment for age, sex, current smoking, comorbidities, and medication. They also had excess risk of cardiovascular death (HR 3.59, 95% CI, 1.91-6.78) and all cause mortality (HR 2.16, 95% CI, 1.47-3.17), which was not associated with increased levels of inflammatory markers, or the presence of *C. pneumoniae* antibodies. In contrast, fibrinogen, IL-6 and CRP were associated with all cause mortality in stroke-free subjects, adjusted for age and sex. Despite an increased inflammatory response several years after index stroke, none of the markers were independent predictors of mortality in long-term ischemic stroke survivors. Mortality risk estimation in stroke survivors may warrant a different approach than in stroke-free subjects at similar age.

## Discussion

### Methodological considerations

#### Study design

The four papers are based on data from individuals with different manifestations of atherosclerotic disease, such as previous myocardial infarction (Study 1), presence of carotid artery plaque(s) (Studies 2 and 3) or previous ischemic stroke (Study 4). Studies 1 and 2 were case-control studies; Study 3 was a cross-sectional population-based study, and Study 4 was a prospective observational cohort study. In Studies 1 and 4, controls were drawn from the Tromsø Study in 1994, whereas cases and controls in Study 2 were recruited from the Tromsø Study in 2001.

#### Internal validity

The internal validity of a study refers to whether the results are representative, true or valid for the source population. Three types of biases may threaten the internal validity of a study; selection bias, information bias and confounding [153]. Any observed association may also occur by chance.

#### Selection bias

Selection bias is a systematic error in a study that stems from the procedures used to select subjects and from factors that influence study participation [153]. In Study 1, cases were patients (both genders, aged 40-60 years) hospitalized because of first time myocardial infarction, and they were identified from hospital computer records. In this geographical area, individuals experiencing acute central chest pain are hospitalized at the same hospital and examined for myocardial infarction. It is unlikely that several subjects with myocardial infarction are missing from the records. Ten patients were excluded due to complex medical disorders, and nine patients did not respond to the invitation. We do not know if these patients were unable to attend due to cardiovascular complications, or other comorbidities.

Avoidance of bias is important when choosing appropriate controls in case-control studies, and population-based controls are often desirable [154]. Selection bias arises if controls are not representative of those at risk of the disease in question. Controls should reflect the background frequency of the exposure in the population, and they should be similar in all important aspects to cases, except that they do not have the disease/disorder in question. Their selection must be independent of exposure [153]. Controls do not need to be healthy, as

exclusion of ill people as controls can distort or bias the results. For example, 18 % of the controls (no carotid pathology) had experienced CVD (Study 2).

Although the overall attendance rate was high (78.5 %) in the fifth Tromsø Study (2001), the age-specific attendance rate was low in women and men below 30 years. Studies have shown that non-attenders are less healthy, they have more CVD and disabilities, and a larger proportion is smokers [155]. This may be an important source of bias, and it is likely that the prevalence of carotid atherosclerosis is underestimated among the oldest participants in Study 3. Selective non-attendence of individuals with unfavourable cardiovascular risk factor profile could lead to underestimation of the relationship between inflammatory markers and measures of carotid artery pathology. However, the association between inflammatory markers and measures of carotid atherosclerosis is less likely to be biased, as this would occur if the relationship is different between attenders and non-attenders.

Studies on elderly (Study 4) may be biased by the healthy participant or selective survival bias, resulting in a high representation of subjects with low levels of or different responses to risk factors. Ischemic stroke survivors and stroke free subjects were followed for 8 years (Study 4) and data on all deaths were collected from death certificates and medical records. No death certificates were missing. Selection bias is usually a minor problem in follow-up studies, because exposure information is ascertained before the development of the outcome, which was mortality in Study 4.

**Information bias**

Information bias may occur if there are systematic measurement errors or misclassification of exposure or outcome between study groups [153]. Differential misclassification occurs when either the misclassification of the exposure differs by the outcome or the misclassification of the outcome differs by exposure status. If either the misclassification of the exposure or the outcome is independent of the status of the other, misclassification is non-differential. Differential misclassification can exaggerate or underestimate, while nondifferrential misclassification tends to dilute an association [153].

The questionnaires might be subjective and imprecise. Self-reported myocardial infarction, diabetes mellitus and stroke show substantial agreement with medical record data [156,157], and TIA is the most common cause of false stroke diagnosis [157]. It is unlikely that study subjects are misclassified due to stroke-status, as a verified ischemic stroke was based on clinical examination, medical records and cerebral CT-scans (Study 4). Misclassification of *C. pneumoniae* antibody status (dichotomous variable) is non-differential,

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as seropositivity was independent of disease status and outcome. The misclassification reffered to the questionnaires is likely to be random (non-differential), with respect to the association of interest, as few of the study participants were aware of the hypothesized relationship between variables under study [158].

In the present papers we chose to use data on current smoking, rather than previous smoking habits, which are prone to recall bias. Most errors related to the ultrasound examinations are random errors (non-differential). Obesity may lead to misclassification in ultrasound studies, as adipose tissue makes the imaging conditions poor. However, subjects with echolucent and echogenic carotid plaques had similar BMI values (Studies 2 and 3). Computer assisted analyses of carotid plaque content is considered a more objective method with better reproducibility than visual classification [148,150]. The inter- and intraobserver agreement on plaque occurrence and computer-assisted GSM classification was substantial with K-values 0.67 (95% CI 0.58-0.76) and 0.80 (95% CI 0.70-0.91) [149], and 0.77 (95% CI 0.73-0.80) and 0.79 (95% CI 0.75-0.84), respectively [150]. The chance of diagnosing a non-stenotic artery as stenotic was low, since a plaque was defined as a protrusion of the vessel wall into the lumen of at least 50% compared to the adjacent IMT (Study 3). Measurement of only one carotid artery in Study 3 may introduce misclassification of individuals with an ipsilateral plaque in the non-plaque group, but there is evidence of bilateral symmetry of carotid atherosclerosis [8,159]. Examination of both carotids was performed in all participants in Study 2.

The serum samples were frozen at -70 °C for 1-4 years until analysis. Antibodies and inflammatory markers were analysed when the serum samples were thawed for the first time. Levels of IgG antibodies, CRP and fibrinogen have been shown to be relatively stable in frozen serum [36,160,161], so the delayed analyses are not likely to represent a major problem. Minor daytime fluctuations have been reported for fibrinogen [162], but not for CRP [33]. IL-6 peaks at late evening times [163], and blood samples were not drawn at this time of the day. WBC and fibrinogen demonstrated minor, and CRP did not show seasonal heterogeniety [162], and WBC, fibrinogen and CRP show long-term stability [33].

Studies on long-term *C. pneumoniae* antibody stability in CVD patients are lacking, but in Finnish laboratory personnel followed for a decade, the IgA antibody persistency was more frequent when analysed with an EIA test than with an *in-house* MIF test, whereas IgG antibody stability was similar [42]. *C. pneumoniae* respiratory tract infections occur more frequently during winter months [102,106], but no *C. pneumoniae* outbreak was detected in the community or in our laboratory during the samplings.

Food consumption does not alter serum fibrinogen or CRP levels [33]. The non-fasting effect on total cholesterol and HDL cholesterol is negligible, but triglycerides may vary greatly. In Studies 1 and 2, fasting lipids were presented as basic characteristics, and total cholesterol and HDL-cholesterol were used as independent variables in the regression analyses in Study 3.

**Confounding and interaction**

When an association between an exposure variable and an outcome variable is distorted due to the effect of a third variable, related to both exposure and outcome, it is called confounding [153]. Confounding may over- or under estimate the association under study. Knowledge about potential confounders can be accounted for in the analyses. Stratification by sex and/or age, and multivariable analysis with inclusion of potential confounders, are strategies to control or to minimize confounding [164]. Multivariable analysis allows us to determine the independent contribution of the various explanatory variables on the outcome or dependent variable [164]. In Studies 3 and 4, inflammatory markers, adjusted for age, sex, smoking, cardiovascular disease, diabetes and medication (statins and ASA), were presented. Stratification by sex was done in Study 3 to describe the sex-dependent associations between inflammatory markers and carotid atherosclerosis. In Study 4, adjustments for age and sex were made in proportional hazards regression models because of small study sample size.

Interaction or effect modification, a difference in effect of one factor according to the level of another factor, can have direct biological and public health relevance [153]. In Study 3 we tested for interaction by inclusion of the interaction terms (exposure variable multiplied with possible effect modifier); interaction was detected between sex and log CRP (p=0.003) and sex and WBC (p=0.04) when modelling plaque area and plaque morphology, respectively.

**External validity (generalizability)**

The study findings are generalizable if the results are applicable to other populations. The age and sex distribution reflect the Tromsø population, and are not substantially different from Western populations regarding the incidence and prevalence of cardiovascular diseases, educational level, social and lifestyle factors. The carotid plaque prevalence in the Tromsø Study population is comparable to populations in Europe and USA [158].

**Discussion of main results**

*C. pneumoniae* **IgA and IgG antibodies**

Our data showed that the *C. pneumoniae* seroprevalence was assay and cut-off dependent (Study 1). The results are consistent with other reports, illustrating the impact of assay choice and cut-off levels on epidemiological studies of atherosclerosis and *C. pneumoniae* seropositivity [61,165,166]. Presence of *C. pneumoniae* IgA and IgG antibodies did not differ between groups with inflammatory or non-inflammatory carotid plaques, and subjects without carotid pathology (Study 2), and antibody presentation did not predict mortality in either long-term ischemic stroke survivors or in stroke free subjects (Study 4).

At present there is no validated serological marker of a persistent *C. pneumoniae* infection, and the measurement of IgA antibodies is discouraged [51,52,54]. However, some researchers argue that presence of IgA antibodies, due to a short circulatory half-life (4-6 days), could be a consequence of *C. pneumoniae* antigen release from the respiratory tract or from vascular tissue [42,58]. Longitudinal studies on *C. pneumoniae* IgA stability in CVD patients are lacking, and IgA persistence and kinetics are assay dependent in healthy laboratory personnel [42]. Cross-reactivity might also influence study results as *C. trachomatis* and other microbial antigens are reported to cross-react with *C. pneumoniae* antigens [52,167]. To avoid IgA and IgG cross-reactivity, sera were incubated with rheumatoid factor-absorbent to remove IgG antibodies. In addition, serum IgA levels increase with age [168,169], and men have higher IgA seroprevalence than women [169].

Confusing results of *C. pneumoniae* antibody status after coronary angioplasty [170,171], lack of correlation between serology and *C. pneumoniae* detection in atheromas [57], and intra- and interlaboratory variations and poor agreements between different *C. pneumoniae* tests [57,172] add to the evidence that *C. pneumoniae* antibody status is not predictive of vascular *C. pneumoniae* infection [51,57,61]. As a consequence of the current understanding of *C. pneumoniae* serology, the drawbacks of the MIF test, and the strength of agreement between the two IgG assays (Kw=0.79) in Study 1, we replaced the MIF-test with the EIA test in the other studies. From my point of view, *C. pneumoniae* seropositivity can only be interpreted as previous exposure to the organism.

*C. pneumoniae* **DNA and CMV DNA**

The main finding in Study 2 was that *C. pneumoniae* DNA or CMV DNA was not detected in PBMCs in subjects with carotid atherosclerosis, regardless of plaque echogenicity, nor in

controls without carotid pathology. All groups were highly IgG seropositive, confirming previous exposure.

Our data are consistent with some reports [95,103,104], but in sharp contrast to Prager et al [91] and Sessa et al [92], who reported 86 % and 72 % *C. pneumoniae* DNA prevalence in PBMCs in patients undergoing carotid endarterectomy. The two groups did also report high positivity rates in carotid atheromas, in contrast to Apfalter et al, who did not detect *C. pneumoniae* DNA in similar specimens [95].

Absence of a standardized PCR-protocol for detection of *C. pneumoniae* in cells or tissue from CVD patients, may possibly explain why published results are contradictory, and may have contributed to the overestimation of CVD attributable to *C. pneumoniae* infection [57,173]. Previous reports with high positivity rates are mainly based on nested PCR technology [91,92], now discouraged due to significant problems with contamination [173].

By adherence to internationally recommended validation criteria [51], we developed sensitive and specific probe-based real-time PCRs, and emphasized to avoid false positive results. Newly designed DNA sequences (primers and probes) were searched by the use of BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/) to check for specificity, and primers and probes were tested against common human pathogens.

Could the negative results be a consequence of inadequate DNA extraction, as kit-dependent DNA recovery variations have been reported [96]? We used a commercial DNA isolation kit designed for blood cells. Extracted DNA was positively verified by real-time PCR targeting a partial sequence of the human chromosome 8. To check for human DNA might be insufficient if the PBMC pathogen concentration is very low. However, leukocyte samples spiked with cultured *C. pneumoniae*, showed no essential inhibition during the process of extraction. We extracted DNA from a high number of leukocytes (about $8 \times 10^5$ cells), as cell count/blood volume seems important for the yield of pathogen DNA [102].

Could the negative results be a consequence of low PCR sensitivity or inhibition? Both PCR assays demonstrated high analytical sensitivity, PCR inhibition was ruled out by amplification of spiked samples with purified DNA and DNA extracted from cultured organisms, and each DNA extract was examined in multiple repeats. No essential inhibition was detected in PBMC samples and inhibition does not seem to be a general problem in clinical specimens [57,96].

A weakness of our study is the relative small number of participants in each group and the low prevalence of manifest CVD. More importantly, from a methodological point of view, we had no access to a positive clinical *C. pneumoniae* PBMC sample for this molecular study.

We could not detect CMV DNA in a single PBMC sample from immunocompetent individuals with or without carotid pathology, which is consistent with some [108], but not all previous reports [106]. Herpes viruses may reactivate and be excreted coincidentally with immunological stress [47], as a possible explanation of the diverging study results.

**Inflammatory markers and atherosclerosis**

Our data show that subjects with carotid plaque(s) had elevated levels of inflammatory markers as compared to individuals without plaques (Paper 3), and that long-term ischemic stroke survivors had increased levels as compared to stroke free subjects several years after the index stroke (Paper 4). The results are consistent with previous reports stating a relationship between inflammatory markers, and carotid pathology [123,124] and stroke survival [174]. The main finding in Study 3 was the sex dependent differences in associations between inflammatory markers and measures of carotid atherosclerosis, and that CRP did not discriminate echolucent from echogenic carotid plaques in either gender.

Few studies focus on the relations between carotid plaque morphology and inflammatory markers. IL-6, but not CRP, was associated with lower plaque echogenicity among selected patients with carotid plaques [175], and neither fibrinogen nor CRP differed between patients with echolucent and echogenic carotid plaques in another study [129]. An ultrasound study on carotid and femoral plaques in 288 men showed no independent associations between fibrinogen and CRP, and plaque echolucency [176].

Few studies report sex-specific associations between inflammatory markers and carotid measurements. Increased levels of CRP associated with advanced carotid atherosclerosis have been reported in male dyslipidemic patients [177]. In population based studies, an association between carotid IMT and CRP was reported in women [124], whereas carotid plaque formation was strongly associated with CRP in men in another study [123]. However, the IMT increase was strongly associated with ageing and traditional cardiovascular risk factors rather than CRP [123]. Study results may differ because of different study populations, methodological issues and carotid measurement size, as IMT thickening and plaque formation may progress at different rates at different locations.

The main finding from Study 4 was that none of the inflammatory markers studied were independent predictors of death in long-term ischemic stroke survivors, even though the multivariable adjusted levels of WBC, fibrinogen, IL-6 and CRP were significantly higher at baseline as compared to a stroke-free group. In contrast, fibrinogen, IL-6 and CRP were independent predictors of all cause mortality among the stroke free subjects.

Measurement of inflammatory markers has been suggested to improve mortality prediction in the elderly [178,179], but low grade chronic inflammation is also associated with common age-related diseases like Alzheimer disease, diabetes mellitus, advanced atherosclerosis, sarcopenia, frailty and malignancies [32,118,180], which may confound the association.

Despite excess risk of cardiovascular and all cause death, there is limited data on preventive strategies in long-term ischemic stroke survivors [181,182], and the role of inflammatory markers is unclear. Increasing age, stroke severity, functional and cognitive impairment is associated with poor outcome [183]. Strength of Study 4 is that blood sampling and measurements of inflammatory markers were performed several years after the index stroke, probably reflecting the subjects' habitual baseline levels. Beamer et al reported elevated levels of fibrinogen in stroke survivors after one year [174], and our results extend their observations.

The impact of common risk factors on mortality was different in the two groups, indicating risk factor modification in long term ischemic stroke survivors [184]. Due to accumulated life long risk burden, these individuals may have reached a level that overshadows the impact of additional inflammatory markers [119]. Our data do not support an additive effect of CRP or other inflammatory markers on mortality among long term ischemic stroke survivors.

Studies 3 and 4 have some limitations worth to mention. Severely ill and disabled individuals at increased CVD risk are probably underrepresented in Study 3, and the cohort of long-term ischemic stroke survivors is highly selective (Study 4). This healthy participant and survival bias may weaken the true association between inflammatory markers and study endpoints.

**Study conclusions**

The results in Study 1 illustrate the difficulties with seroepidemiology and focus on the importance of choice of assay when evaluating a possible relationship between *C. pneumoniae* seropositivty and CHD [165,166]. In Studies 2 and 4, we replaced the MIF test with the EIA test, and interpreted presence of antibodies as previous exposure. This is in line with a consensus report, stating that there is no validated serological marker of a persistent or chronic *C. pneumoniae* infection [51], and that presence of IgA and/or IgG antibodies does not predict vascular infection [57,61].

Detection of leukocyte pathogen DNA has gained popularity as a surrogate marker of vascular infection, but reported positivity rates vary greatly, indicating methodological difficulties [57,95,173], and correlation with pathogen DNA in atheromas is inconsistent [57,91,95]. In Study 2, we selected subjects on basis of carotid plaque echogenicity, a marker of plaque inflammation, and we did not detect *C. pneumoniae* DNA or CMV DNA in a single PBMC sample by means of probe-based, highly sensitive and specific real-time PCR technology. Whether circulating nucleic acids of CMV and/or *C. pneumoniae* represent true CVD risk factors, needs to be explored on a larger scale with standardised real-time PCR protocols, with emphasis on analyses of PBMCs and atheromatous tissue sampled from the same individual at the same time.

In 1886, Robert Koch developed criteria to prove that a microorganism caused a specific disease. Whether the postulates are appropriate criteria for *C. pneumoniae* to cause a chronic disease like atherosclerosis is questioned [185]. The criteria are;

1. The pathogen must be present in nearly all cases of disease.
2. The pathogen must be isolated from the diseased host and grown in culture.
3. The disease must be reproduced when the culture/organism is introduced into a susceptible host.
4. The organism must be recovered from the experimentally infected host.

From my point of view, the four traditional criteria are not entirely fulfilled. *C. pneumoniae* is not present in all atheromas, and all CVD patients do not present antibodies (first postulate). The organism has a tropism for vascular endothelium, but has rarely been cultured from vascular tissue, and reported *C. pneumoniae* DNA positivity varies greatly due to methodological limitations (second postulate). Data from animal studies supporting the third postulate are conflicting [79,186], but the organism has been isolated from experimentally infected animals (fourth postulate) [185]. A beneficial effect of antibiotic therapy on CVD end points could have provided additional evidence, but this is not the case with the current available antichlamydial therapy. Atherosclerosis is a multifactorial disorder, and there is not enough evidence to support that *C. pneumoniae* cause atherosclerosis in man [185].

It is not known whether inflammatory markers are true atherosclerosis risk factors or merely indicators of an ongoing inflammatory activity within lesions. The continuing influx of activated monocytes/macrophages contributes to echolucent plaque growth and instability [18,149], whereas HDL cholesterol and statin therapy seem to reverse the process and

stabilize plaques [187,188]. Identifying individuals with echolucent plaques is important because the presence of vulnerable plaques is associated with increased risk of ischemic events [4]. Due to the paucity of published reports, we studied the relationship between inflammatory markers and carotid plaque area and morphology (Study 3). The main finding in our study was the sex dependent differences in associations between inflammatory markers and measures of carotid atherosclerosis, and that none of the inflammatory markers studied were associated with carotid plaque echolucency, and that CRP did not discriminate echolucent from echogenic carotid plaques in either gender. The results are consistent with other cross-sectional studies reporting no association between CRP and plaque echolucency [129,175,176], but prospective studies are lacking.

The fact that men have increased carotid plaque area and more echolucent plaques (Study 3)[25,26], has been linked to the sex-dependent differences in CVD presentations [9,11,13,26]. There are sex differences in the inflammatory response to injury [189], and a weaker association between CRP and fibrinogen, and CVD mortality in women may reflect different gender pathophysiology [179]. Although Study 3 has no clinical end points, the results indicate sex dependent associations between CRP and measures of carotid atherosclerosis, supporting the importance of sex-specific analyses in future studies.

American Heart Association and Centers for Disease Control recommend that repeated measurements of CRP could add value in predicting coronary events [36]. However, the predictive ability of CRP seems to be less useful in old-age than in middle-age, the evidence is mainly based on studies on young to middle aged populations, and there is no age, sex or ethnicity specific recommendation [118,190].

Epidemiological studies provide evidence that inflammatory markers predict ischemic events and death in population based studies. Stroke survivors have an increased risk of death [183], and the role of inflammatory markers in mortality risk prediction is unclear. Even though the inflammatory markers were significantly elevated several years after the index stroke, none of the markers predicted mortality (Study 4). Interestingly, the levels of inflammatory markers were similar in stroke subjects having experienced their index stroke more than 7 or less than 7 years ago, probably reflecting an increased baseline activity. Our data do not support an additive effect of CRP or other inflammatory markers on mortality, and mortality prediction in long term ischemic stroke survivors may warrant a different approach than in stroke free subjects at similar age. More research is needed to establish the relevance of inflammatory markers in the secondary prevention setting.

**Further research and concluding remarks**

The cardiovascular infectious disease hypothesis seems less popular now, as the antibiotic intervention trials revealed no beneficial effect of antibiotics on clinical CVD endpoints [113]. Both serological and molecular techniques to determine a persistent *C. pneumoniae* infection suffer from lack of standardization [51,61,173]. The improvement and accessibility of PCR technology offer researchers an opportunity to reinvestigate the association between microorganisms and atherosclerosis, or even detect pathogen mRNA as evidence of viable organisms. Until we have standardized protocols, the true role *C. pneumoniae* in chronic diseases will be questioned [54].

Atherosclerosis is recognized as a systemic inflammatory disorder with focal manifestations [3]. Population based studies provide evidence that inflammatory markers predict first ever ischemic events and mortality. The major challenge is to identify the "vulnerable individual" at increased risk of plaque instability and rupture [3]. Combinations of inflammatory and endothelial markers, and/or conventional risk factors, and/or non-invasive vascular imaging may improve risk prediction in the future.

Ultrasound imaging allows assessment of carotid atherosclerosis and plaque composition. Whether carotid plaque echogenicity is representative of plaque composition elsewhere in the vascular bed is unclear, but recent reports indicate that plaques at different locations share morphological characteristics, suggesting plaque instability to be a widespread process [27,191]. More research is needed to validate the prognostic strength of presence or absence of carotid artery plaques as a marker of coronary artery stability [192].

Immune modulation in atherosclerosis is a growing and fascinating research field; the anti-inflammatory effects of statins [193], the recommendation of influenza vaccination as a secondary CVD prevention strategy [194], the atheroprotective effect of experimental immunization with oxidized LDL and heat shock proteins in mice [hansson195], and intervention with anti-CRP substances is shown to reduce myocardial infarction size in rats [196]. Knowledge of the direct effect of CRP in atherosclerosis, and the interplay between coagulation and the inflammatory response may open for new therapeutic strategies [23]. As the population ages, important research fields should include studies on the inflammatory response in the elderly, and the gender specific inflammatory response in particular.

There is a growing understanding of atherosclerosis as a systemic inflammatory disorder. However, the clinical utility is not established for any of the novel inflammatory markers [197], and approaches to prevent atherosclerotic disease can be based on similar principles worldwide with a focus on traditional risk factors [141,198].

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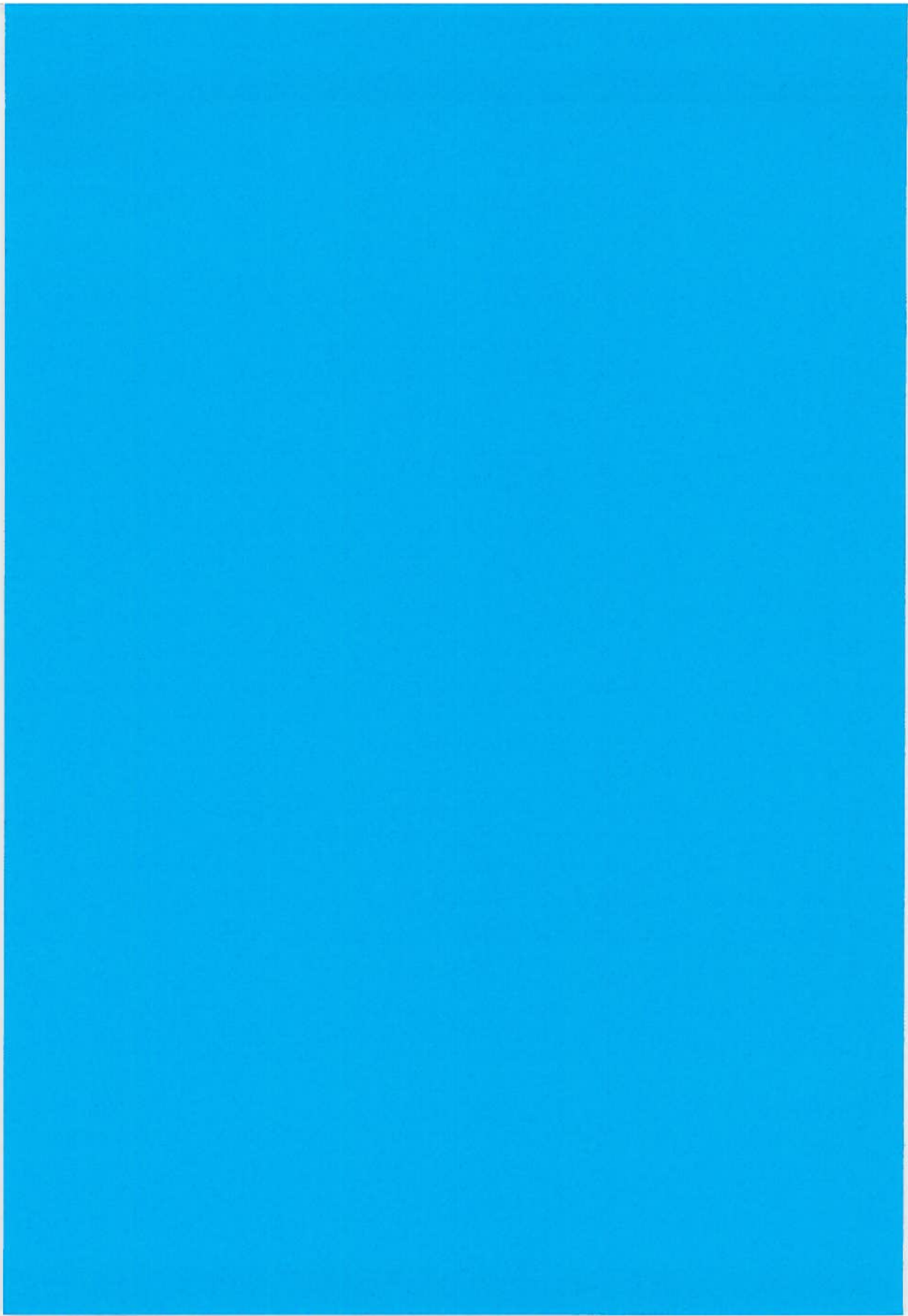
# *Chlamydia pneumoniae* IgA- and IgG antibodies in young survivors of myocardial infarction. A comparison of antibody detection by a microimmunofluorescence test and an enzyme immunoassay

D. S. HALVORSEN[1], T. BØRVIK[2], I. NJØLSTAD[3], T. J. GUTTEBERG[1], L. H. VORLAND[1] & J.-B. HANSEN[2]

*From the [1]Department of Microbiology, University Hospital of Tromsø, Tromsø, [2]Department of Medicine, Institute of Clinical Medicine; and [3]Institute of Community Medicine, University of Tromsø, Norway*

**Abstract.** Halvorsen DS, Børvik T, Njølstad I, Gutteberg TJ, Vorland LH, Hansen J-B (University Hospital of Tromsø; and Institute of Community Medicine, Tromsø, Norway). *Chlamydia pneumoniae* IgA- and IgG antibodies in young survivors of myocardial infarction. A comparison of antibody detection by a microimmunofluorescence (MIF) test and an enzyme immunoassay (EIA). *J Intern Med* 2002; 251: 142–147.

**Objectives.** Chronic *Chlamydia pneumoniae* infection is considered as a cardiovascular risk factor and antibodies are commonly analysed by the subjective microimmunofluorescence (MIF) test. We wanted to investigate the *C. pneumoniae* IgA- and IgG seroprevalence in young survivors of myocardial infarction and matched controls, and to compare the agreement of detecting antibodies between a MIF test and an enzyme immunoassay (EIA).

**Design.** A total of 61 patients hospitalized as a result of myocardial infarction, 51 patients hospitalized with chest pain and negative exercise-ECG and 61 age and sex matched controls (mean age 53.3 years, range 40–60 years) were included in this case–control study. Serological comparisons were expressed as sensitivity, specificity and interrater agreement (K or Kw) of the EIA test related to the MIF test.

**Results.** Presence of IgA (cut off = 16) antibodies was significantly higher in coronary heart patients compared with controls for both assays ($P = 0.02$ by the MIF and $P = 0.05$ by the EIA test). The presence of IgG (cut off = 32) antibodies was significantly higher amongst patients ($P = 0.05$) when analysed by the MIF-test, but not with the EIA-test ($P = 0.16$). The strength of agreement between the assays was good for both IgA- (Kw = 0.67) and IgG (Kw = 0.79) analyses. However, only 52.8% of the IgA samples classified as strong positive (cut-off = 32) by the MIF test were strong positive by the EIA test (K = 0.56). Only 73.2% of the negative IgG samples (<32) by the MIF-test turned out negative by the EIA-test (K = 0.73).

**Conclusions.** Dependent on assay and cut-off level, there is an increased *C. pneumoniae* IgA- and IgG seroprevalence in young survivors of myocardial infarction compared with controls. Despite the subjective interpretation of MIF-titres, the strength of agreement between the EIA and MIF tests was good for both antibody classes. However, misclassification of highly positive IgA samples and negative IgG samples by the MIF test may influence study conclusions. We conclude that the choice of serological method is of major importance when evaluating a possible relationship between *C. pneumoniae* and coronary heart disease.

**Keywords:** *Chlamydia pneumoniae* antibodies, EIA test, MIF test, seroprevalence.

## Introduction

There is increasing data implicating *Chlamydia pneumoniae* in the pathogenesis of atherosclerosis. Cross-sectional and case–control studies have shown that the presence of *C. pneumoniae* IgA- and/or IgG antibodies is associated with clinical or angiographically demonstrated cardiovascular disease [1–4].

*Chlamydia pneumoniae* is also detected in human atheromatous tissue [5–7], but prospective studies are still few and offer conflicting results [1, 8–10].

In most studies, *C. pneumoniae* IgA- and IgG antibodies are analysed by a microimmunofluorescence (MIF) test. Although the MIF test is labour demanding, subjective and antigen dependent it is recommended as the method of choice for determining *C. pneumoniae* seroprevalence [11–13]. Different incubation-procedures and cut-off levels in different study-populations for both immunoglobulin classes, may also hamper the interpretation of the results. In order to standardize test procedures and to ease study comparisons, a more objective serological method is desirable. A species-specific *C. pneumoniae* enzyme immunoassay (EIA) test is now commercially available and according to the manufacturer the *C. pneumoniae* antigen is identical with the antigen in the MIF test.

In the present case–control study, we wanted to examine the *C. pneumoniae* seroprevalence in young survivors of myocardial infarction and matched healthy controls, and to compare the agreement of these two serological methods, in order to investigate the reliability of detecting *C. pneumoniae* IgA- and IgG antibodies.

## Patients and methods

### Study population

All patients (both genders, age 40–60 years) hospitalized because of first time acute myocardial infarction or chest pain with negative exercise-ECG at the University Hospital of Tromsø during 1993–1995, were identified from computer-records. Patients met the internationally accepted International classification of diseases-ninth revision (ICD-9) criteria (code 410 and 786.5). Age and sex matched controls to patients with myocardial infarction were randomly drawn from the population survey of Tromsø (1994–1995) in this case–control study. A total of 10 patients suffering from inherited hypercholesterolemia, malignant hypertension, diabetes mellitus, coagulation disorders, manifest chronic inflammation, renal failure, liver disease, thyroidea or malignant disorders were excluded. A total of 70 patients with acute myocardial infarction, 70 age and sex matched controls and 55 patients with chest pain and negative exercise-ECG were invited.

A total of 61 patients surviving acute myocardial infarction, 51 patients hospitalized with chest pain and negative exercise-ECG and 62 age and sex matched controls responded on the invitation. Final study population was reduced to 61 age and sex matched controls because of shortage of serum. Blood samples were obtained 2–3.5 years after hospitalization of the acute myocardial infarction during spring 1997, and neither patients with chest pain, nor controls had experienced an acute coronary event until blood sampling. Blood samples were drawn after 12 h overnight fasting for all participants. All sera were stored at –70°C and antibody analyses were performed by one investigator unaware of sample identification. Informed written consent was obtained from each participant and the study was approved by the Regional Board of Research Ethics.

### Detection of Chlamydia pneumoniae *antibodies*

A total of 173 serum samples were available for antibody analyses. Lipopolysaccharide depleted elementary bodies serve as antigen in the MIF test obtained from Labsystems OY (Helsinki, Finland). For the IgA-analyses, all sera were incubated overnight at 4°C with rheumatoid factor-absorbent (Serion Immundiagnostica, Wurzburg, Germany) to remove IgG. Sera were incubated on slide for 30 (IgG) and 60 (IgA) min, respectively, and after washing, fluorescein isothiocyanate conjugated goat antihuman IgA or IgG was added for another 30 min The slides were washed and dried before immunofluorescence reading. IgA samples were examined at dilutions 1 : 16, 1 : 32 and 1 : 64, whereas all IgG samples were screened at the dilution 1 : 32, and positive samples were further diluted 1 : 64, 1 : 128, 1 : 256 and 1 : 512. The *C. pneumoniae* EIA test (Labsystems OY, Helsinki, Finland) is based on the same antigen preparation as in the MIF test and was performed according to the manufacturer. The reaction was read photometrically at 450 nm and the end product is proportional to the concentration of *C. pneumoniae* antibodies, expressed as enzyme immunounits (EIU). According to the manufacturer, the EIA kit is scaled and calibrated in such a way that the EIU corresponds to the inverted titre of the MIF test, so that direct titre comparisons can be made.

*Statistical analyses*

Statistical calculations were carried out using the computer programme EpiInfo [14]. Between group differences were assessed by the $\chi^2$ test and McNemar's test was applied for comparison between patients with myocardial infarction and controls. Only comparisons between patients with myocardial infarction and healthy matched controls are presented. A *P*-value < 0.05 was considered significant. Sensitivity and specificity were calculated according to Galen *et al.* [15], and interrater agreement was estimated by kappa (K) and weighted kappa (Kw) according to Altman [16] and Skogen *et al.* [17], in which the MIF test served as reference test. The interrater agreement was estimated as poor (<0.20), fair (0.21–0.40), moderate (0.41–0.60), good (0.61–0.80) or very good (0.81–1.00) [16]. The agreement was calculated for two different cut-off levels: between negative and weak positive and between weak positive and strong positive.

## Results

Basic characteristics of the study groups are described in Table 1. Patients with myocardial infarction had significantly increased body mass index (BMI) and elevated levels of triglycerides, whereas controls had a significantly higher level of HDL-cholesterol. Patients with myocardial infarction were significantly more frequent smokers and used medication with statins and acetylsalicylate to a greater extent. Presence of IgA-(cut off = 16)

antibodies was significantly higher in patients with myocardial infarction compared with matched controls for both the MIF test (*P* = 0.02) and the EIA test (*P* = 0.05). However, presence of IgG (cut off = 32) antibodies was significantly increased in patients with myocardial infarction compared with matched controls for the MIF test (*P* = 0.05), but not for the EIA test (*P* = 0.16). Stratified analyses for smoking and nonsmoking revealed no differences concerning antibody-status between groups. A dot-plot of corresponding titres in all dilution steps, analysed by the MIF- and EIA test is illustrated in Fig. 1. The titre distribution for both assays, according to the commonly used cut-off levels is given in Table 2. The agreement between EIA and MIF, expressed as Kw, was 0.67 for IgA- and 0.79 for IgG analyses. The highest agreement (K = 0.83) was found for IgG analyses with cut-off 64, whilst the lowest (K = 0.56) was found for IgA analyses with cut-off 32. The sensitivity for EIA IgA was low (52.8%) when cut off was 32. The specificity for EIA IgG was 73.3% when cut-off was 32. All other test estimates were greater than 80% (Table 3).

## Discussion

A reliable test for detecting *C. pneumoniae* IgA- and IgG antibodies is important when performing seroepidemiological studies to determine whether seropositivity represents a cardiovascular risk factor. A species-specific *C. pneumoniae* EIA test is now commercially available, and according to the manufacturer the *C. pneumoniae* antigen is identical

**Table 1** Basic characteristics of the study groups

| | Patients hospitalized with | | | |
| --- | --- | --- | --- | --- |
| | Myocardial infarction (*n* = 61) | Chest pain and negative exercise-ECG (*n* = 51) | Controls (*n* = 61) | P-value[a] |
| Female/male | 14/47 | 21/30 | 14/47 | |
| Age, years[b] | 53.5 (5.5) | 52.9 (5.8) | 53.5 (5.4) | |
| Current smokers, % | 41.0 | 56.9 | 22.9 | 0.03 |
| Body mass index, kg m$^{-2}$ | 28.3 (4.4) | 27.4 (3.5) | 26.1 (2.8) | 0.002 |
| Total cholesterol, mmol L$^{-1}$ | 6.06 (1.09) | 6.41 (1.29) | 6.17 (1.03) | 0.54 |
| HDL-cholesterol, mmol L$^{-1}$ | 1.25 (0.23) | 1.36 (0.36) | 1.40 (0.33) | 0.008 |
| Triglycerides, mmol L$^{-1}$ | 1.76 (0.89) | 1.76 (1.19) | 1.26 (0.58) | 0.002 |
| Current use of statins, % | 45.9 | 7.8 | 4.9 | <0.001 |
| Current use of ASA[c], % | 82.0 | 3.9 | 3.3 | <0.001 |

[a]Matched analyses (McNemar's test and paired *t*-test) between patients with myocardial infarction and controls.
[b]Continuous variables are presented as mean (SD).
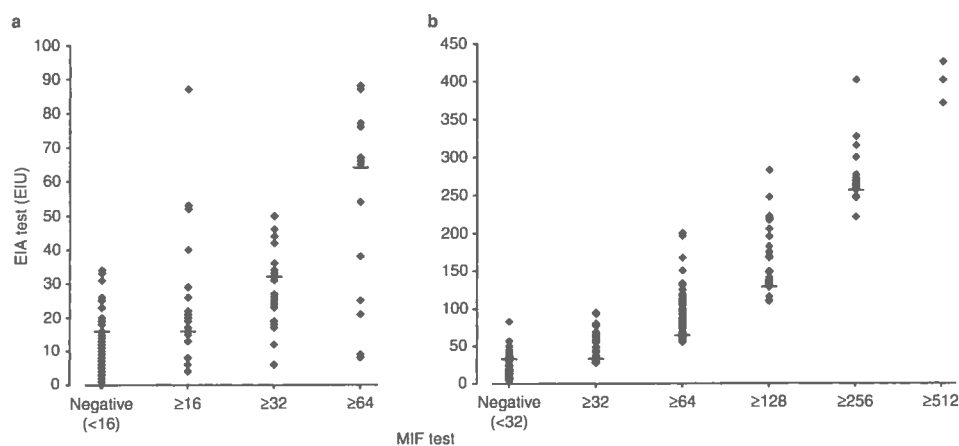[c]Acetylsalicylate.

**Fig. 1** Distribution of *Chlamydia pneumoniae* IgA- (a) and IgG-antibody titres (b) at different cut-off levels. All sera (*n* = 173) were analysed by a microimmunofluorescence (MIF) test and enzyme immunoassay (EIA) test.

with the antigen in the MIF test. The EIA reactions are read photometrically compared with the subjective immunofluorescence reading of the MIF test. To our knowledge, this is the first report evaluating *C. pneumoniae* MIF and EIA titres in young survivors

of myocardial infarction compared with healthy matched controls.

Our data demonstrate that the *C. pneumoniae* seroprevalence and the level of significance are both cut-off and assay dependent. The prevalence of IgA

**Table 2** Distribution of *Chlamydia pneumoniae* IgA and IgG antibody titres, analysed by a microimmunofluorescence (MIF) test and an enzyme immunoassay (EIA) test

| IgA | Negative (<16) | | | Weak positive (≥16) | | | Strong positive (≥32) | | |
|---|---|---|---|---|---|---|---|---|---|
| | *n* | % | 95% CI[a] | *n* | % | 95% CI | *n* | % | 95% CI |
| MIF | 111 | 64.16 | 56.87–71.45 | 26 | 15.03 | 9.59–20.46 | 36 | 20.81 | 14.64–26.98 |
| EIA | 109 | 63.01 | 55.67–70.35 | 39 | 22.54 | 16.19–28.84 | 25 | 14.45 | 9.10–19.78 |
| IgG | Negative (<32) | | | Weak positive (≥32) | | | Strong positive (≥64) | | |
| | *n* | % | 95% CI | *n* | % | 95% CI | *n* | % | 95% CI |
| MIF | 41 | 23.70 | 17.23–30.15 | 30 | 17.34 | 11.58–23.08 | 102 | 58.96 | 51.48–66.42 |
| EIA | 35 | 20.23 | 14.12–26.34 | 28 | 16.18 | 10.58–21.76 | 110 | 63.58 | 56.27–70.89 |

[a] Confidence interval.

**Table 3** Sensitivity, specifisity and interrater agreement for 173 sera using a microimmunofluorescence (MIF) test and an enzyme immunoassay (EIA) test for determination of *Chlamydia pneumoniae* IgA- and IgG antibodies, with the MIF test as reference test

| | Sensitivity (%) | Specificity (%) | Agreement (K)[a] | 95% CI[b] for K |
|---|---|---|---|---|
| IgA | | | | |
| Cut-off 16 | 53/62 (85.5) | 100/111 (90.1) | 0.74 | 0.63–0.85 |
| Cut-off 32 | 19/36 (52.8) | 131/137 (95.6) | 0.56 | 0.39–0.73 |
| IgG | | | | |
| Cut-off 32 | 127/132 (96.2) | 30/41 (73.2) | 0.73 | 0.60–0.86 |
| Cut-off 64 | 99/102 (97.1) | 60/71 (84.5) | 0.83 | 0.74–0.92 |

[a] Agreement expressed as Kappa.
[b] Confidence interval.

(cut-off = 16) and IgG (cut-off = 32 and 64) antibodies was significantly increased in coronary heart patients compared with healthy matched controls, when analysed by the MIF test. This observation is consistent with previous reports [3, 10]. The EIA-test confirmed only the different level of seroprevalence between groups for IgA-antibodies and for IgG-antibodies at cut-off 64. The EIA-analyses revealed less differences between groups and veiled the statistical associations described with the MIF test.

There is controversy regarding how to define a chronic *C. pneumoniae* infection serologically [18], in terms of choosing an appropriate IgA- and IgG cut-off level. Persisting IgA seropositivity is emphasized as a marker of chronic *C. pneumoniae* infection [19], but longitudinal studies on the IgA stability is lacking. At present there is no validated serologic marker of persistent or chronic *C. pneumoniae* infection [20, 21]. The manufacturer argues for an EIA IgA cut-off as low as 8, but test properties are defined in patients with acute respiratory illness and not in patients with persisting infections, nor in patients with cardiovascular diseases.

In the present study, samples were examined with the most commonly used cut-off levels for IgA- (cut-off = 16 or 32) and IgG antibodies (cut-off = 32 or 64) [18]. Our data suggest that the overall strength of agreement between the two assays is good for both *C. pneumoniae* IgA- (Kw = 0.67) and IgG (Kw = 0.79) antibodies. A better agreement for the IgG-analyses compared with the IgA-analyses is consistent with the findings of Schumacher *et al.* [22] and Gnarpe *et al.* [23]. Messmer *et al.* [24] also reported a good correlation between MIF and EIA for IgG-detection. The discrepancy between the MIF- and EIA test might be a consequence of different antigen manipulation. The elementary bodies are fixed on glass surfaces in the MIF test, whilst the elementary bodies are attached to plastic wells in the EIA test. Prolonged incubation time and different antigen concentration in the tests might also influence the antibody-level [25].

The strength of agreement was better for IgA cut-off 16 (K = 0.74) than for cut-off 32 (K = 0.56). Only 52.8% of the IgA samples classified as strong positive (≥32) by the MIF test tested strong positive by the EIA test. Dependent on the IgA cut-off level, misclassification of highly positive IgA-samples by the MIF test may influence study conclusions, as IgA seropositivity is reported as an independent cardiovascular risk factor [26,27]. The strength of agree-

ment was better for IgG cut-off 64 (K = 0.83) than cut-off 32 (K = 0.73). Only 73.2% of the negative (cut-off < 32) IgG samples analysed by the MIF test turned out negative by the EIA test. Dependent on the IgG cut-off level, misclassification of negative IgG samples may displace the seroprevalence level in different study populations.

An objection to our study is the relative small number of participants in each group and the unequal distribution of serum samples at the different cut-off levels, as the majority of samples were IgA negative and/or strong IgG positive. Although sensitivity and specificity are independent of the seroprevalence in the study-population, the interrater agreement is dependent on the proportions of sera classified as negative, weak positive and strong positive. However, these samples reflect the distribution of antibody titres in such a study population.

The results illustrate the difficulties with seroepidemiology and focus on the importance of choice of serological method when evaluating a possible relationship between *C. pneumoniae* and coronary heart disease. In future epidemiological studies, an agreement on different cut-off levels and the use of a more standardized *C. pneumoniae* antibody test would be desirable. Further comparative studies on a larger scale are needed until a firm recommendation of replacing the MIF test can be given. Despite the subjective interpretation of MIF titres, we conclude that the strength of agreement between the EIA and MIF tests is good for both *C. pneumoniae* IgA- and IgG analyses.

## Acknowledgements

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*Correspondence:* Dag S. Halvorsen, Department of Microbiology, University Hospital of Tromsø, N-9038 Tromsø, Norway (fax: +47-77627015; e-mail: mlabdsh@rito.no).
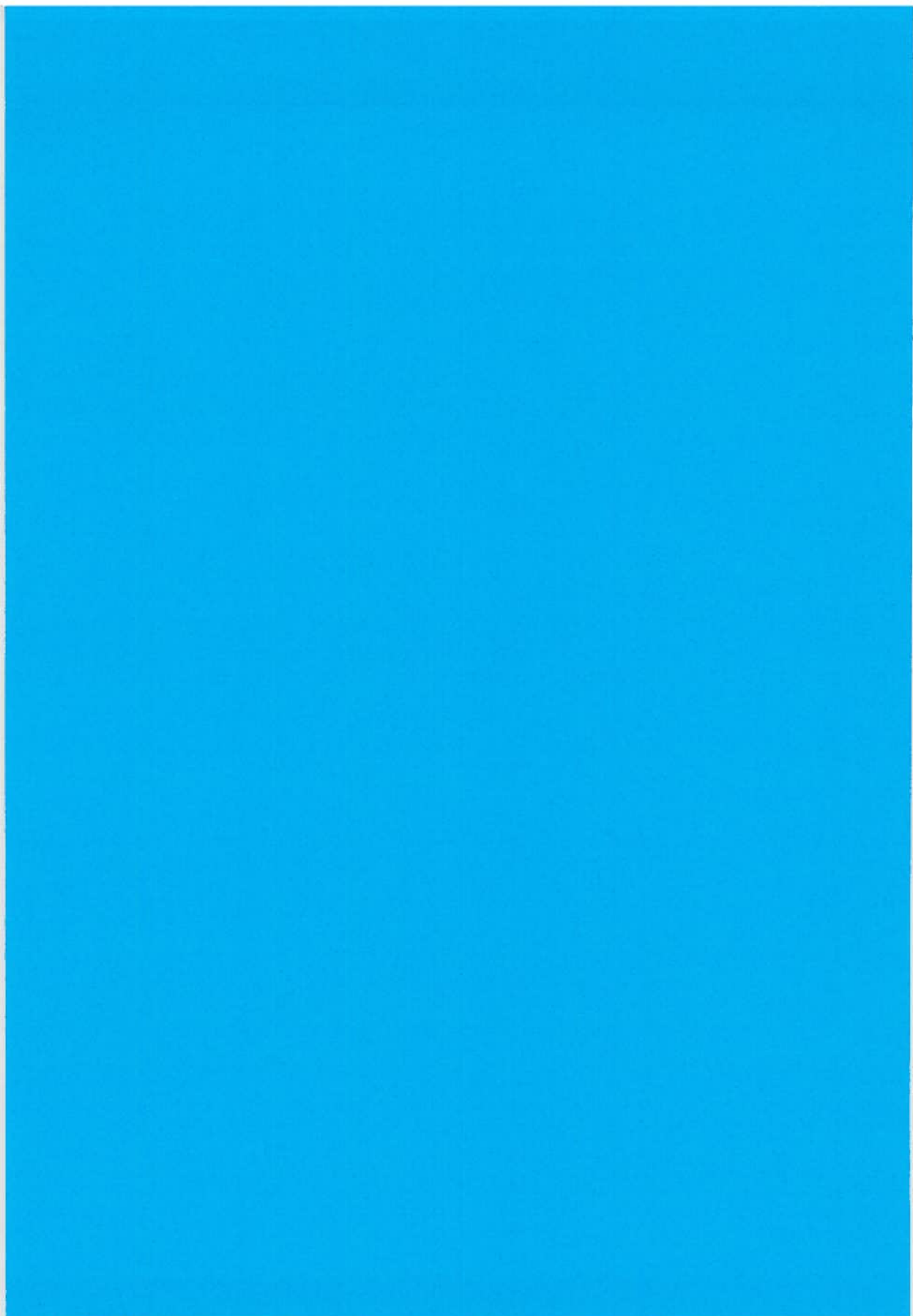
# No detectable *Chlamydia pneumoniae* and cytomegalovirus DNA in leukocytes in subjects with echolucent and echogenic carotid artery plaques [☆]

Dag S. Halvorsen [a,*], Joachim Karlsen [a], Ann-Trude W. Notø [b], Ellisiv B. Mathiesen [c], Inger Njølstad [d], Tore J. Gutteberg [a,e], Lars H. Vorland [e], John-Bjarne Hansen [b]

[a] *Department of Microbiology, University Hospital of North Norway, N-9038 Tromsø, Norway*
[b] *Center for Atherothrombotic Research in Tromsø, Institute of Clinical Medicine, University of Tromsø, N-9037 Tromsø, Norway*
[c] *Institute of Clinical Medicine, University of Tromsø, N-9037 Tromsø, Norway*
[d] *Institute of Community Medicine, University of Tromsø, N-9037 Tromsø, Norway*
[e] *Department of Microbiology and Virology, University of Tromsø, N-9037 Tromsø, Norway*

## Abstract

*Background:* Controversy exists whether persistent *Chlamydia pneumoniae* or cytomegalovirus infections cause initiation or progression of atherosclerosis. *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMC) has been proposed to be a more reliable marker of cardiovascular risk than are *C. pneumoniae* antibodies. Reported prevalences of *C. pneumoniae* DNA among cardiovascular patients vary greatly, indicating methodological limitations. There is an increasing concern that published results may have been biased by extensive use of less specific polymerase chain reaction (PCR) technology.
*Methods:* *C. pneumoniae* DNA and cytomegalovirus DNA were determined by probe-based real-time PCR technology in PBMCs among subjects with echolucent ($n=29$) or echogenic ($n=28$) carotid artery plaques, and in controls without carotid plaques ($n=38$), all recruited from a population-based study. Samples were examined in multiple repeats with PCR assays targeting two different sequences of the genome for both microorganisms.
*Results and conclusion:* IgG seropositivity was frequent in all three groups, confirming previous exposure, but *C. pneumoniae* DNA or cytomegalovirus DNA was not detected in a single PBMC sample by means of probe-based, highly sensitive, and specific real-time PCR assays. Our results indicate that persistent *C. pneumoniae* or CMV infection is not a common phenomenon in subjects with carotid atherosclerosis.
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*Keywords:* Chlamydia pneumoniae; Cytomegalovirus; DNA; Carotid atherosclerosis

## 1. Introduction

Inflammation plays a major role in the pathogenesis of atherosclerosis [1]. Whether cytomegalovirus (CMV) and/or *Chlamydia pneumoniae* (*C. pneumoniae*) are involved in this process remains unclear [2,3]. The methods to determine a chronic or persistent infection suffer from lack of standardization [4], and this may possibly explain why previous results are contradictory. In cross-sectional studies, the presence of *C. pneumoniae* and CMV antibodies has been associated with

carotid and coronary atherosclerosis, whereas prospective studies offer conflicting results concerning antibody presentation and cardiovascular events [3,5].

Both microorganisms have been detected in carotid atheroma, but the prevalence varies from 0 to 82% for *C. pneumoniae* [6,7] and from 0 to 53% for CMV [8,9]. DNA positivity in atheromatous tissue is reported to correlate with DNA extracted from peripheral blood mononuclear cells (PBMC), and the presence of pathogen DNA in PBMCs has therefore been proposed to be a more reliable marker of chronic or persistent infection than are antibodies [7,10]. However, positivity rates of circulating *C. pneumoniae* and CMV DNA in cardiovascular patients range from 0 to 86% for *C. pneumoniae* [7,11] and from 0 to 17% for CMV [12,13], indicating methodological difficulties. Poor reproducibility in intra- and interlaboratory studies and a high rate of false-positive results have been reported for *C. pneumoniae* [14].

Ultrasound imaging allows assessment of carotid atherosclerosis and plaque composition. Plaques that appear echolucent have a thin fibrous cap overlaying a lipid core with numerous macrophages, and are recognized as inflammatory plaques. In contrast, echogenic or non-inflammatory plaques contain more fibrous tissue, and less lipids and inflammatory cells [15]. Identifying subjects with echolucent plaques is clinically important, because the presence of these vulnerable plaques is associated with an increased risk of ischemic events, as compared to echogenic plaques [15–17]. Subjects with echolucent plaques have increased levels of circulating interleukin-6, suggesting a systemic inflammatory response [18]. The continuing influx of activated monocytes/macrophages contributes to echolucent plaque growth and instability, subsequently leading to ischemic events [19,20].

Transmigration of infected monocytes has been postulated as a vector for atherosclerosis progression [21,22]. In vitro and animal studies support that *C. pneumoniae* and CMV disseminate systemically, and exert direct atherogenic effects, accelerate macrophage foam cell formation and modulate plaque structure [22,23]. It is not known whether echolucent plaques are more often infected than echogenic plaques.

In this study, we selected subjects on the basis of carotid plaque echogenicity, a marker of plaque inflammation. To address whether inflammatory plaques are more often associated with persistent infections, we analysed CMV DNA and *C. pneumoniae* DNA in PBMCs in subjects with echolucent and echogenic carotid artery plaques, and in controls, by means of highly sensitive and specific PCR methodology.

## 2. Materials and methods

### 2.1. Study participants

The participants were recruited from the population-based Tromsø study in 2001, which included ultrasound examination of the right carotid artery [16], with morphological classifica-

tion of plaques in four categories, based on echogenicity [24]. Persons aged 56–80 years were eligible for the plaque group if they had one plaque or more in the carotid bifurcation or internal carotid artery at the screening visit, with a plaque thickness of ≥2.5 mm and plaque morphology classified as echolucent (grade 1) or echogenic (grade 4). Persons of the same age without carotid plaques served as controls. We excluded persons with inherited hypercholesterolemia, malignant hypertension, coagulation disorders, manifest chronic inflammation, renal failure, liver disease, thyroid and malignant disorders, or use of lipid-lowering drugs or anticoagulants. A medical history was obtained, and cardiovascular disease was defined as: prior or present angina pectoris, myocardial infarction, peripheral vascular disease, transient ischemic attacks, stroke, and amaurosis fugax. Eligible persons were invited to a second visit in which an ultrasound examination of both carotid arteries was performed. Fifty-eight persons with plaques in the right carotid artery and 47 without plaques at the screening examination were recruited to the study. All participants gave informed written consent and the Regional Board of Research Ethics recommended the study. The investigation conforms to the principles outlined in the Declaration of Helsinki.

### 2.2. Ultrasound examination

Details of the ultrasound measurements have been described previously [16,20]. One experienced examiner performed all ultrasound examinations of both carotid arteries. A plaque was defined as a localised protrusion of the vessel wall into the lumen, and was recorded from the near and far walls of the common carotid, the bifurcation, and the internal carotid arteries on both sides (12 locations). Computer-assisted analysis of grey scale content and calculation of grey scale median (GSM) was used to assess echogenicity. This is considered to be a more objective method with better reproducibility [15]. The grey level distribution and the GSM of each plaque were calculated, and the standardization of GSM was performed as described previously [20]. In persons with more than one plaque, the standardized GSM of the total plaque area was estimated as a weighted mean of the GSM value of each single plaque. In each person, the area of each plaque was divided by the individual's total area of plaques, and this fraction was multiplied with each plaque's standardized GSM value. All scores were added to calculate the total GSM score for each person.

The median of the GSM was used to define an echogenic group (GSM>65.0) and an echolucent group (GSM≤65.0). At the second ultrasound examination, no plaque could be detected in two of the 58 persons with plaques, and these were excluded from the study. Six persons with no plaques in the right carotid artery had plaques on the left side and were allocated to the plaque group. Eight persons were excluded because they used anticoagulants or lipid-lowering drugs. Thus, there were 29 persons in the echolucent group, 28 in the echogenic group and 38 persons in the control group.

### 2.3. Blood collection and measurements

Blood samples were drawn after an overnight fasting for standard biochemical analysis and PBMC studies at the time of recruitment. Serum was prepared by clotting whole blood in a glass tube at room temperature for 1 h and then centrifuged at 2000 g for 15 min at 22 °C. Aliquots of 1 ml were transferred into sterile cryovials (Greiner Laboratechnik, Nürtringen, Germany), flushed with nitrogen, and frozen at −70 °C until analysis. High sensitive CRP was measured by a particle-enhanced immunoturbidimetric assay from Roche Diagnostics (Mannheim, Germany).

### 2.4. Preparation of peripheral blood mononuclear cells

The isolation, freezing and thawing procedures have been described in detail [25]. Aliquots of 1 ml mononuclear cells ($4 \times 10^6$) were dispersed into sterile cryovials (Greiner Labortechnik, Frickenhausen, Germany) and finally stored at -135 °C in a Bio-freezer (Queue Cryostar, Queue Systems, Parkenberg, WV) until analysis.

### 2.5. Antibody detection

CMV IgG antibodies were analysed by microparticle enzyme immunoassay (MEIA) technique from Abbott Laboratories (Illinois, USA). C. pneumoniae IgA and IgG antibodies were analysed by an enzyme immunoassay (EIA) test from Labsystems OY (Helsinki, Finland). In our laboratory, the strength of agreement is good for this objective species specific EIA test compared with the microimmunofluorence (MIF) test from the same manufacturer [26]. For the IgA analyses, all sera were incubated

overnight at 4 °C with rheumatoid factor-absorbent (Serion Immundiagnostica, Wurzburg, Germany) to remove IgG.

### 2.6. DNA extraction

DNA was extracted from 200 μl PBMC suspension by means of GenoPrep DNA isolation kit from blood (Qiagen, Hilden, Germany) on a BioRobot M48 (Qiagen, Hilden, Germany) and eluted in 200 μl ddH$_2$O. Extracted DNA were positively verified by real-time PCR, targeting a partial sequence of the human chromosome 8 (Table 1) and quantified on a TBS-380 Fluorometer (Turner BioSystems, Sunnyvale, CA, USA).

### 2.7. Real-time PCR

Table 1 shows details concerning primer/probe sequences and concentrations, amplicon length and Gen-Bank accession numbers. CMV DNA was detected by a real-time PCR SYBR green assay targeting a 135 base pair (bp) sequence of the immediate early (IE) gene. A CMV dual probe-based real-time PCR assay was designed, targeting a 211 bp sequence of the TRL10 gene of human CMV [27]. A dual labelled probe with reporter dye 6-carboxy-fluorescein (FAM) at the 5′ end and Eclipse Dark Qencher at the 3′ end was used (Eurogentec, Seraing, Belgium). Real-time PCR on extracted DNA of 10 folded serial dilutions of cultured CMV strain AD-169 showed a PCR sensitivity of 0.2 plaque forming units/μl template. Positive CMV samples from immunocompromised patients also served as positive controls. C. pneumoniae DNA was detected by a real-time PCR SYBR green assay targeting a 51 bp sequence of the ompA gene. A C. pneumoniae dual

Table 1

Targets, primer and probe sequences used for PCR detection of cytomegalovirus (CMV), Chlamydia pneumoniae (C. pneumoniae) and human chromosome 8 in peripheral blood mononuclear cells among subjects with echolucent and echogenic carotid artery plaques

| Target | Primer/probe sequence (5′–3′) | Concentration (nM) | Amplicon length (bp)[a] | GenBank accession number |
|---|---|---|---|---|
| CMV IE[b] | F[c] GGAGATGTGGATGGCTTGTATTAA | 300 | 135 | AF099584 |
| CMV IE | R[d] TAGCACATATACATCATCTTTCTCCTAAGTT | 900 | | |
| CMV gp[e] TRL10 | F TGATAGTCCTGCTGGTGGTCTTC | 50 | 211 | X17403 |
| CMV gp TRL10 | R CCCGTCGGTTCGATCAAC | 50 | | |
| CMV gp TRL10 | P[f] CAACACGGGCACCGAGGTAGATCAA | 50 | | |
| C. pneumoniae ompA[g] | F AAAGTCTGCGACCATCAATTATCA | 300 | 51 | AF347608 |
| C. pneumoniae ompA | R GATAGAGAGGCTCCTACTTGCCATT | 300 | | |
| C. pneumoniae hypothetical protein | F GTATCGGAGTCTGACGCAGAGTT | 900 | 201 | NC002179 |
| C. pneumoniae hypothetical protein | R ATGTCAAAGCCCCCGTCTTC | 300 | | |
| C. pneumoniae hypothetical protein | P[f] AGAGGGCGAAGATCCCATGGAATGG | 250 | | |
| Human chromosome 8 | F TCTTACCCTCTCTGCCATTCACA | 300 | 77 | AC018796 |
| Human chromosome 8 | R AAGAGCTAGAAGGACACCGAAGATT | 300 | | |

[a] bp, Base pair.
[b] IE, Immediate early.
[c] F, Forward primer.
[d] R, Reverse primer.
[e] gp, Glycoprotein.
[f] P, Dual labelled probe with reporter dye FAM at the 5′ end and Eclipse dark Quencher at the 3′ end.
[g] ompA, Outer membrane protein A.

Table 2
Basic characteristics of the study participants with echolucent and echogenic carotid artery plaques and persons without carotid artery plaques

|  | Echolucent plaques ($n=29$) | Ecbogenic plaques ($n=28$) | Controls ($n=38$) |
|---|---|---|---|
| Female/male | 13/16 | 13/15 | 19/19 |
| Age, years [a] | 68.7 (66.2–71.2) | 70.2 (68.0–72.5) | 68.0 (66.0–70.0) |
| Current smokers, % | 18 | 29 | 24 |
| Body mass index, kg/m$^2$ | 25.5 (24.1–26.9) | 26.4 (24.8–28.0) | 27.4 (25.8–27.3) |
| Total cholesterol, mmol/l | 6.45 (5.91–7.00) | 6.43 (5.98–6.88) | 6.07 (5.69–6.46) |
| LDL cholesterol, mmol/l | 4.14 (3.76–4.51) | 4.17 (3.80–4.54) | 3.76 (3.46–4.06) |
| HDL cholesterol, mmol/l | 1.59 (1.36–1.82) | 1.74 (1.52–1.95) | 1.77 (1.63–1.91) |
| Triglycerides, mmol/l | 1.27 (0.99–1.56) | 1.08 (0.83–1.33) | 1.13 (0.96–1.30) |
| White blood cell count, ×10$^9$/l | 6.34 (5.64–7.03) | 6.03 (5.32–6.74) | 5.89 (5.29–6.49) |
| Neutrophils | 3.58 (2.99–4.17) | 3.31 (2.84–3.78) | 3.19 (2.80–3.78) |
| Monocytes | 0.55 (0.48–0.61) | 0.53 (0.46–0.59) | 0.50 (0.45–0.55) |
| Lymphocytes | 1.79 (1.62–1.97) | 1.94 (1.65–2.22) | 1.87 (1.62–2.13) |
| High sensitive CRP [b], g/l | 1.20 (0.27, 12.75) | 1.46 (0.15, 10.96) | 1.21 (0.34, 29.44) |
| Cardiovascular disease, % | 17 | 25 | 18 |
| Current use of acetylsalicylate, % | 10 | 25 | 13 |

[a] Normally distributed, continuous variables are presented as mean (95% CI).

[b] Skewed distribution, presented as median (minimum, maximum).

probe-based real-time PCR assay was designed, targeting a 201 bp sequence described by Read et al. [28]. By use of quantitative controls of *C. pneumoniae* DNA (strain CDC-CWL-011, Advanced Biotechnologies Incorporated, Maryland USA), the sensitivity of the real-time PCR assays was 1.3 copies/µl template. Cultured *C. pneumoniae* (strain TW-183, Washington Research Foundation, Seattle, USA) also served as positive controls. All real-time PCRs were performed in 96-well MicroAmp optical plates (Applied Biosystems, Foster City, CA, USA) with a total reaction volume of 25 µl consisting of 5 µl of template DNA, 12.5 µl SYBR green mastermix or 12.5 µl probe assay mastermix (both Eurogentec) and primers and probes. The thermal cycling conditions were 50 °C for 2 min for the activation of uracil-*N*-glycosylase (UNG), hot GoldStar Activation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting analyses were performed after 40 cycles when applying the SYBR green assay. PBMC samples were run in quintets (CMV) and quartets (*C. pneumoniae*) on an ABI 7900 HT sequence detection system (Applied Biosystems). Each run contained non-template controls, ddH$_2$O and spiked controls with CMV or *C. pneumoniae* strains or DNA, respectively. The new primers and probes were designed using Primer Express 2.0 (Applied Biosystems) and primers and probes were searched by Basic Local Allignment Sequence Tool (BLAST) (http://www.ncbi.nlm.nih.gov/ BLAST/) to check the specificity of the primers and probes for both CMV and *C. pneumoniae*. In addition, primers and probes were validated against a panel of common human bacterial and viral pathogens.

### 2.8. Statistical analysis

Statistical calculations were carried out using the computer programme SAS version 8 (SAS Institute, Cary, NC, USA). Between group differences were assessed by the

$\chi^2$ test and analysis of variance. A $p$-value <0.05 was considered significant.

## 3. Results

Table 2 shows basic characteristics of the study participants. Subjects with echolucent plaques had lower HDL cholesterol levels, whereas subjects with echogenic plaques were older and used medication with acetylsalicylate to a greater extent. White blood cell count and leukocyte fractions did not differ between groups. CMV DNA and *C. pneumoniae* DNA were not detected in a single PBMC sample from any participant regardless of plaque status (Table 3). PBMC samples were examined in five (CMV) and

Table 3
Presence (%) of cytomegalovirus- and *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells and serum antibodies in participants with echolucent and echogenic carotid artery plaques and in persons without carotid artery plaques

|  | Echolucent plaques ($n=29$) | Echogenic plaques ($n=28$) | Controls ($n=38$) | $p$-value [a] |
|---|---|---|---|---|
| Cytomegalovirus DNA | 0 (0) | 0 (0) | 0 (0) |  |
| *C. pneumoniae* DNA | 0 (0) | 0 (0) | 0 (0) |  |
| Cytomegalovirus IgG [b] | 25 (86.2) | 25 (89.3) | 30 (78.9) | 0.49 |
| *C. pneumoniae* IgA (cut-off≥ 16) [c] | 17 (58.6) | 16 (57.1) | 16 (42.1) | 0.32 |
| *C. pneumoniae* IgA (cut-off≥ 32) | 9 (31.0) | 7 (25.0) | 5 (13.2) | 0.20 |
| *C. pneumoniae* IgG (cut-off≥ 32) | 19 (65.5) | 22 (78.6) | 30 (78.9) | 0.39 |
| *C. pneumoniae* IgG (cut-off≥ 64) | 14 (48.3) | 15 (53.6) | 21 (55.3) | 0.85 |

[a] Between group differences were assessed by the $\chi^2$ test.

[b] Cytomegalovirus IgG antibodies were detected with microparticle enzyme immunoassay (MEIA) technique.

[c] *C. pneumoniae* IgA and IgG antibodies were detected with an enzyme immunoassay (EIA) test.

four repeats (*C. pneumoniae*), respectively, and the negative findings were confirmed with both PCRs for both micro-organisms. All groups had a high CMV and *C. pneumoniae* IgG seroprevalence, confirming previous exposure (Table 3). Presence of *C. pneumoniae* IgA antibodies was higher in the echolucent group for both cut-off levels (cut-off= 16 and 32), but differences between groups were nonsignificant (*p*-value 0.32 and 0.20, respectively).

## 4. Discussion

Our main finding was that DNA from *C. pneumoniae* and CMV were not detected in PBMCs in subjects with carotid atherosclerosis, regardless of echogenicity, nor in controls without carotid pathology. All groups were highly IgG seropositive, confirming previous exposure to both micro-organisms. To our knowledge, this is the first report investigating circulating nucleic acids of *C. pneumoniae* and CMV in relation to carotid plaque echogenicity, a marker of plaque inflammation.

Our results indicate that chronic CMV or *C. pneumoniae* infection is not a common phenomenon in persons with carotid atherosclerosis. Interestingly, there were no differences between subjects with inflammatory and non-inflammatory plaques. A possible explanation could be that carotid plaque echogenicity is not a companion to atherosclerotic plaque composition elsewhere in the vascular bed. However, studies indicate that plaques in the carotid arteries and in other vascular territories share morphological characteristics, suggesting plaque instability to be a widespread and systemic process [17,29]. Our observations are in line with data from randomised antichlamydial antibiotic interventions, which do not support the hypothesis that chronic *C. pneumoniae* infection is a major cause of cardiovascular disease [30].

All PBMC specimens were analysed by means of highly sensitive and specific probe-based real-time PCR technology. At present there is no validated serologic marker, nor any standardized PCR protocol, to determine a chronic infection in cardiovascular patients [4]. Our results are consistent with some, but not all previous findings. Tondella et al. reported 0.4% *C. pneumoniae* DNA prevalence in PBMCs in patients with ischemic stroke [31]. In a recent paper, *C. pneumoniae* DNA was absent in carotid atheroma and only four of 75 patients undergoing carotid endarterectomy had weak positive PBMC samples [6]. All four patients had a previous history of respiratory tract infection. Both research groups performed probe-based real-time PCR methodology.

Our results are in sharp contrast to Prager et al. [7], who reported 86 and 82%, and Sessa et al. [10], who reported 72 and 44% *C. pneumoniae* DNA prevalence in PBMCs and carotid atheroma in patients undergoing carotid endarterectomy. Another study reported 29% DNA positivity in PBMCs among patients with carotid artery stenosis [32], whereas Mitusch et al. [33] reported 13% DNA

positivity among patients with increased carotid intima-media thickening, in a cross-sectional population study. Except for one [32], all studies were based on nested PCR methodology.

CMV is linked to post-angioplasty restenosis and accelerated post-transplantation atherosclerosis [2] and there are few reports describing CMV DNA in PBMCs among cardiovascular patients. Borgia et al. [12] could not detect CMV DNA, whereas Smieja et al. [13] reported 17% CMV DNA positivity in PBMCs among patients undergoing coronary angiography.

Controversy exists whether PBMC DNA positivity predicts vascular infection [6,7,11]. The variation in reported prevalence may be related to differences in means of specimen collection and processing, nucleic acid extraction procedures, choice of primers and target sequences, type of polymerase-enzyme used, amplification product detection, number of repeats tested and the prevention and identification of false-positive and false-negative results [4,14,34]. The majority of published reports are based on nested PCR technology and gel-based product visualisation. The main drawback of the two-step nested PCR is the increased risk of carryover contamination, when amplicons from the first PCR reaction are reamplified using a second, inner primer pair. Vials potentially containing millions of DNA copies have to be opened to set up the second PCR reaction, as well as for product detection [4,34]. Incorporating UNG as a decontamination strategy cannot be used in nested assays, since the enzyme would destroy the template for the second PCR reaction [34]. Real-time PCR technology, however, is less prone to contamination due to the closed system, and there are no vial manipulations, sample transfers, reagent additions or gel separations once the PCR reaction is set up [6,31,34]. Further advantages are rapidity, automation and a non-nested format including template inactivation. Replacing ethidium bromide with SYBR green as a detection dye has improved sensitivity, and the introduction of sequence specific probes has improved specificity [31].

Lack of correlation between *C. pneumoniae* DNA detection and antibodies has also been noted [3]. Besides methodological issues, the discrepancy may reflect different populations being sampled during times of different *C. pneumoniae* activity, as *C. pneumoniae* respiratory tract infections occur epidemically during winter months [13]. In our study, samples were drawn from April to August 2003, and no *C. pneumoniae* outbreak was detected in the community or in our laboratory. Lack of association could also be related to disease state, the short time frame of PBMC infection or effective clearance of organisms due to protective antibodies and circulating phagocytes [35].

We developed *C. pneumoniae* and CMV PCR assays with probe-based real-time PCR technology to achieve the highest available diagnostic accuracy. According to inter-nationally recommended validation criteria [4], the PCR assays included extraction controls, positive and negative

controls, and amplification controls. Newly designed DNA sequences (primers and probes) were searched by the use of BLAST to check for specificity, and no hits were detected. In addition, primers and probes were tested against common human pathogens, and no PCR products were detected. Further, it is recommended to repeat testing of PBMC samples and to perform PCR inhibition control [4,34]. Each DNA extract from each PBMC sample was examined in five (CMV) and four (*C. pneumoniae*) repeats. PCR inhibition was ruled out by amplification of spiked PBMC samples with cultured *C. pneumoniae*, with purified *C. pneumoniae* DNA, with cultured CMV AD169, and with known CMV-positive samples from immunocompromised patients. No essential inhibition was detected in PBMC samples and inhibition does not seem to be a general problem in clinical specimens [3,14].

A weakness of our study is the relative small number of participants in each group and the low prevalence of manifest cardiovascular disease among the subjects. The negative findings of this study need to be confirmed in a larger cohort of individuals with sampling all through the year. There is increasing concern that previous publications may have been biased by false-positive PCR results due to the use of nested PCR technology [3,14,34]. Whether circulating nucleic acids of CMV and/or *C. pneumoniae* represent true cardiovascular risk factors or only reflect traces of a passing infection needs to be explored on a larger scale with standardized real-time PCR protocols.

## Acknowledgments

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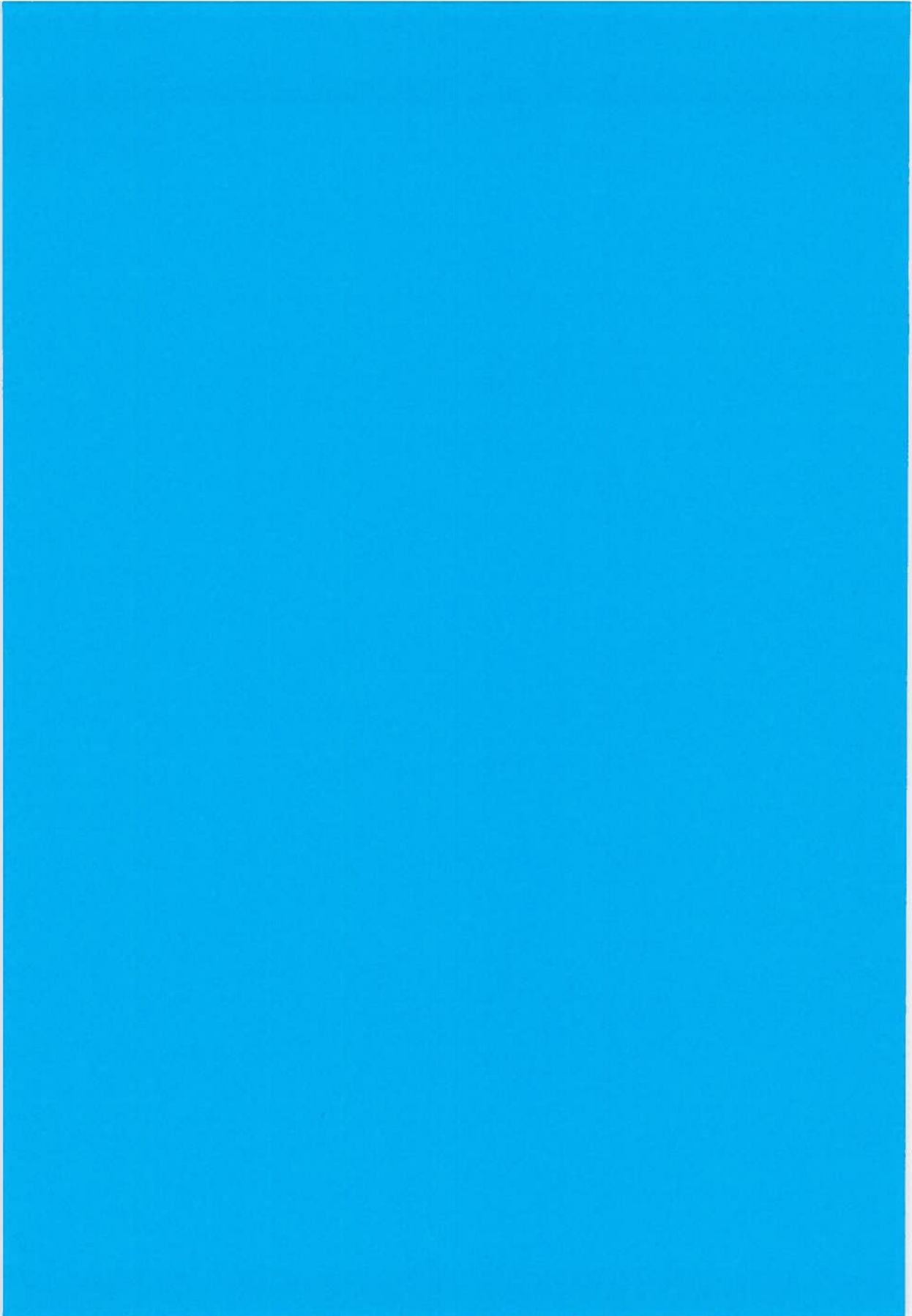
Paper 3

# The association between white blood cells, fibrinogen and C-reactive protein, and carotid atherosclerosis is sex dependent. The Tromsø Study.

Dag S. Halvorsen [a,*], Stein H. Johnsen [b], Ellisiv B. Mathiesen [b,d], Inger Njølstad [c]

Halvorsen et al. Inflammation and carotid atherosclerosis

[a] Department of Microbiology and [b] Department of Neurology, University Hospital of North Norway, N-9038 Tromsø, Norway, [c] Institute of Community Medicine and [d] Institute of Clinical Medicine, University of Tromsø, N-9037 Tromsø, Norway

*Corresponding author: Dag S. Halvorsen MD, Department of Microbiology, University Hospital of North Norway, N-9038 Tromsø, Norway. Telephone +47 (776) 26000
Email: dag.seeger.halvorsen@unn.no

**Conflict of interest** None

**Word count** 3832

**Abstract**

**Background** The presence of echolucent artery plaques is associated with increased risk of cardiovascular events as compared to echogenic plaques. Our aim was to investigate the relationship between inflammatory markers and carotid plaque morphology in a general population.

**Design** 5341 individuals were examined with ultrasonography of the right carotid artery in a cross-sectional health survey.

**Methods** Carotid plaque area ($mm^2$) and plaque morphology, expressed as computer assisted gray scale median (GSM), were determined in 3205 individuals with carotid plaque(s). Beside assessment of cardiovascular risk factors, white blood cell count (WBC), fibrinogen and C-reactive protein (CRP) were analysed. We used multiple linear and logistic regression models to determine the relationship between plaque area and morphology, and inflammatory markers.

**Results** Women and men with carotid plaque(s) had significantly elevated levels of WBC and fibrinogen, but not CRP, as compared to subjects without plaques. WBC was significantly associated with higher plaque echogenicity in women, whereas fibrinogen was associated with higher plaque echogenicity in age-adjusted models in men only.

**Conclusion** This cross-sectional study showed sex dependent differences in associations between measures of carotid atherosclerosis and inflammatory markers. CRP did not discriminate echolucent from echogenic carotid plaques in either gender. Our data highlights the importance of sex-specific analyses in future studies.

**Word count** 206

**Key words** inflammation, carotid atherosclerosis, plaque echolucency

**Introduction**

Besides traditional risk factors, inflammation plays a major role in the pathogenesis of atherosclerosis [1]. Cross-sectional associations between inflammatory markers and subclinical carotid atherosclerosis have been reported [2-6]. In prospective studies, increased levels of white blood cell count (WBC) [7], fibrinogen [8] and C-reactive protein (CRP) [9-12] predict first-ever myocardial infarction and ischemic stroke. However, the gender specific effects of inflammatory markers are not clear, as most studies present data unstratified by gender.

Ultrasound imaging allows assessment of carotid atherosclerosis and plaque composition. Plaques that appear echolucent have a thin fibrous cap overlaying a lipid core with numerous macrophages, and are recognized as inflammatory plaques [13]. The presence of echolucent plaques is associated with higher risk of coronary and cerebrovascular ischemic events than echogenic plaques [13-15]. Low levels of HDL cholesterol and increased levels of triglyceride rich proteins are associated with echolucent plaques [16,17].

Among the inflammatory markers studied, interleukin 6 (IL-6) [18], in contrast to fibrinogen and CRP [19], has been reported to be associated with carotid plaque echolucency. Due to the paucity of published reports, we studied the association between WBC, fibrinogen and CRP, and ultrasound assessed carotid plaque area and echogenicity in 5341 subjects in the Tromsø study.

**Materials and methods**

**Study participants**

The Tromsø Study is a single-centre, population-based prospective study with repeated health surveys of inhabitants in Tromsø, Norway. A total of 8130 attended the survey in 2001, and ultrasound examination of the right carotid artery was performed in 5454 persons. A total of 5341 persons (2982 women and 2359 men) were enrolled in this study, as 83 subjects did not consent to medical research, and 30 subjects were excluded due to technical and data management difficulties. Finally, 3205 (1613 women and 1592 men) subjects had carotid plaque(s), and their data are included in the regression models. The Norwegian Data Inspectorate licensed all data. The Regional Board of Research Ethics recommended the study. Written informed consent was obtained from all participants.

**Cardiovascular risk factors**

A questionnaire on previous myocardial infarction and stroke, present angina pectoris and diabetes mellitus (yes/no), use of blood-pressure and lipid-lowering drugs (yes/no) and current smoking (yes/no) was enclosed in the letter of invitation. Coronary heart disease (CHD) was defined as prevalent angina pectoris or previous myocardial infarction. Cardiovascular disease (CVD) was defined as CHD and/or previous stroke.

We measured height, weight, blood pressure, non-fasting lipids, WBC, fibrinogen and CRP. Fibrinogen was assayed with a clothing method in a STA-compact analyzer (Diagnostica Stago, Asnieres, France); the detection limit was 0.6 g/l and the coefficient of variation (CV) was 4.0 %. CRP was measured by a particle-enhanced immunoturbidimetric assay from Roche Diagnostics (Mannheim, Germany); with detection limit of 0.12 mg/l and the CV was 1.4 %. Blood samples were analysed at the Department of Clinical Chemistry, University Hospital of North Norway.

**Ultrasound examination and assessment of plaque morphology**

The measures of carotid atherosclerosis used in this study were carotid plaque area ($mm^2$) and carotid plaque morphology, expressed as computer assisted gray scale median (GSM). High-resolution ultrasonography (Acuson Xp10 128, ART upgraded, with a 7.5-MHz linear-array transducer, aperture size 38 mm) of the right carotid artery was done as previously described [20]. The sonographers were blinded to the laboratory data and questionnaires. We defined a plaque as a localised protrusion of the vessel wall into the lumen of at least 50% compared to the adjacent intima-media thickness (IMT). In each subject, a maximum of 6 plaques were registered in the near and far walls of common carotid, bifurcation, and internal carotid, respectively. For each plaque a still image was recorded with the transducer parallel to the vessel wall and vertical to the point of maximum plaque thickness, with the regional expansion selection set to 38mm x 20mm. All recordings were done on a Panasonic 7650 video player with Super VHS tape. Still images were digitalized off-line using a PC with the Matrox Meteor II frame grabber card and Matrox Intellicam v2.07 software, at a resolution of 768 x 576 pixels [21]. The histogram function in Adobe Photoshop was used to generate GSM values of each plaque, a measure of plaque echogenicity. The plaque GSM was standardized using the GSM of the lumen and media-adventitia interface as reference structures [21]. In subjects with more than one plaque, the areas of all plaques were summarized to give the total plaque area. The GSM of the total plaque area was estimated as a weighted mean of the GSM value of each single plaque.

**Statistical analysis**

We used the SAS software package, v8.2 (SAS Institute, Cary, NC, USA) for data management and data analysis. A p-value < 0.05 was considered significant. We used student's t-test, $\chi^2$ test and analysis of covariance (ANCOVA) to test differences between

groups. CRP was log transformed to correct for skewed distributions. We performed sex stratified analyses due to interactions between sex and log CRP (p=0.003) and sex and WBC (p=0.04) when modelling plaque area and plaque morphology, respectively.

When subjects without carotid plaque(s) (total plaque area = 0 mm$^2$) were included in the analyses, the distribution of plaque area was skewed and did not satisfy the model criteria for linear or for ordinal logistic regression analyses. Therefore, in a separate analysis we compared the levels of inflammatory markers in subjects with and without plaques. Within the plaque group, the relationship between inflammatory markers and total plaquea area and plaque echogenicity were tested in regression analyses.

We used multiple regression analysis to test the independent association between plaque area and inflammatory markers. Plaque area was used as dependent variable, with inflammatory markers as continuous independent variables. We controlled for age, current smoking, total cholesterol and HDL cholesterol, systolic blood pressure, diabetes mellitus, cardiovascular disease and treatment with lipid lowering drugs, known to be associated with atherosclerosis [12]. Linear trend for plaque area and plaque morphology (GSM) across quartiles of the inflammatory markers were tested by linear regression.

The independent relations between plaque morphology (GSM) and inflammatory markers were tested in multiple logistic regression analysis (cumulative ordinal logit model), where quartiles of GSM were used as dependent variable. The model calculates the OR for being in a higher quartile of the dependent variable. The score test for the proportional odds assumption was > 0.05 in all logistic regression models for both genders. In additional logistic regression analyses, GSM was dichotomized, where GSM quartile 1 was defined as echolucent plaque and quartiles 2 to 4 as echogenic plaque, and we controlled for all covariates mentioned above.

**Results**

Table 1 shows basic characteristics of the study participants. Age, proportion of current smoking, body mass index, systolic blood pressure and fibrinogen levels were similar for both genders. Men had higher levels of WBC, CRP, triglycerides, diastolic blood pressure, lower levels of total cholesterol and HDL cholesterol, more prevalent CHD and used more lipid lowering drugs than did women (Table 1). Men had more carotid plaques (p < 0.0001), larger plaque area (p < 0.0001) and lower plaque echogenicity than women (p < 0.0001) (Table 2). Women and men with carotid plaque(s) had elevated levels of WBC (both <0.0001) and fibrinogen (p=0.04 and p=0.003) as compared to individuals without carotid plaques (Table 3), whereas CRP showed borderline between group difference among men only (p=0.05).

In men with carotid plaques, there was a linear relation between WBC (p < 0.0001), fibrinogen (p =0.0004) and CRP (p < 0.0001) and carotid plaque area (Figure 1). In women with plaques, significant linear trends were present for WBC (p=0.0002) and fibrinogen (p=0.009) only (Figure 1). In multiple regression models, WBC, fibrinogen and CRP were associated with carotid plaque area after adjustment for other covariates among men, whereas this was not the case in women (Table 4). Excluding individuals on lipid lowering drugs did not alter the associations. In women and men, systolic blood pressure, smoking, diabetes, CVD and lipid-lowering medication were independent risk factors of carotid plaque area (data not shown). Total cholesterol and HDL-cholesterol were independent risk factors in women only (data not shown).

There was a linear trend for higher plaque echogenicity across WBC quartiles in women (p=0.004) and across fibrinogen quartiles in men (p=0.05)(Figure 2). In logistic regression analyses, WBC in women (p=0.003) and fibrinogen in men (p=0.02) were associated with higher plaque echogenicity in age-adjusted models (Table 5). Among women, WBC was associated with plaque echogenicity after adjustment for other covariates.

However, this was not the case when excluding individuals on medication with lipid lowering drugs (OR=1.08, 95% CI; 0.96-1.21, p=0.21). Current smoking was associated with higher plaque echogenicity in all models among both genders (data not shown). Repeated analyses in logistic regression models of plaque morphology as a dichotomized variable showed similar ORs by the inflammatory markers (data not shown).

**Discussion**

This study showed sex dependent differences in associations between inflammatory markers and carotid plaque area, and plaque morphology. WBC, fibrinogen and CRP were associated with total carotid plaque area among men in models adjusted for traditional CVD risk factors. In women, WBC and fibrinogen demonstrated significant associations in age adjusted models, but the associations were attenuated when other covariates were introduced in the models. WBC was predictive of higher carotid plaque echogenicity in women only, and CRP did not discriminate echolucent from echogenic carotid plaques in neither women nor men.

Inflammatory processes are involved in the pathogenesis of atherosclerotic plaque development and progression [1]. The continuing influx of activated monocytes/macrophages contributes to echolucent plaque growth and instability, subsequently leading to ischemic events [20,22]. On the contrary, HDL cholesterol and statin therapy seem to reverse the inflammatory process and to stabilize plaques [23,24]. Therefore, it is important to study risk factors predictive of plaque morphology.

To our knowledge, this is the largest population-based study of ultrasound assessed carotid plaque echogenicity and inflammatory markers. Yamagami et al. [18] reported increased levels of IL-6 associated with lower plaque echogenecity among selected patients with carotid atherosclerosis. IL-6 is the main inducer of hepatic CRP secretion, but CRP was not associated with plaque morphology in the Japanese study. IL-6 and CRP have different

sources of origin and do not always move in parallel [25,26]. Unfortunately, IL-6 was not measured in our study. Kofoed et al. [19] reported that neither fibrinogen nor CRP differed between patients with echolucent and echogenic carotid plaques, but both markers were significantly increased among the 318 patients compared to population based controls free of ischemic cerebrovascular or coronary heart disease in the Copenhagen City Heart Study. Our results extend their observations, and support a relationship between carotid plaque burden and CRP in men, but not between carotid plaque morphology and CRP. Carotid plaque area reflects the extent of atherosclerosis. Whether plaque echogenicity in the carotids is representative of plaque composition elsewhere in the vascular bed is unclear, but recent reports indicate that plaques at different locations share morphological characteristics, suggesting plaque instability to be a widespread process [27].

It is not known whether inflammatory markers are true atherosclerosis risk factors or merely indicators of an ongoing inflammatory activity within atherosclerotic lesions [1,12]. In most studies, the effects of inflammatory markers are attenuated after controlling for traditional CVD risk factors [28-30], and few studies report sex stratified associations. A significant association between CRP and advanced carotid atherosclerosis has been reported in male dyslipidemic patients [31]. In population based studies, an association between carotid IMT measurement and CRP was reported in women [6], whereas carotid plaque formation was strongly associated with CRP in men in another study [4]. The disagreements may be explained by study design, study populations, methodological issues and carotid measurement site, as IMT thickening and plaque formation may progress at different rates at different carotid sites.

Women develop atherosclerotic disease later in life than men, and the difference has been attributed to the specific effects of sex steroid hormones on lipid metabolism, the vasculature, the coagulation and fibrinolytic system, and the inflammatory cascade

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[26,32,33]. In addition, race, age, cigarette smoking, adiposity and drug interactions affect the levels of circulating inflammatory markers [25,34-37]. WBC, fibrinogen and CRP seem to increase modestly with age, but there is less evidence for a continuing CRP increase in women or above the age of seventy for both genders [36]. American Heart Association and Centers for Disease Control recommend that repeated measurements of CRP could add value in predicting coronary events [11]. However, the predictive ability of CRP seems to be less useful in old-age than in middle-age, the evidence is mainly based on studies on young to middle aged populations, and there are no sex specific recommendations [36]. Although our study has no clinical endpoints, the results indicate sex dependent associations between CRP and measures of carotid atherosclerosis.

Our study has strengths; we examined an unselected cohort of middle to old age individuals within a large sample size (n=5341 subjects). Total carotid plaque area seems to be a better marker for carotid atherosclerosis than separate or localised measurements. Furthermore, computerized GSM analysis, weighted for plaque area, appears to be a more objective and quantitative method of assessing carotid plaque echogenicity than visual characterization [21,38].

Study limitations include the cross-sectional design, measurement of only one carotid artery and single measurements of inflammatory markers. Severely ill and disabled individuals at increased CVD risk are probably underrepresented in the study. Although several steps were taken to reduce measurement error, all ultrasound measurements are operator dependent and prone to measurement variability. However, adjustment for plaque area did not change the ORs in logistic regression models addressing plaque morphology. Both healthy participant bias and imprecise ultrasound measurements may weaken the true associations between carotid atherosclerosis and inflammatory markers.

We conclude that CRP, WBC and fibrinogen were independently related to carotid plaque burden in men, but not in women. WBC was significantly associated with higher carotid plaque echogenicity in women, even after adjustment for traditional CVD factors, whereas fibrinogen was significantly associated in age-adjusted models among men only. CRP did not discriminate echolucent from echogenic carotid plaques in either gender. Our data extends the results from previous reports, and highlights the importance of sex-specific analyses in future studies investigating inflammatory markers and carotid atherosclerosis.

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**Legends to figures**

Figure 1

Total carotid plaque area (mm$^2$) in quartiles of white blood cell (WBC) count, fibrinogen, and C-reactive protein (CRP) in women (n=1613) and men (1592). Values are age-adjusted. The Tromsø Study 2001.

Figure 2

Mean carotid plaque GSM (Gray Scale Median) in quartiles of white blood cell (WBC) count, fibrinogen, and C-reactive protein (CRP) in women (n=1613) and men (n=1592). Values are age-adjusted. The Tromsø Study 2001.

**Table 1 Characteristics of the 5341 participants. The Tromsø Study 2001.**

| | Women (n=2982) | Men (n=2359) | |
| --- | --- | --- | --- |
| | Mean (SD) | Mean (SD) | p-value* |
| Age (years) | 65.6 (9.7) | 66.0 (9.3) | 0.18 |
| Current smoking, % | 24.3 | 23.8 | 0.64 |
| Body mass index (kg/m²) | 27.0 (4.7) | 26.9 (3.5) | 0.53 |
| Systolic blood pressure (mmHg) | 143.4 (21.2) | 144.1 (19.4) | 0.22 |
| Diastolic blood pressure (mmHg) | 81.8 (12.5) | 83.5 (11.9) | < 0.0001 |
| Serum lipids (mmol/l) | | | |
| Total cholesterol | 6.50 (1.18) | 6.06 (1.13) | < 0.0001 |
| HDL cholesterol | 1.59 (0.40) | 1.35 (0.37) | < 0.0001 |
| Triglycerides | 1.50 (0.81) | 1.63 (0.98) | < 0.0001 |
| Inflammatory markers | | | |
| White blood cell count (x $10^9$/l) | 6.31 (1.78) | 6.42 (1.90) | 0.03 |
| Fibrinogen (g/l) | 3.21 (0.79) | 3.22 (0.83) | 0.91 |
| C-Reactive protein (mg/l) | 1.55 (0.17, 97.25)† | 1.63 (0.12, 220.51)† | 0.02 |
| Comorbidities, % | | | |
| Diabetes mellitus | 4.4 | 5.0 | 0.33 |
| Coronary heart disease | 10.3 | 20.2 | < 0.0001 |
| Previous stroke | 3.6 | 5.3 | 0.002 |
| Cardiovascular disease | 12.6 | 23.5 | < 0.0001 |
| Current medication, % | | | |
| Antihypertensive drugs | 24.4 | 25.5 | 0.34 |
| Lipid lowering drugs | 12.1 | 17.6 | < 0.0001 |

* P-values for differences between groups by t-test and $\chi^2$.
† Median (minimum, maximum) due to skewed distribution.

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**Table 2 Plaque characteristics of study participants (n=5341). The Tromsø Study 2001.**

| | Women (n=2982) | Men (n=2359) | p-value[*] |
|---|---|---|---|
| | n (%) | n (%) | |
| Distribution of plaques | | | |
| 0 | 1369 (45.9) | 767 (32.5) | |
| 1 | 805 (27.0) | 688 (29.2) | |
| 2 | 514 (17.2) | 508 (21.5) | |
| 3-6 | 294 (9.9) | 396 (16.8) | |
| Mean number of plaques | 1.72 (1.68-1.77) | 1.94 (1.89-1.99) | < 0.0001 |
| Mean plaque area, mm$^2$ | 20.20 (19.29-21.12) | 27.93 (27.01-28.85) | < 0.0001 |
| Mean GSM[†] value | 53.51 (52.41-54.61) | 50.02 (48.92-51.13) | < 0.0001 |

[*] Age adjusted means (95% CI), among participants with ≥ 1 plaque(s)(n=1613 women and n=1592 men).

[†] Computerized Gray Scale Median value of plaque morphology (weighted for total plaque area). Values range from 4 to 200.

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**Table 3 Age adjusted inflammatory markers in individuals with and without carotid plaques. The Tromsø Study 2001.**

| | With carotid plaque(s) | No carotid plaques | p-value |
|---|---|---|---|
| **Women** | n=1613 | n=1369 | |
| White blood cell count (x10⁹/l) | 6.46 (6.36-6.56) | 6.12 (6.02-6.23) | < 0.0001 |
| Fibrinogen (g/l) | 3.25 (3.20-3.29) | 3.18 (3.13-3.22) | 0.04 |
| C-reactive protein (mg/l)* | 1.75 (1.66-1.84) | 1.62 (1.53-1.72) | 0.08 |
| **Men** | n=1592 | n=767 | |
| White blood cell count (x10⁹/l) | 6.56 (6.46-6.66) | 6.15 (6.01-6.30) | < 0.0001 |
| Fibrinogen (g/l) | 3.26 (3.21-3.30) | 3.14 (3.08-3.20) | 0.003 |
| C-reactive protein (mg/l)* | 1.86 (1.76-1.96) | 1.69 (1.57-1.82) | 0.05 |

Continuous variables presented as mean (95% CI) or antilog (95% CI)* due to skewed distributions

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**Table 4 Regression coefficients for carotid plaque area (mm$^2$) in multiple linear regression analyses with inflammatory markers as independent variables. The Tromsø Study 2001.**

| | Model I | | Model II | |
| --- | --- | --- | --- | --- |
| | β* (SE) | p | β* (SE) | p |
| **Women (n=1613)** | | | | |
| White blood cell count | 1.43 (0.41) | 0.0006 | 0.26 (0.45) | 0.57 |
| Fibrinogen | 1.28 (0.40) | 0.001 | 0.72 (0.41) | 0.08 |
| Log C-reactive protein | 0.61 (0.91) | 0.50 | -0.68 (0.94) | 0.47 |
| **Men (n=1592)** | | | | |
| White blood cell count | 2.63 (0.49) | <0.0001 | 1.63 (0.57) | 0.004 |
| Fibrinogen | 1.82 (0.55) | 0.0009 | 1.25 (0.57) | 0.03 |
| Log C-reactive protein | 5.99 (1.18) | <0.0001 | 4.47 (1.24) | 0.0003 |

*Values are regression coefficients (SE) expressed in mm$^2$ for 1SD change in continuous variables and presence vs absence of categorical variables
Model I, adjusted for age
Model II, adjusted for age, current smoking, total and HDL cholesterol, systolic blood pressure, diabetes mellitus, cardiovascular disease and treatment with lipid lowering drugs

**Table 5**
**Cumulative ordinal logistic regression model for plaque echogenicity by inflammatory markers. The Tromsø Study 2001.**

| | Model I | | | Model II | | |
|---|---|---|---|---|---|---|
| | OR* | 95%CI | p | OR | 95%CI | p |
| **Women (n=1613)** | | | | | | |
| White blood cell count | 1.15 | 1.05-1.26 | 0.003 | 1.12 | 1.01-1.25 | 0.03 |
| Fibrinogen | 1.05 | 0.96-1.14 | 0.31 | 1.02 | 0.93-1.13 | 0.63 |
| Log C-reactive protein | 1.04 | 0.85-1.27 | 0.71 | 1.05 | 0.84-1.30 | 0.68 |
| **Men (n=1592)** | | | | | | |
| White blood cell count | 1.01 | 0.93-1.11 | 0.76 | 0.99 | 0.90-1.09 | 0.79 |
| Fibrinogen | 1.11 | 1.02-1.22 | 0.02 | 1.10 | 0.99-1.21 | 0.07 |
| Log C-reactive protein | 1.16 | 0.95-1.42 | 0.14 | 1.07 | 0.87-1.33 | 0.52 |

*Adjusted odds ratio predicts the probability of being in a higher category of GSM-assessed plaque morphology for 1 SD change in continuous variables and presence vs absence of categorical variables
Model I was adjusted for age
Model II was adjusted for age, current smoking, total and HDL cholesterol, systolic blood pressure, diabetes mellitus, cardiovascular disease and treatment with lipid lowering drugs

**Women**                                         **Men**

WBC

Fibrinogen

CRP



Mean plaque area (mm²) by quartile (Q1–Q4) of WBC, Fibrinogen, and CRP for Women and Men.

p for linear trend values:
- WBC, Women: p for linear trend = 0.0002
- WBC, Men: p for linear trend = < 0.0001
- Fibrinogen, Women: p for linear trend = 0.009
- Fibrinogen, Men: p for linear trend = 0.0004
- CRP, Women: p for linear trend = 0.48
- CRP, Men: p for linear trend = < 0.0001

**Women** | **Men**

WBC — mean plaque GSM — Q1, Q2, Q3, Q4 — p for linear trend = 0.004 (Women); p for linear trend = 0.71 (Men)

Fibrinogen — mean plaque GSM — Q1, Q2, Q3, Q4 — p for linear trend = 0.47 (Women); p for linear trend = 0.05 (Men)

CRP — mean plaque GSM — Q1, Q2, Q3, Q4 — p for linear trend = 0.52 (Women); p for linear trend = 0.36 (Men)

**Paper 4**

**Persistent inflammatory response does not predict mortality in long-term ischemic stroke survivors**

Dag S. Halvorsen MD, MPH [a],*, Torgeir Engstad MD, PhD [b], Jan Brox MD, PhD [c], Bjarne Østerud PhD[d], Inger Njølstad MD, PhD[e]

[a] Department of Microbiology, [b] Department of Geriatric Medicine and [c] Department of Clinical Chemistry, University Hospital of North Norway, N-9038 Tromsø, Norway, [d] Institute of Medical Biology and [e] Institute of Community Medicine, University of Tromsø, N-9037 Tromsø, Norway

*Corresponding author: Dag S. Halvorsen, Department of Microbiology, University Hospital of North Norway, N-9038 Tromsø, Norway
Email: dag.seeger.halvorsen@unn.no
Telephone +47 (776) 26000, Facsimile +47 (776) 27015

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## Abstract

**Background and purpose** - Inflammatory markers are independent risk predictors for cardiovascular events and death in population-based studies, but uncertainty remains of their predictive role in secondary prevention, particularly among long-term stroke survivors.

**Methods** - In 1997, 187 ischemic stroke survivors (median 7.0 years after index stroke) and 243 stroke-free subjects were recruited from a population-based study. Beside clinical examination and assessment of common risk factors, serum inflammatory markers and *C. pneumoniae* antibodies were analysed. In Cox proportional hazards regression models we examined the relationship between inflammatory markers and risk of death during an 8 year follow-up.

**Results** - Stroke survivors had significantly elevated levels of white blood cell counts, fibrinogen, interleukin-6 (IL-6) and C-reactive protein (CRP) at baseline, as compared to stroke-free subjects, adjusted for age, sex, current smoking, comorbidities, and medication. They also had higher risk of cardiovascular mortality (HR=3.59; 95% CI, 1.91-6.78) and all cause mortality (HR=2.16; 95% CI, 1.47-3.17), which was not associated with increased levels of inflammatory markers. In contrast, fibrinogen, IL-6 and CRP were associated with all cause mortality in stroke-free subjects, adjusted for age and sex. Presence of *C. pneumoniae* antibodies did not predict mortality in either group.

**Conclusions** - Among long-term ischemic stroke-survivors, elevated levels of inflammatory markers were not associated with mortality. In contrast, fibrinogen, IL-6 and CRP were independent predictors of mortality in stroke-free subjects, which could indicate a modified risk of inflammatory markers in long-term stroke survivors. To establish the inflammatory markers' relevance in secondary prevention strategies, further studies are required.

**Key words**: inflammation, long-term ischemic stroke survivors, mortality

**Introduction**

Inflammation plays a major role in the pathogenesis of atherosclerosis.[1] Whether microorganisms, such as *Chlamydia pneumoniae* (*C. pneumoniae*), are involved in this process remains unclear.[2,3] The host inflammatory response occurs not only in the affected vessel wall, but also systemically, as indicated by elevated levels of acute phase reactants.[4-6] In prospective studies, inflammatory markers predict first-ever myocardial infarction and ischemic stroke.[7-9] In addition, fibrinogen, interleukin-6 (IL-6) and CRP are associated with mortality among healthy middle-aged and elderly individuals.[8,10-15]

The levels of circulating inflammatory markers correlate with acute brain infarction volume and stroke severity.[10,16,17] Stroke patients with an increased CRP level on admission or at discharge are associated with a worse outcome.[10,17-19] Stroke-survivors have a continuing high risk of death from cardiovascular diseases,[20,21] and secondary prevention strategies include control of hypertension, hyperglycaemia and atherogenic lipoproteins, and cessation of smoking.[22-25]

Few reports focus on the inflammatory response and the predictive ability of inflammatory markers in long-term stroke survivors.[10,26] Most studies recruited patients shortly after the index-stroke, when the risk of recurrence and mortality is higher, and the follow-up period is usually short. To test the hypothesis whether inflammatory markers or presence of *C. pneumoniae* antibodies predict mortality, we followed 187 long-term ischemic stroke survivors and 243 stroke-free subjects for 8 years.

**Materials and methods**

**Study participants**

Two hundred and sixty-nine individuals with a self-reported history of stroke and 262 individuals without a history of stroke or transient ischemic attack (TIA) were recruited from the Tromsø Study (1994), and re-examined in 1997, which is defined as baseline in the present study. Details on stroke criteria, the selection process, the response and attendance rate, and the validity of a self-reported stroke have been published previously.[27] In 1997, a previous stroke was verified in 221 individuals, and our final study population comprised 187 individuals with a previous ischemic stroke, as we excluded 20 subjects with a subarachnoid haemorrhage and 14 subjects with an intracerebral haemorrhage.[28] The median elapsed time from ischemic stroke onset to the re-examination in 1997 was 7.0 (range 0-43) years. The final control group consisted of 243 population-based individuals without a history of stroke or TIA.[28] The number of stroke-free subjects was larger, as they were drawn on basis of a tentative larger and more heterogenic stroke-cohort than the final study sample of long-term ischemic stroke survivors. All participants gave informed written consent at study entry, and the Regional Committee of Research Ethics recommended the study. We followed all participants for 8 years, from August 1997 until December 1, 2005. The clinical end point was all cause mortality, and we collected data from hospital medical records and death certificates for both groups.

For those deceased, a copy of the death certificate was retrieved from the Community Health Services of Tromsø or from the hospital medical records. The causes of death were validated and classified according to the International Classification of Diseases, 10th revision by 2 physicians (DSH, TE), based on all available information from the medical records, including the death certificates. Causes of death were grouped into four categories; 1) death due to fatal myocardial infarction or stroke, 2) malignancy, 3) other (infectious diseases,

trauma, Alzheimer disease, and renal or respiratory end stage disease) or 4) sudden death of unknown cause outside hospital lacking conclusive information on the death certificates.

**Risk factors**

Data on all participants were recorded at baseline in 1997. We measured height, weight and blood pressure. Details on previous myocardial infarction and stroke, present angina pectoris and diabetes mellitus (yes/no), use of blood-pressure and lipid-lowering drugs (yes/no) and current smoking (yes/no) were recorded. Coronary heart disease (CHD) was defined as prevalent angina pectoris or previous myocardial infarction. Outdoor physical, social and domestic indoor activities were assessed with the Frenchay Activity Index.[28]

**Blood collection and measurements**

All blood samples were drawn at baseline in 1997 and stored at −70 °C until analysis. Fibrinogen was assayed in 1998 with a clothing method in a STA-compact analyzer (Diagnostica Stago, Asnieres, France); the detection limit was 0.6 g/l and the coefficient of variation (CV) was 4.0 %. High sensitive CRP, IL-6 and *C. pneumoniae* IgA and IgG antibodies were analyzed in 2001. CRP was measured by a particle-enhanced immuno-turbidimetric assay from Roche Diagnostics (Mannheim, Germany). The detection limit was 0.175 mg/l and the CV was 4.0 %. IL-6 was analyzed with an ELISA (Quantikine Immunoassay) with detection limit 0.70µg/l and CV 4.2 %, from R&D Systems (Abingdon, UK). *C. pneumoniae* IgA- and IgG antibodies were analysed by an enzyme immunoassay (EIA) test from Labsystems OY (Helsinki, Finland).[29] For the IgA-analyses, all sera were incubated overnight at 4 °C with rheumatoid factor-absorbent (Serion Immundiagnostica, Wurzburg, Germany) to remove IgG.[29]

**Statistical analysis**

The SAS software package, v8.2 (SAS Institute, Cary, NC, USA) was used for both data management and data analysis. A p-value <0.05 was considered significant. CRP and IL-6 was log transformed to correct for skewed distributions. We used student's t-test, $\chi^2$ test and analysis of covariance (ANCOVA) to test differences between groups of subjects with and without ischemic stroke, controlling for age, sex, smoking (yes/no), comorbidities (diabetes and/or previous myocardial infarction) and medication with statins or acetylsalicylate.

In Cox proportional hazards model we examined the effect of inflammatory markers or presence of *C. pneumoniae* antibodies on mortality for stroke subjects and stroke free subjects separately. The effect of stroke on mortality was examined in a common model with stroke status (yes/no) as an explanatory variable. The inflammatory markers were analysed both as continuous variables and grouped into tertiles. *C. pneumoniae* antibodies were analysed as a dichotomous variable based on the commonly used cut-off values for IgA- ($\geq$ 16) and IgG ($\geq$ 32) antibodies.[29] Due to a high correlation between CRP and fibrinogen (Pearsons, r=0.66), these variables were not jointly included in the same regression models.

**Results**

Stroke survivors had a higher HBA1c level, suffered more comorbidities, used more medication, and had a lower Frenchay activity index score than did stroke-free subjects (Table 1). At baseline, long-term stroke survivors had significantly higher levels of fibrinogen, IL-6 and CRP as compared to stroke free subjects when adjusted for age and sex (Table 2). The estimates of all inflammatory markers were marginally attenuated when controlling for current smoking, comorbidities and medication with statins and acetylsalicylate (data not shown). Both groups had high *C. pneumoniae* IgG (cut-off $\geq$ 32) seroprevalence (81.6 % vs 77.4 %; p=0.28), confirming previous exposure. Presence of *C. pneumoniae* IgA (cut-off $\geq$

16) antibodies was significantly more prevalent (63.8 % vs 51.9 %; p=0.01) in stroke subjects compared to stroke free subjects.

In the stroke free group, the deceased subjects had significantly higher levels of all inflammatory markers compared to the survivors at baseline. This was not the case within the stroke group (Table 3). The inflammatory markers were similar in stroke subjects having experienced the index stroke for $\geq$ 7 years ago (n=98) as compared to those having experienced it less than 7 years ago (n=89)(data not shown).

Sixty-nine of the 187 ischemic stroke survivors (36.9 %) and 42 of the 243 stroke-free subjects (17.3 %) died during follow-up, and the mortality rates per 1000 person-years were 56.1 and 23.6, respectively (p<0.001). Thirty-seven stroke subjects and 13 stroke free subjects experienced fatal myocardial infarction or stroke, whereas eight stroke subjects and 14 stroke free subjects died from malignancy. Eighteen stroke subjects and 12 stroke free subjects died from other causes, mainly infectious diseases, and six stroke subjects and three stroke free subjects died from an unknown cause outside hospital. A history of ischemic stroke increased the risk of cardiovascular death (hazard ratio [HR]= 3.59; 95% CI, 1.91-6.78; p<0.0001), and all cause mortality (HR=2.16; 95% CI, 1.47-3.17; p<0.0001), when adjusted for age and sex.

Among stroke survivors, none of the inflammatory markers predicted death during the 8-year follow up (Table 4). By contrast, in stroke free subjects there was a highly significant association between CRP, IL-6 and fibrinogen, and death, adjusted for age and sex in Cox proportional hazards models (Table 4). Presence of *C. pneumoniae* IgA- and IgG antibodies were not associated with mortality in either group (data not shown). When comparing the last to the first tertile of IL-6 and CRP, significant associations were observed in the stroke free group (HR= 2.58; 95% CI, 1.07-6.20; p=0.03 and HR= 2.43; 95% CI, 1.14-5.16; p=0.02), but this was not the case for ischemic stroke survivors. A 5-point increased Frenchay Activity

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Index score was associated with reduced risk of death in both groups (HR= 0.77; 95% CI, 0.69-0.87; p<0.0001 and HR=0.69; 95% CI, 0.57-0.82; p<0.0001).

**Discussion**

The main finding from this study was that none of the inflammatory markers studied were independent predictors of death in long-term ischemic stroke survivors, even though the multivariable adjusted levels of WBC, fibrinogen, IL-6 and CRP were significantly higher at baseline as compared to a stroke-free group. In contrast, fibrinogen, IL-6 and CRP were independent predictors of all cause mortality among the stroke free subjects. *C. pneumoniae* IgA- or IgG antibodies did not predict mortality in either group. To our knowledge this is the first report describing inflammatory markers in a cohort of long term ischemic stroke survivors. Beamer et al reported elevated levels of fibrinogen in stroke survivors after one year,[26] and our results extend their observations, and support an ongoing inflammatory response in advanced atherosclerotic disease.

The stroke group had increased risk of cardiovascular death and all cause mortality as compared to the stroke-free group in our study. Measurement of inflammatory markers has been suggested to improve mortality prediction.[12] However, low grade chronic inflammation is also attributable to common age-related diseases like Alzheimer disease, diabetes mellitus, advanced atherosclerosis, sarcopenia, frailty and malignancies.[30-32] A high proportion of the stroke subjects in our study presented comorbidities, and ischemic stroke survivors often present advanced atherosclerosis in other vascular locations.[33] A larger endothelial involvement is consistent with chronic low grade inflammation inducing platelet reactivity and hypercoagulability, triggering pro-thrombotic mechanisms.[34,35] Whether the inflammatory markers have causal or counter-regulatory functions remains unclear, but it is postulated that

individuals genetically predisposed to a weak inflammatory response are less likely to develop age-related diseases and suffer premature death.[36]

In population-based studies, fibrinogen, IL-6 and CRP predict mortality in apparently healthy individuals,[10-15] and IL-6 seems to be a better predictor among the oldest, in particular.[11-13] The strong association between IL-6 and mortality might reflect age related physiological deterioration and immunoscenesence, as aging is associated with a dysregulated acute phase response promoting enhanced production of proinflammatory cytokines.[31,32]

Despite excess risk of cardiovascular and all cause death, there is limited data on preventive strategies in long-term ischemic stroke survivors.[23,37] Increasing age, stroke severity, functional and cognitive impairment is associated with poor outcome.[28] Besides carotid revascularization, secondary prevention strategies includes immune response modulation through antiplatelet therapy, cessation of smoking and control of hyperglycaemia. Statins have anti-inflammatory effects beyond lipid-lowering,[38] and may stabilize atherosclerotic plaques.[39] Therapy with a high dose atorvastatin reduced the incidence of cardiovascular events and stroke recurrence, when patients were enrolled shortly after index stroke, but had no effect on overall mortality.[25] Unfortunately, data on inflammatory markers were not presented. In our study, the levels of inflammatory markers were unaffected by statin therapy, as only a very small proportion in the stroke group used statins.

Our study has some limitations. Subjects suffering acute myocardial infarction and subsequent sudden death outside the hospital were classified as "unknown cause of death" due to poor quality of death certificate. Due to a limited number of deaths in the stroke-free group, possible effects of inflammatory markers on cardiovascular death were not detected. Severely ill and disabled stroke subjects at increased risk of death are probably under-represented in this study. Enrolment of subjects at different times post stroke may conceal recurrent vascular risks. Consequently, a single measurement of inflammatory markers may

mask a potential time-dependency. However, the levels of inflammatory markers were similar in stroke subjects having experienced their index stroke more than seven years ago, as compared to those having experienced it less than seven years ago. The inflammatory cascade may also have other origins than artery atherosclerosis, such as systemic inflammation or local infections. At study entry, no subjects had clinical signs of ongoing infections. Our cohort of *long-term* ischemic stroke survivors is highly selective, and this survival bias may weaken the true association between inflammatory markers and mortality risk prediction in ischemic stroke survivors.

The impact of common risk factors for death was different in the two groups, indicating risk factor modification in long-term ischemic stroke survivors.[22,23,40] It is not unlikely that risk factors for a first vascular event might be less predictive for recurrent events due to selection, incident comorbidities, and advanced atherosclerosis.[22,23,40] Due to accumulated life-long risk burden, these individuals may have reached a level of total risk that overshadows the impact of additional inflammatory markers.[10]


**Summary**

Long-term ischemic stroke-survivors had increased risk of death compared to stroke free subjects. Despite elevated levels of inflammatory markers several years after index stroke, none of these markers were associated with mortality. Mortality risk estimation in stroke subjects may warrant a different approach than in stroke free subjects at similar age. More research is needed to establish the relevance of inflammatory markers in secondary prevention strategies.

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**Table 1. Basic characteristics of individuals with and without ischemic stroke.**

|  | Ischemic stroke (n=187) | No Stroke (n=243) | p-value |
|---|---|---|---|
| *Male sex, % | 57.8 | 56.0 | 0.58 |
| *Age, years (SD) | 68.3 (12.4) | 65.2 (13.8) | 0.35 |
| Body mass index (kg/m²) | 26.9 (26.32-27.60) | 26.7 (26.16-27.28) | 0.05 |
| Systolic blood pressure (mmHg) | 151.5 (147.81-155.18) | 147.2 (145.91-152.37) | 0.90 |
| Diastolic blood pressure (mmHg) | 81.8 (80.10-83.43) | 79.5 (78.08-80.99) | 0.02 |
| Total cholesterol (mmol/L) | 6.49 (6.31-6.67) | 6.50 (6.35-6.66) | |
| HBA1c | 5.43 (5.33-5.54) | 5.26 (5.17-5.36) | |
| | | | |
| Current smoking (yes/no), % | 30.0 | 31.7 | 0.69 |
| | | | |
| Morbidity in 1997, % | | | |
| Myocardial infarction | 16.0 | 7.8 | 0.008 |
| Diabetes mellitus | 11.2 | 5.4 | 0.03 |
| Myocardial infarction or Diabetes | 25.1 | 12.8 | 0.001 |
| | | | |
| Registered drug use in 1997, % | | | |
| Antihypertensive medication | 55.1 | 24.3 | <0.0001 |
| Statins | 12.4 | 2.5 | <0.0001 |
| Acetylsalicylate | 53.5 | 8.2 | <0.0001 |
| | | | |
| Frenchay Activity Index | 38.4 (37.2-39.6) | 43.0 (42.0-44.0) | <0.0001 |

Values are age and sex adjusted means (95% CI) and percentages
* Unadjusted values

**Table 2. Age and sex adjusted inflammatory markers in individuals with and without ischemic stroke at baseline.**

| | Ischemic stroke (n=187) | | No Stroke (n=243) | | p-value |
|---|---|---|---|---|---|
| White blood cell count (x10$^9$/l) | 6.97 | (6.72-7.22) | 6.46 | (6.23-6.68) | 0.003 |
| Fibrinogen (g/l) | 3.65 | (3.53-3.77) | 3.33 | (3.22-3.43) | 0.0001 |
| Interleukin-6* (µg/l) | 1.80 | (1.58-2.04) | 1.40 | (1.25-1.57) | 0.004 |
| CRP* (mg/l) | 2.22 | (1.89-2.62) | 1.63 | (1.42-1.88) | 0.006 |

Values are age and sex adjusted means or *geometric means (95% CI)

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**Table 3.**

**Age and sex adjusted inflammatory markers in survivors and deceased individuals with and without ischemic stroke at baseline.**

| | Ischemic stroke (n=187) | | | No stroke (=243) | | |
|---|---|---|---|---|---|---|
| | Survivors (n=118) | Deceased (n=69) | p | Survivors (n=201) | Deceased (n=42) | p |
| WBC (x10⁹/l) | 6.79 (6.45-7.12) | 7.16 (6.71-7.62) | 0.21 | 6.38 (6.14-6.62) | 7.03 (6.47-7.59) | 0.04 |
| Fibrinogen (g/l) | 3.64 (3.46-3.82) | 3.77 (3.52-4.02) | 0.43 | 3.25 (3.15-3.35) | 3.56 (3.32-3.80) | 0.03 |
| Interleukin-6* (µg/l) | 1.74 (1.49-2.04) | 2.01 (1.62-2.49) | 0.32 | 1.27 (1.12-1.44) | 2.12 (1.58-2.84) | 0.002 |
| CRP* (mg/l) | 2.17 (1.75-2.68) | 2.47 (1.85-3.29) | 0.50 | 1.49 (1.28-1.75) | 2.27 (1.57-3.28) | 0.05 |

Values are age and sex adjusted means or *geometric means (95% CI)

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**Table 4.**
**Predictors of mortality in individuals with and without ischemic stroke in age and sex-adjusted Cox proportional hazards models.**

| | Ischemic stroke (n=69) | | No stroke (n=42) | |
|---|---|---|---|---|
| | HR (95 % CI) | p-value | HR (95 % CI) | p-value |
| *Sex (M/F) | 1.42 (0.87-2.32) | 0.16 | 1.19 (0.64-2.20) | 0.58 |
| *Age (1) | 1.08 (1.05-1.11) | <0.0001 | 1.13 (1.09-1.17) | <0.0001 |
| Smoking (yes/no) | 1.75 (1.06-2.89) | 0.03 | 2.20 (1.13-4.30) | 0.02 |
| History of | | | | |
| Myocardial infarction (yes/no) | 1.03 (0.57-1.86) | 0.93 | 2.08 (0.95-4.57) | 0.07 |
| Diabetes (yes/no) | 1.32 (0.69-2.53) | 0.39 | 3.16 (1.39-7.19) | 0.006 |
| Myocardial infarction or diabetes (yes/no) | 1.16 (0.70-1.93) | 0.57 | 2.61 (1.36-5.02) | 0.004 |
| Inflammatory markers | | | | |
| White blood cell count (1SD) | 1.16 (0.92-1.46) | 0.21 | 1.30 (0.90-1.88) | 0.16 |
| Fibrinogen (1SD) | 1.15 (0.93-1.42) | 0.19 | 1.65 (1.17-2.33) | 0.004 |
| Log Interleukin-6 | 1.98 (0.96-4.08) | 0.07 | 3.37 (1.37-8.32) | 0.008 |
| Log C-reactive protein | 1.29 (0.80-2.09) | 0.31 | 2.42 (1.31-4.45) | 0.005 |

*Age and sex are adjusted only for one another.
The values within brackets in the left column are the units used in the Cox proportional hazards regression models

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**Appendices**
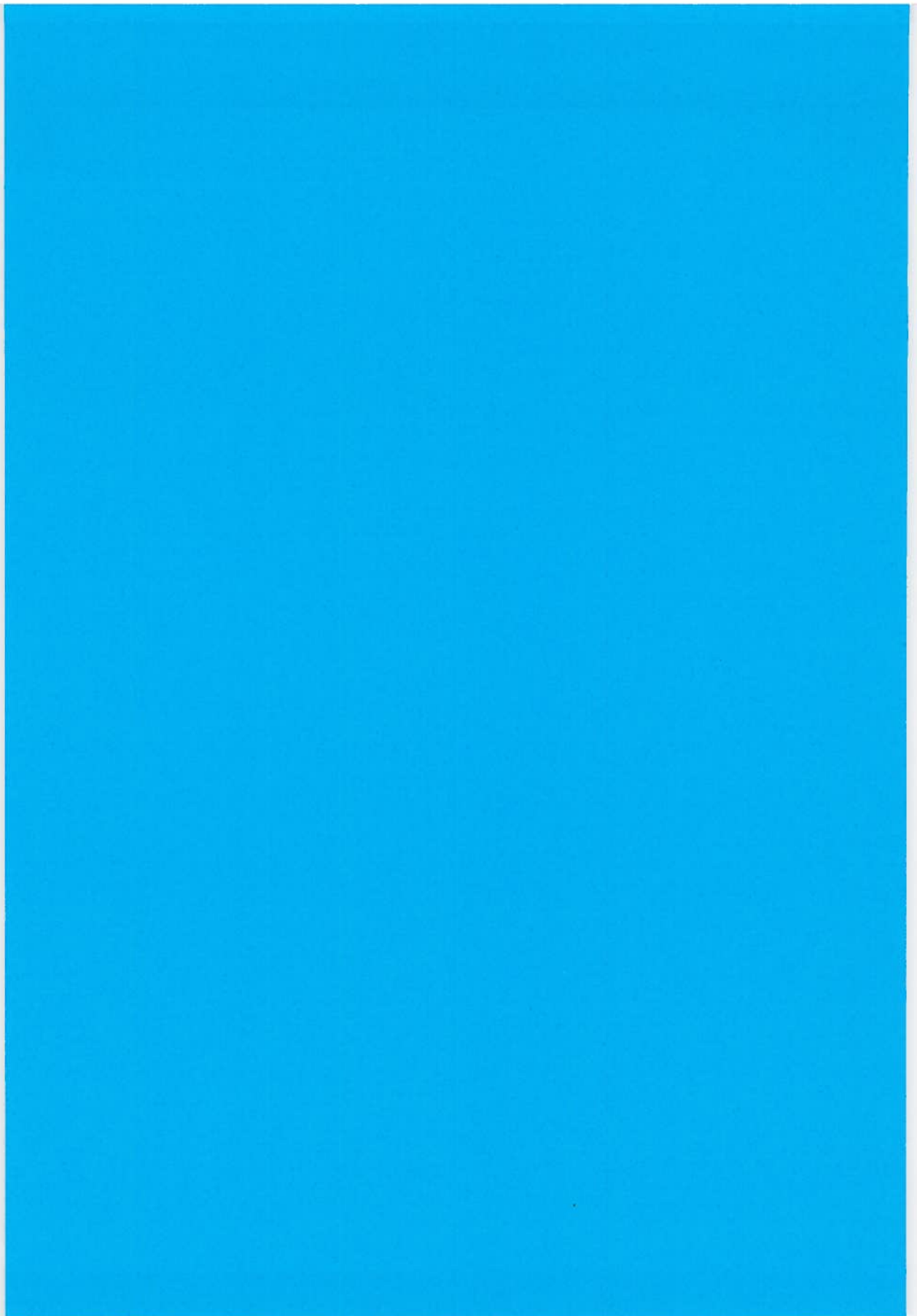
# Helse–
## undersøkelsen

## Personlig innbydelse

## 1. EGEN HELSE

**1.1 Hvordan er helsen din nå?** *(Sett bare ett kryss)*

| Dårlig | Ikke helt god | God | Svært god |
|---|---|---|---|
| ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 |

**1.2 Har du, eller har du hatt?:**

| | JA | NEI | Alder første gang |
|---|---|---|---|
| Astma | ☐ | ☐ | |
| Høysnue | ☐ | ☐ | |
| Kronisk bronkitt/emfysem | ☐ | ☐ | |
| Diabetes (sukkersyke) | ☐ | ☐ | |
| Benskjørhet (osteoporose) | ☐ | ☐ | |
| Fibromyalgi/kronisk smertesyndrom | ☐ | ☐ | |
| Psykiske plager som du har søkt hjelp for | ☐ | ☐ | |
| Hjerteinfarkt | ☐ | ☐ | |
| Angina pectoris (hjertekrampe) | ☐ | ☐ | |
| Hjerneslag/hjerneblødning | ☐ | ☐ | |

**1.3 Har du merket anfall med plutselig endring i pulsen eller hjerterytmen siste året?**  JA ☐  NEI ☐

**1.4 Får du smerter eller ubehag i brystet når du:**  JA ☐  NEI ☐
Går i bakker, trapper eller fort på flat mark?  ☐ ☐

**1.5 Hvis du får slike smerter, pleier du da å:**

| Stoppe? | Sakne farten? | Fortsette i samme takt? |
|---|---|---|
| ☐ 1 | ☐ 2 | ☐ 3 |

**1.6 Dersom du stopper, forsvinner smertene da etter mindre enn 10 minutter?**  JA ☐  NEI ☐

**1.7 Kan slike smerter opptre selv om du er i ro?**  JA ☐  NEI ☐

## 2. MUSKEL OG SKJELETTPLAGER

**2.1 Har du vært plaget med smerter og/eller stivhet i muskler og ledd i løpet av de siste 4 ukene?**
*(Varighet angis bare hvis du har hatt plager)*

| | Ikke plaget | En del plaget | Sterkt plaget | Varighet inntil 2 uker | 2 uker eller mer |
|---|---|---|---|---|---|
| Nakke/skuldre | ☐ | ☐ | ☐ | ☐ | ☐ |
| Armer, hender | ☐ | ☐ | ☐ | ☐ | ☐ |
| Øvre del av ryggen | ☐ | ☐ | ☐ | ☐ | ☐ |
| Korsryggen | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hofter, ben, føtter | ☐ | ☐ | ☐ | ☐ | ☐ |
| Andre steder | ☐ | ☐ | ☐ | ☐ | ☐ |
| | 1 | 2 | 3 | 1 | 2 |

**2.2 Har du noen gang hatt:**  JA  NEI   Alder siste gang
Brudd i håndledd/underarm?  ☐ ☐

Lårhalsbrudd?  ☐ ☐

## 3. ANDRE PLAGER

**3.1 Under finner du en liste over ulike problemer. Har du opplevd noe av dette den siste uken (til og med i dag)?**
*(Sett ett kryss for hver plage)*

| | Ikke plaget | Litt plaget | Ganske mye | Veldig mye |
|---|---|---|---|---|
| Plutselig frykt uten grunn | ☐ | ☐ | ☐ | ☐ |
| Føler deg redd eller engstelig | ☐ | ☐ | ☐ | ☐ |
| Matthet eller svimmelhet | ☐ | ☐ | ☐ | ☐ |
| Føler deg anspent eller oppjaget | ☐ | ☐ | ☐ | ☐ |
| Lett for å klandre deg selv | ☐ | ☐ | ☐ | ☐ |
| Søvnproblemer | ☐ | ☐ | ☐ | ☐ |
| Nedtrykt, tungsindig | ☐ | ☐ | ☐ | ☐ |
| Følelse av å være unyttig, lite verd | ☐ | ☐ | ☐ | ☐ |
| Følelse av at alt er et slit | ☐ | ☐ | ☐ | ☐ |
| Følelse av håpløshet mht. framtida | ☐ | ☐ | ☐ | ☐ |
| | 1 | 2 | 3 | 4 |

## 4. BRUK AV HELSETJENESTER

**4.1 Hvor mange ganger de siste 12 månedene har du selv brukt:**
*(Sett ett kryss for hver linje)*

| | ingen | 1-3 ganger | 4 eller flere |
|---|---|---|---|
| Allmennpraktiserende lege | ☐ | ☐ | ☐ |
| Bedriftslege | ☐ | ☐ | ☐ |
| Psykolog eller psykiater (privat eller på poliklinikk) | ☐ | ☐ | ☐ |
| Annen spesialist (privat eller på poliklinikk) | ☐ | ☐ | ☐ |
| Legevakt (privat eller offentlig) | ☐ | ☐ | ☐ |
| Sykehusinnleggelse | ☐ | ☐ | ☐ |
| Hjemmesykepleie | ☐ | ☐ | ☐ |
| Fysioterapeut | ☐ | ☐ | ☐ |
| Kiropraktor | ☐ | ☐ | ☐ |
| Tannlege | ☐ | ☐ | ☐ |
| Alternativ behandler | ☐ | ☐ | ☐ |

## 5. OPPVEKST OG TILHØRIGHET

**5.1 Hvor lenge har du samlet bodd i fylket?**  år
*(Sett 0 hvis mindre enn et halvt år)*

**5.2 Hvor lenge har du samlet bodd i kommunen?**  år
*(Sett 0 hvis mindre enn et halvt år)*

**5.3 Hvor bodde du det meste av tiden før du fylte 16 år?**
*(kryss av for ett alternativ og spesifiser)*

Samme kommune  ☐ 1

Annen kommune i fylket  ☐ 2  Hvilken: _____

Annet fylke i Norge  ☐ 3  Hvilket: _____

Utenfor Norge  ☐ 4  Land: _____

**5.4 Har du flyttet i løpet av de siste fem årene?**

| Nei | Ja, en gang | Ja, flere ganger |
|---|---|---|
| ☐ 1 | ☐ 2 | ☐ 3 |

## 6. VEKT

**6.1 Anslå din vekt da du var 25 år gammel:**  hele kg

**7.1 Hvor ofte spiser du vanligvis disse matvarene?**
(Sett ett kryss pr. linje)

| | Sjelden aldri | 1-3 g pr mnd | 1-3 g pr uke | 4-6 g pr uke | 1-2 g pr.dag | 3 g el mer pr.dag |
|---|---|---|---|---|---|---|
| Frukt, bær | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ost (alle typer) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Poteter | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kokte grønnsaker | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rå grønnsaker/salat | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Feit fisk (f.eks. laks, ørret, makrell, sild) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| | 1 | 2 | 3 | 4 | 5 | 6 |

**7.2 Hva slags fett bruker du oftest?** (Sett ett kryss pr. linje)

| | Bruker ikke | Meieri smør | Hard margarin | Mykt/lett margarin | Olje | Annet |
|---|---|---|---|---|---|---|
| På brødet | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| I matlagingen | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| | 1 | 2 | 3 | 4 | 5 | 6 |

**7.3 Bruker du følgende kosttilskudd:**

| | Ja, daglig | Iblant | Nei |
|---|---|---|---|
| Tran, trankapsler, fiskeoljekapsler? | ☐ | ☐ | ☐ |
| Vitamin- og/eller mineraltilskudd? | ☐ | ☐ | ☐ |

**7.4 Hvor mye drikker du vanligvis av følgende?**
(Sett ett kryss pr. linje)

| | Sjelden aldri | 1-6 glass pr uke | 1 glass pr.dag | 2-3 glass pr.dag | 4 glass el mer pr.dag |
|---|---|---|---|---|---|
| Helmelk, kefir, yoghurt | ☐ | ☐ | ☐ | ☐ | ☐ |
| Lettmelk, cultura, lettyoghurt | ☐ | ☐ | ☐ | ☐ | ☐ |
| Skummet melk (sur/søt) | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ekstra lettmelk | ☐ | ☐ | ☐ | ☐ | ☐ |
| Fruktjuice | ☐ | ☐ | ☐ | ☐ | ☐ |
| Vann | ☐ | ☐ | ☐ | ☐ | ☐ |
| Farris, Ramlosa e.l | ☐ | ☐ | ☐ | ☐ | ☐ |
| Cola-holdig leskedrikk | ☐ | ☐ | ☐ | ☐ | ☐ |
| Annen brus/leskedrikk | ☐ | ☐ | ☐ | ☐ | ☐ |
| | 1 | 2 | 3 | 4 | 5 |

**7.5 Drikker du vanligvis brus/cola:** Med sukker ☐1  Uten sukker ☐2

**7.6 Hvor mange kopper kaffe og te drikker du daglig?** Antall kopper
(Sett 0 for de typene du ikke drikker daglig)

Filterkaffe

Kokekaffe/trykkanne

Annen kaffe

Te

**7.7 Omtrent hvor ofte har du i løpet av det siste året drukket alkohol?**
(Lettøl og alkoholfritt øl regnes ikke med)

| Har aldri drukket alkohol | Har ikke drukket alkohol siste år | Noen få ganger siste år | Omtrent 1 gang i måneden |
|---|---|---|---|
| ☐1 | ☐2 | ☐3 | ☐4 |

| 2-3 ganger pr måned | ca. 1 gang i uka | 2-3 ganger i uka | 4-7 ganger i uka |
|---|---|---|---|
| ☐5 | ☐6 | ☐7 | ☐8 |

Til dem som har drukket siste år

**7.8 Når du har drukket alkohol, hvor mange glass eller drinker har du vanligvis drukket?** Antall

**7.9 Omtrent hvor mange ganger i løpet av det siste året har du drukket så mye som minst 5 glass eller drinker i løpet av ett døgn?** Antall ganger

**7.10 Når du drikker, drikker du da vanligvis:** (Sett ett eller flere kryss)

| Øl | Vin | Brennevin |
|---|---|---|
| ☐ | ☐ | ☐ |

**8.1 Hvor lenge er du vanligvis daglig tilstede i røykfylt rom?** Antall hele timer

**8.2 Røykte noen av de voksne hjemme da du vokste opp?**  JA ☐  NEI ☐

**8.3 Bor du, eller har du bodd, sammen med noen dagligrøykere etter at du fylte 20 år?**  ☐ ☐

**8.4 Har du røykt/røyker du daglig?**  Ja, nå ☐  Ja, tidligere ☐  Aldri ☐
Hvis ALDRI: Hopp til spørsmål 9 (UTDANNING OG ARBEID)

**8.5 Hvis du røyker daglig nå, røyker du:**  JA  NEI

Sigaretter? ☐ ☐

Sigarer/sigarillos? ☐ ☐

Pipe? ☐ ☐

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

**8.7 Hvis du røyker daglig nå eller har røykt tidligere:**

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

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

Hvor mange år til sammen har du røykt daglig? Antall år

**9.1 Hvor mange års skolegang har du gjennomført?** Antall år
(Ta med alle år du har gått på skole eller studert)

**9.2 Er du i inntektsgivende arbeid?**

Ja, full tid ☐1   Ja, deltid ☐2   Nei ☐3

**9.3 Beskriv virksomheten på det arbeidsstedet (avdelingen) der du utførte inntektsgivende arbeid i lengst tid de siste 12 mnd.** (F.eks. regnskapsbyrå, ungdomsskole, barneavd. på sykehus, snekkerverksted, bilverksted, bank, dagligvarehandel e.l.)

Virksomhet: _____
Hvis pensjonert, skriv tidligere hovedvirksomhet og yrke
Gjelder også 9.4

**9.4 Hvilket yrke/tittel har eller hadde du på dette arbeidsstedet?** (F.eks. sekretær, lærer, industriarbeider, barnepleier, møbelsnekker, avdelingsleder, selger, sjåfor e.l.)

Yrke: _____

**9.5 Arbeider du i ditt hovedyrke som selvstendig, som ansatt eller som familiemedlem uten fast avtalt lønn?**

| Selvstendig | Ansatt | Familiemedlem |
|---|---|---|
| ☐ | ☐ | ☐ |

**9.6 Mener du at du står i fare for å miste ditt nåværende arbeid eller inntekt de nærmeste 2 årene?**  JA ☐  NEI ☐

**9.7 Mottar du noen av følgende ytelser?**  JA  NEI

Sykepenger (er sykmeldt) ☐ ☐

Alderstrygd, førtidspensjon (AFP) eller etterlattepensjon ☐ ☐

Rehabiliterings-/attføringspenger ☐ ☐

Uførepensjon (hel eller delvis) ☐ ☐

Dagpenger under arbeidsledighet ☐ ☐

Sosialhjelp/-stønad ☐ ☐

Overgangsstønad for enslige forsørgere ☐ ☐

## 10. MOSJON OG FYSISK AKTIVITET

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

| | Timer pr. uke | | | |
| --- | --- | --- | --- | --- |
| | Ingen | Under 1 | 1-2 | 3 og mer |
| Lett aktivitet *(Ikke svett/andpusten)* | ☐ | ☐ | ☐ | ☐ |
| Hard fysisk aktivitet *(Svett/andpusten)* | ☐1 | ☐2 | ☐3 | ☐4 |

**10.2 Angi bevegelse og kroppslig anstrengelse i din fritid. Hvis aktiviteten varierer meget f.eks. mellom sommer og vinter, så ta et gjennomsnitt. Spørsmålet gjelder bare det siste året.**
*(Sett kryss i den ruta som passer best)*

Leser, ser på fjernsyn eller annen stillesittende beskjeftigelse? .................. ☐1

Spaserer, sykler eller beveger deg på annen måte minst 4 timer i uka?........................ ☐2
*(Her skal du også regne med gang eller sykling til arbeidsstedet, søndagsturer m.m.)*

Driver mosjonsidrett, tyngre hagearbeid e.l.?................ ☐3
*(Merk at aktiviteten skal vare minst 4 timer i uka)*

Trener hardt eller driver konkurranseidrett regelmessig og flere ganger i uka? ................ ☐4

## 11. FAMILIE OG VENNER

**11.1 Bor du sammen med:** 
| | JA | NEI |
| --- | --- | --- |
| Ektefelle/samboer? ....................... | ☐ | ☐ |

**11.2 Hvor mange gode venner har du?** *Antall venner*

*Regn med de du kan snakke fortrolig med og som kan gi deg hjelp dersom du trenger det. Tell ikke med de du bor sammen med, men ta med andre slektninger*

**11.3 Hvor stor interesse viser folk for det du gjør?**
*(Sett bare ett kryss)*

| Stor interesse | Noe interesse | Litt interesse | Ingen interesse | Usikkert |
| --- | --- | --- | --- | --- |
| ☐1 | ☐2 | ☐3 | ☐4 | ☐5 |

**11.4 Hvor mange foreninger, lag, grupper, kirkesamfunn e.l. deltar du i på fritiden?** *Antall*
*(Skriv 0 hvis ingen)*

**11.5 Føler du at du kan påvirke det som skjer i lokalsamfunnet der du bor?** *(Sett bare ett kryss)*

| Ja, i stor grad | Ja, en del | Ja, i liten grad | Nei | Har ikke forsøkt |
| --- | --- | --- | --- | --- |
| ☐1 | ☐2 | ☐3 | ☐4 | ☐5 |

## 12. SYKDOM I FAMILIEN

**12.1 Har en eller flere av dine foreldre eller søsken hatt hjerteinfarkt (sår på hjertet) eller angina pectoris (hjertekrampe)?** .........

| | JA | NEI | VET IKKE |
| --- | --- | --- | --- |
| | ☐ | ☐ | ☐ |

**12.2 Kryss av for de slektningene som har eller har hatt noen av sykdommene:** *(Sett kryss for hver linje)*

| | Mor | Far | Bror | Søster | Barn | Ingen av disse |
| --- | --- | --- | --- | --- | --- | --- |
| Hjerneslag eller hjerneblødning...... | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hjerteinfarkt før 60 års alder ............. | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Astma ................. | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kreftsykdom ......... | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Diabetes (sukkersyke) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

**12.3 Hvis noen slektninger har diabetes, i hvilken alder fikk de diabetes (hvis for eks. flere søsken, før opp den som fikk det tidligst i livet):**

| Vet ikke, ikke aktuelt | Mors alder | Fars alder | Brors alder | Søsters alder | Barns alder |
| --- | --- | --- | --- | --- | --- |
| ☐ | | | | | |

## 13. BRUK AV MEDISINER

*Med medisiner mener vi her medisiner kjøpt på apotek. Kosttilskudd og vitaminer regnes ikke med her.*

**13.1 Bruker du?**

| | Nå | Før, men ikke nå | Aldri brukt |
| --- | --- | --- | --- |
| Medisin mot høyt blodtrykk ... .. | ☐ | ☐ | ☐ |
| Kolesterolsenkende medisin . . . . . . . . | ☐ | ☐ | ☐ |

**13.2 Hvor ofte har du i løpet av de siste 4 ukene brukt følgende medisiner?** *(Sett ett kryss pr. linje)*

| | Ikke brukt siste 4 uker | Sjeldnere enn hver uke | Hver uke men ikke daglig | Daglig |
| --- | --- | --- | --- | --- |
| Smertestillende uten resept ... | ☐ | ☐ | ☐ | ☐ |
| Smertestillende på resept ... | ☐ | ☐ | ☐ | ☐ |
| Sovemedisin ...... | ☐ | ☐ | ☐ | ☐ |
| Beroligende medisin ........... | ☐ | ☐ | ☐ | ☐ |
| Medisin mot depresjon ..... | ☐ | ☐ | ☐ | ☐ |
| Annen medisin på resept... .. ... | ☐1 | ☐2 | ☐3 | ☐4 |

**13.3 For de medisinene som du har krysset av for i pkt. 13.1 og 13.2, og som du har brukt i løpet av de siste 4 ukene:**

Angi navnet og hvilken grunn det er til at du tar/har tatt disse (sykdom eller symptom)·
*(Kryss av for hvor lenge du har brukt medisinen)*

| Navn på medisinen (ett navn pr. linje) | Grunn til bruk av medisinen | Hvor lenge har du brukt medisinen? | |
| --- | --- | --- | --- |
| | | Inntil 1 år | Ett år eller mer |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |

*Dersom det ikke er nok plass her, kan du fortsette på eget ark som du legger ved.*

## 14. RESTEN AV SKJEMAET SKAL BARE BESVARES AV KVINNER

**14.1 Hvor gammel var du da du fikk menstruasjon eller første gang?** *Alder i år*

**14.2 Hvis du ikke lenger får menstruasjon, hvor gammel var du da den sluttet?** *Alder i år*

**14.3 Er du gravid nå?**

| Ja | Nei | Usikker | Over fruktbar alder |
| --- | --- | --- | --- |
| ☐1 | ☐2 | ☐3 | ☐4 |

**14.4 Hvor mange barn har du født?** *Antall barn*

**14.5 Bruker du, eller har du brukt?** *(Sett ett kryss for hver linje)*

| | Nå | Før, men ikke nå | Aldri |
| --- | --- | --- | --- |
| P-pille/minipille/p-sprøyte....... | ☐ | ☐ | ☐ |
| Hormonspiral *(ikke vanlig spiral)* | ☐ | ☐ | ☐ |
| Østrogen *(tabletter eller plaster)* | ☐ | ☐ | ☐ |
| Østrogen *(krem eller stikkpiller)* | ☐ | ☐ | ☐ |

**14.6 Hvis du bruker/har brukt reseptpliktig østrogen:** Hvor lenge har du brukt dette? *Antall år*

**14.7 Hvis du bruker p-pille, minipille, p-sprøyte, hormonspiral eller østrogen; hvilket merke bruker du?**

E

# Helse–
## undersøkelsen

Hvordan er helsen din nå? *(Sett bare ett kryss)*

| Dårlig | Ikke helt god | God | Svært god |
|--------|---------------|-----|-----------|
| ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 |

Har du, eller har du hatt?:

Alder første gang

| | JA | NEI |
|---|---|---|
| Astma ................................................ | ☐ | ☐ |
| Kronisk bronkitt/emfysem ...................... | ☐ | ☐ |
| Diabetes (sukkersyke) ............................ | ☐ | ☐ |
| Benskjørhet (osteoporose) ...................... | ☐ | ☐ |
| Fibromyalgi/kronisk smertesyndrom ........ | ☐ | ☐ |
| Psykiske plager som du har søkt hjelp for | ☐ | ☐ |
| Hjerteinfarkt ........................................ | ☐ | ☐ |
| Angina pectoris (hjertekrampe) .............. | ☐ | ☐ |
| Hjerneslag/hjerneblødning ..................... | ☐ | ☐ |

Får du smerter eller ubehag i brystet når du:

| | JA | NEI |
|---|---|---|
| Går i bakker, trapper eller fort på flat mark? ............. | ☐ | ☐ |

Hvis du får slike smerter, pleier du da å:

| Stoppe? | Saktne farten? | Fortsette i samme takt? |
|---------|----------------|-------------------------|
| ☐ 1 | ☐ 2 | ☐ 3 |

| | JA | NEI |
|---|---|---|
| Dersom du stopper, forsvinner smertene da etter mindre enn 10 minutter? ................ | ☐ | ☐ |
| Kan slike smerter opptre selv om du er i ro? ...... | ☐ | ☐ |

Har en eller flere av dine foreldre eller søsken hatt:

| | JA | NEI | Vet ikke |
|---|---|---|---|
| Hjerteinfarkt (sår på hjertet) eller angina pectoris (hjertekrampe)? ................ | ☐ | ☐ | ☐ |

Kryss av for de slektningene som har eller har hatt noen av sykdommene: *(Sett kryss for hver linje)*

| | Mor | Far | Bror | Søster | Barn | Ingen av disse |
|---|---|---|---|---|---|---|
| Hjerneslag eller hjerneblødning... | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Hjerteinfarkt før 60 års alder ............................ | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Astma ........................... | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kreftsykdom ................. | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Diabetes (sukkersyke) | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |

Hvis noen slektninger har diabetes, i hvilken alder fikk de diabetes (hvis for eks. flere søsken, før opp den som fikk det tidligst i livet):

| Vet ikke, ikke aktuelt | Mors alder | Fars alder | Brors alder | Søsters alder | Barns alder |
|---|---|---|---|---|---|
| ☐ | | | | | |

Under finner du en liste over ulike problemer.
Har du opplevd noe av dette den siste uken
(til og med i dag)?
*(Sett ett kryss for hver linje)*

| | Ikke plaget | Litt plaget | Ganske mye | Veldig mye |
|---|---|---|---|---|
| Plutselig frykt uten grunn ............... | ☐ | ☐ | ☐ | ☐ |
| Føler deg redd eller engstelig ........ | ☐ | ☐ | ☐ | ☐ |
| Matthet eller svimmelhet ................ | ☐ | ☐ | ☐ | ☐ |
| Føler deg anspent eller oppjaget .... | ☐ | ☐ | ☐ | ☐ |
| Lett for å klandre deg selv .............. | ☐ | ☐ | ☐ | ☐ |
| Søvnproblemer ............................... | ☐ | ☐ | ☐ | ☐ |
| Nedtrykt, tungsindig ...................... | ☐ | ☐ | ☐ | ☐ |
| Følelse av å være unyttig, lite verd | ☐ | ☐ | ☐ | ☐ |
| Følelse av at alt er et slit ............... | ☐ | ☐ | ☐ | ☐ |
| Følelse av håpløshet mht. framtida . | ☐ | ☐ | ☐ | ☐ |
| | 1 | 2 | 3 | 4 |

Hvor mange tenner har du mistet/trukket? *Antall tenner*
*(Se bort fra melketenner og visdomstenner)*

Har du vært plaget med smerter og/eller stivhet
i muskler og ledd i løpet av de siste 4 ukene?

| | Ikke plaget | En del plaget | Alvorlig plaget |
|---|---|---|---|
| Nakke/skuldre ........................ | ☐ | ☐ | ☐ |
| Armer, hender ........................ | ☐ | ☐ | ☐ |
| Øvre del av ryggen ................ | ☐ | ☐ | ☐ |
| Korsryggen ............................ | ☐ | ☐ | ☐ |
| Hofter, ben, føtter .................. | ☐ | ☐ | ☐ |
| Andre steder .......................... | ☐ | ☐ | ☐ |

Har du noen gang hatt:

Alder siste gang

| | JA | NEI |
|---|---|---|
| Brudd i håndledd/underarm? ................... | ☐ | ☐ |
| Lårhalsbrudd? ......................................... | ☐ | ☐ |

Har du falt i løpet av det siste året? *(Sett bare ett kryss)*

| Nei | Ja, 1-2 ganger | Ja, mer enn 2 ganger |
|-----|----------------|----------------------|
| ☐ 1 | ☐ 2 | ☐ 3 |

Hvordan har din fysiske aktivitet vært det siste året?
*Tenk deg et ukentlig gjennomsnitt for året*
*Besvar begge spørsmålene.*

Timer pr. uke

| | Ingen | Under 1 | 1-2 | 3 og mer |
|---|---|---|---|---|
| Lett aktivitet (ikke svett/andpusten) .............. | ☐ | ☐ | ☐ | ☐ |
| Hard fysisk aktivitet (svett/andpusten) ................... | ☐ | ☐ | ☐ | ☐ |
| | 1 | 2 | 3 | 4 |

Anslå din vekt da du var 25 år gammel:                hele kg

## E7. UTDANNING

Hvor mange års skolegang har du
gjennomført?                    *Antall år*
*(Ta med alle år du har gått på skole eller studert)*

## E8. MAT OG DRIKKE

Hvor ofte spiser du vanligvis disse matvarene?
*(Sett ett kryss for hver linje)*

| | Sjelden /aldri | 1-3 g pr.mnd | 1-3 g pr.uke | 4-6 g pr.uke | 1-2 g pr.dag | 3 g. el mer pr.dag |
|---|---|---|---|---|---|---|
| Frukt, bær | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ost *(alle typer)* | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Poteter | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Kokte grønnsaker | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Rå grønnsaker/salat | ☐ | ☐ | ☐ | ☐ | ☐ | ☐ |
| Feit fisk *(f.eks. laks, ørret, makrell, sild)* | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 | ☐ 6 |

Bruker du kosttilskudd:

| | Ja, daglig | Iblant | Nei |
|---|---|---|---|
| Tran, trankapsler, fiskeoljekapsler | ☐ | ☐ | ☐ |
| Vitamin- og/eller mineraltilskudd | ☐ | ☐ | ☐ |

Hvor mye drikker du vanligvis av følgende?
*(Sett ett kryss for hver linje)*

| | Sjelden /aldri | 1-6 glass pr.uke | 1 glass pr.dag | 2-3 glass pr.dag | 4 glass el. mer pr.dag |
|---|---|---|---|---|---|
| Helmelk, kefir, yoghurt | ☐ | ☐ | ☐ | ☐ | ☐ |
| Lettmelk, cultura, lettyoghurt | ☐ | ☐ | ☐ | ☐ | ☐ |
| Skummet melk (sur/søt) | ☐ | ☐ | ☐ | ☐ | ☐ |
| Ekstra lettmelk | ☐ | ☐ | ☐ | ☐ | ☐ |
| Fruktjuice | ☐ | ☐ | ☐ | ☐ | ☐ |
| Vann | ☐ | ☐ | ☐ | ☐ | ☐ |
| Brus, mineralvann | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 | ☐ 5 |

Hvor mange kopper kaffe og te drikker du daglig?
*(Sett 0 for de typene du ikke drikker daglig)*     *Antall kopper*

Filterkaffe

Kokekaffe/trykkanne

Annen kaffe

Te

Omtrent hvor ofte har du i løpet av det siste året drukket
alkohol? *(Lettøl og alkoholfritt øl regnes ikke med)*

| Har aldri drukket alkohol | Har ikke drukket alkohol siste år | Noen få ganger siste år | Omtrent 1 gang i måneden |
|---|---|---|---|
| ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 |
| 2-3 ganger pr. måned | ca. 1 gang i uka | 2-3 ganger i uka | 4-7 ganger i uka |
| ☐ 5 | ☐ 6 | ☐ 7 | ☐ 8 |

Til dem som har drukket siste år:
Når du har drukket alkohol, hvor mange glass
eller drinker har du vanligvis drukket?     *Antall*

Omtrent hvor mange ganger i løpet av det siste
året har du drukket så mye som minst 5 glass
eller drinker i løpet av ett døgn?     *Antall ganger*

## E9. RØYKING

Hvor lenge er du vanligvis daglig
tilstede i et røykfylt rom?     *Antall hele timer*

| | JA | NEI |
|---|---|---|
| Røykte noen av de voksne hjemme da du vokste opp? | ☐ | ☐ |
| Bor du, eller har du bodd, sammen med noen dagligrøykere etter at du fylte 20 år? | ☐ | ☐ |

| | Ja, nå | Ja, tidligere | Aldri |
|---|---|---|---|
| Har du røykt/røyker du daglig? | ☐ | ☐ | ☐ |

Hvis du **ALDRI** har røykt daglig;
Hopp til spørsmål E11 (FUNKSJON OG TRYGGHET)

Hvis du røyker daglig nå, røyker du:

| | JA | NEI |
|---|---|---|
| Sigaretter? | ☐ | ☐ |
| Sigarer/sigarillos? | ☐ | ☐ |
| Pipe? | ☐ | ☐ |

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

Hvis du røyker daglig nå eller har røykt
tidligere:

Hvor mange sigaretter røyker eller røykte
du vanligvis daglig?     *Antall sigaretter*

Hvor gammel var du da du begynte å
røyke daglig?     *Alder i år*

Hvor mange år til sammen har du
røykt daglig?     *Antall år*

## E10. FUNKSJON OG TRYGGHET

Ville du følt deg trygg ved å ferdes alene
på kveldstid i nærområdet der du bor?

| Ja | Litt utrygg | Svært utrygg |
|---|---|---|
| ☐ 1 | ☐ 2 | ☐ 3 |

Når det gjelder førlighet, syn og hørsel, kan du:
*(Sett ett kryss for hver linje)*

| | Uten problemer | Med litt problemer | Med store problemer | Nei |
|---|---|---|---|---|
| Gå en 5 minutters tur i noenlunde raskt tempo? | ☐ | ☐ | ☐ | ☐ |
| Lese vanlig tekst i aviser, evt. med briller? | ☐ | ☐ | ☐ | ☐ |
| Høre hva som blir sagt i en normal samtale? | ☐ 1 | ☐ 2 | ☐ 3 | ☐ 4 |

Har du på grunn av varige helseproblemer vansker
med å: *(Sett ett kryss for hver linje)*

| | Ingen vansker | Noen vansker | Store vansker |
|---|---|---|---|
| Bevege deg rundt i egen bolig? | ☐ | ☐ | ☐ |
| Komme deg ut av boligen på egen hånd? | ☐ | ☐ | ☐ |
| Delta i foreningsliv eller andre fritidsaktiviteter? | ☐ | ☐ | ☐ |
| Bruke offentlige transportmidler? | ☐ | ☐ | ☐ |
| Utføre nødvendige daglige ærend? | ☐ | ☐ | ☐ |

Hvor mange ganger de siste 12 månedene
har du selv brukt:
*(Sett ett kryss for hver linje)*

| | Ingen | 1-3 ganger | 4 eller flere |
|---|---|---|---|
| Allmennpraktiserende lege ............... | ☐ | ☐ | ☐ |
| Spesialist *(privat eller på poliklinikk)* | ☐ | ☐ | ☐ |
| Legevakt *(privat eller offentlig)* ......... | ☐ | ☐ | ☐ |
| Sykehusinnleggelse ........................ | ☐ | ☐ | ☐ |
| Hjemmesykepleie ......................... | ☐ | ☐ | ☐ |
| Fysioterapeut ................................. | ☐ | ☐ | ☐ |
| Kiropraktor ................................... | ☐ | ☐ | ☐ |
| Kommunal hjemmehjelp .................. | ☐ | ☐ | ☐ |
| Tannlege ..................................... | ☐ | ☐ | ☐ |
| Alternativ behandler ........................ | ☐ | ☐ | ☐ |

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

JA ☐  NEI ☐₂  Vet ikke ☐₃

## E12. FAMILIE OG VENNER

Bor du:  Hjemme? ☐₁  Institusjon/bolellesskap? ☐₂

Bor du sammen med:   JA NEI

Ektefelle/samboer? ...................... ☐ ☐

Andre personer? .......................... ☐ ☐

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

Hvor stor interesse viser folk for det du gjor?
*(Sett bare ett kryss)*

| Stor interesse | Noe interesse | Litt interesse | Ingen interesse | Usikkert |
|---|---|---|---|---|
| ☐₁ | ☐₂ | ☐₃ | ☐₄ | ☐₅ |

Hvor mange foreninger, lag, grupper,
kirkesamfunn e.l. deltar du i ?      Antall
*(Skriv 0 hvis ingen)*

Hvor lenge har du samlet bodd i fylket?      år

Hvor lenge har du samlet bodd i kommunen?      år

Hvor bodde du det meste av tiden for du fylte 16 år?
*(Kryss av for ett alternativ og spesifiser)*

Samme kommune ......... ☐₁

Annen kommune
i fylket ......................... ☐₂ Hvilken:_____

Annet fylke i Norge........ ☐₃ Hvilket:_____

Utenfor Norge............... ☐₄ Land:_____

Har du flyttet i løpet av de siste fem årene?

| Nei | Ja, en gang | Ja, flere ganger |
|---|---|---|
| ☐₁ | ☐₂ | ☐₃ |

## E14. BRUK AV MEDISIN

*Med medisiner mener vi her medisiner kjopt på apotek.*
*Kosttilskudd og vitaminer regnes ikke med her.*

Bruker du?
*(Sett ett kryss for hver linje)*

| | Nå | For, men ikke nå | Aldri brukt |
|---|---|---|---|
| Medisin mot høyt blodtrykk .................. | ☐ | ☐ | ☐ |
| Kolesterolsenkende medisin ................ | ☐ | ☐ | ☐ |
| Medisin mot osteoporose (benskjørhet) | ☐ | ☐ | ☐ |
| Insulin ........................................ | ☐ | ☐ | ☐ |
| Tabletter mot sukkersyke ..................... | ☐ | ☐ | ☐ |

Hvor ofte har du i løpet av de siste 4 ukene brukt
følgende medisiner?
*(Sett ett kryss for hver linje)*

| | Ikke brukt siste 4 uker | Sjeldnere enn hver uke | Hver uke, men ikke daglig | Daglig |
|---|---|---|---|---|
| Smertestillende uten resept ....... | ☐ | ☐ | ☐ | ☐ |
| Smertestillende på resept .......... | ☐ | ☐ | ☐ | ☐ |
| Sovemedisin ........................... | ☐ | ☐ | ☐ | ☐ |
| Beroligende medisin ................. | ☐ | ☐ | ☐ | ☐ |
| Medisin mot depresjon ............... | ☐ | ☐ | ☐ | ☐ |
| Annen medisin på resept .......... | ☐₁ | ☐₂ | ☐₃ | ☐₄ |

Angi navnet på de medisinene du bruker nå, og hva grunnen
er til at du tar medisinene (sykdom eller symptom):
*(Kryss av for hvor lenge du har brukt medisinen)*

| Navn på medisinen: (ett navn pr. linje): | Grunn til bruk av medisinen: | Hvor lenge har du brukt medisinen? | |
|---|---|---|---|
| | | Inntil 1 år | Ett år eller mer |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |
| | | ☐ | ☐ |

*Dersom det ikke er nok plass her, kan du fortsette på eget ark som du legger ved*

## E15. SPØRSMÅLENE PÅ DENNE SIDEN SKAL BARE BESVARES AV KVINNER

Hvor gammel var du da du fikk
menstruasjon eller første gang?      *Alder i år*

Hvor gammel var du da
menstruasjonen sluttet?      *Alder i år*

Hvor mange barn har du født?      *Antall barn*

Bruker du, eller har du brukt østrogenmedisin?      *I antall år totalt*

| | Aldri | For | Nå |
|---|---|---|---|
| Tabletter eller plaster ................. | ☐ | ☐ | ☐ |
| Krem eller stikkpiller ................. | ☐ | ☐ | ☐ |

Hvis du bruker østrogen: hvilket merke bruker du nå?

_____

| | JA | NEI |
|---|---|---|
| Har du noen gang brukt P-pille? .............. | ☐ | ☐ |

**ISM SKRIFTSERIE - FØR UTGITT:**

1.      Bidrag til belysning av medisinske og sosiale forhold i
        Finnmark fylke, med særlig vekt på forholdene blant
        finskættede i Sør-Varanger kommune.
        **Av Anders Forsdahl, 1976. (nytt opplag 1990)**

2.      Sunnhetstilstanden, hygieniske og sosiale forhold i Sør-
        Varanger kommune 1869-1975 belyst ved medisinal-
        beretningene.
        **Av Anders Forsdahl, 1977.**

3.      Hjerte-karundersøkelsen i Finnmark - et eksempel på en
        populasjonsundersøkelse rettet mot cardiovasculære
        sykdommer. Beskrivelse og analyse av
        etterundersøkelsesgruppen.
        **Av Jan-Ivar Kvamme og Trond Haider, 1979.**

4.  D.  The Tromsø Heart Study: Population studies of coronary
        risk factors with special emphasis on high density
        lipoprotein and the family occurrence of myocardial
        infarction.
        **Av Olav Helge Førde og Dag Steinar Thelle, 1979.**

5.  D.  Reformer i distriktshelsetjenesten III: Hypertensjon i
        distriktshelsetjenesten.
        **Av Jan-Ivar Kvamme, 1980.**

6.      Til professor Knut Westlund på hans 60-års dag, 1983.

7.*     Blodtrykksovervåkning og blodtrykksmåling.
        **Av Jan-Ivar Kvamme, Bernt Nesje og Anders Forsdahl, 1983.**

8.*     Merkesteiner i norsk medisin reist av allmennpraktikere.-
        og enkelte utdrag av medisinalberetninger av
        kulturhistorisk verdi.
        **Av Anders Forsdahl, 1984.**

9.      "Balsfjordsystemet." EDB-basert journal, arkiv og
        statistikksystem for primærhelsetjenesten.
        **Av Toralf Hasvold, 1984.**

10. D.  Tvunget psykisk helsevern i Norge. Rettsikkerheten ved
        slikt helsevern med særlig vurdering av
        kontrollkommisjonsordningen.
        **Av Georg Høyer, 1986.**

11. D.  The use of self-administered questionnaires about food
        habits. Relationships with risk factors for coronary heart
        disease and associations between coffee drinking and
        mortality and cancer incidence.
        **Av Bjarne Koster Jacobsen, 1988.**

12.*    Helse og ulikhet. Vi trenger et handlingsprogram for
        Finnmark.
        **Av Anders Forsdahl, Atle Svendal, Aslak Syse og
        Dag Thelle, 1989.**

13. D. Health education and self-care in dentistry - surveys and
       interventions.
       **Av Anne Johanne Søgaard, 1989.**

14.    Helsekontroller i praksis. Erfaringer fra prosjektet
       helsekontroller i Troms 1983-1985.
       **Av Harald Siem og Arild Johansen, 1989.**

15.    Til Anders Forsdahls 60-års dag, 1990.

16. D. Diagnosis of cancer in general practice. A study of delay
       problems and warning signals of cancer, with implications
       for public cancer information and for cancer diagnostic
       strategies in general practice.
       **Av Knut Holtedahl, 1991.**

17. D. The Tromsø Survey. The family intervention study.
       Feasibility of using a family approach to intervention on
       coronary heart disease. The effect of lifestyle
       intervention of coronary risk factors.
       **Av Synnøve Fønnebø Knutsen, 1991.**

18.    Helhetsforståelse og kommunikasjon. Filosofi for
       klinikere.
       **Av Åge Wifstad, 1991.**

19. D. Factors affecting self-evaluated general health status -
       and the use of professional health care services.
       **Av Knut Fylkesnes, 1991.**

20. D. Serum gamma-glutamyltransferase: Population determinants
       and diagnostic characteristics in relation to
       intervention on risk drinkers.
       **Av Odd Nilssen, 1992.**

21. D. The Healthy Faith. Pregnancy outcome, risk of disease,
       cancer morbidity and mortality in Norwegian
       Seventh-Day-Adventists.
       **Av Vinjar Fønnebø, 1992.**

22. D. Aspects of breast and cervical cancer screening.
       **Av Inger Torhild Gram, 1992.**

23. D. Population studies on dyspepsia and peptic ulcer disease:
       Occurrence, aetiology, and diagnosis. From The Tromsø
       Heart Study and The Sørreisa Gastrointestinal Disorder
       Studie.
       **Av Roar Johnsen, 1992.**

24. D. Diagnosis of pneumonia in adults in general practice.
       **Av Hasse Melbye, 1992.**

25. D. Relationship between hemodynamics and blood lipids in
       population surveys, and effects of n-3 fatty acids.
       **Av Kaare Bønaa, 1992.**

26. D. Risk factors for, and 13-year mortality from
cardiovascular disease by socioeconomic status.
A study of 44690 men and 17540 women, ages 40-49.
**Av Hanne Thürmer, 1993.**

27. Utdrag av medisinalberetninger fra Sulitjelma 1891-1990.
**Av Anders Forsdahl, 1993.**

28. Helse, livsstil og levekår i Finnmark. Resultater fra
Hjerte-karundersøkelsen i 1987-88. Finnmark III.
**Av Knut Westlund og Anne Johanne Søgaard, 1993.**

29. D. Patterns and predictors of drug use.
A pharmacoepidemiologic study, linking the analgesic drug
prescriptions to a population health survey in Tromsø,
Norway.
**Av Anne Elise Eggen, 1994.**

30. D. ECG in health and disease. ECG findings in relation to CHD
risk factors, constitutional variables and 16-year
mortality in 2990 asymptomatic Oslo men aged 40-49 years
in 1972.
**Av Per G. Lund-Larsen, 1994.**

31. D. Arrhythmia, electrocardiographic signs, and physical
activity in relation to coronary heart risk factors and
disease. The Tromsø Study.
**Av Maja-Lisa Løchen, 1995.**

32. D. The Military service: mental distress and changes in
health behaviours among Norwegian army conscript.
**Av Edvin Schei, 1995.**

33. D. The Harstad injury prevention study: Hospital-based injury
recording and community-based intervention.
**Av Børge Ytterstad, 1995.**

34.* D. Vilkår for begrepsdannelse og praksis i psykiatri.
En filosofisk undersøkelse.
**Av Åge Wifstad, 1996.** (utgitt Tano Aschehoug forlag 1997)

35. Dialog og refleksjon. Festskrift til professor Tom
Andersen på hans 60-års dag, 1996.

36. D. Factors affecting doctors´ decision making.
**Av Ivar Sønbø Kristiansen, 1996.**

37. D. The Sørreisa gastrointestinal disorder study. Dyspepsia,
peptic ulcer and endoscopic findings in a population.
**Av Bjørn Bernersen, 1996.**

38. D. Headache and neck or shoulder pain. An analysis of
musculoskeletal problems in three comprehensive
population studies in Northern Norway.
**Av Toralf Hasvold, 1996.**

39.        Senfølger av kjernefysiske prøvespreninger på øygruppen
           Novaya Semlya i perioden 1955 til 1962. Rapport etter
           programmet "Liv". Arkangelsk 1994.
           **Av A.V. Tkatchev, L.K. Dobrodeeva, A.I. Isaev,
           T.S. Podjakova, 1996.**

40.        Helse og livskvalitet på 78 grader nord. Rapport fra en
           befolkningsstudie på Svalbard høsten 1988.              **Av
           Helge Schirmer, Georg Høyer, Odd Nilssen, Tormod Brenn og
           Siri Steine, 1997.**

41.* D.    Physical activity and risk of cancer. A population based
           cohort study including prostate, testicular, colorectal,
           lung and breast cancer.
           **Av Inger Thune, 1997.**

42.        The Norwegian – Russian Health Study 1994/95. A cross-
           sectional study of pollution and health in the border
           area.
           **Av Tone Smith-Sivertsen, Valeri Tchachtchine, Eiliv Lund,
           Tor Norseth, Vladimir Bykov, 1997.**

43.  D.    Use of alternative medicine by Norwegian cancer patients
           **Av Terje Risberg, 1998.**

44   D.    Incidence of and risk factors for myocardial infarction,
           stroke, and diabetes mellitus in a general population. The
           Finnmark Study 1974–1989.
           **Av Inger Njølstad, 1998.**

45.  D.    General practitioner hospitals: Use and usefulness.
           A study from Finnmark County in North Norway.
           **Av Ivar Aaraas, 1998.**

45B        Sykestuer i Finnmark. En studie av bruk og nytteverdi.
           **Av Ivar Aaraas, 1998.**

46.  D.    No går det på helsa laus. Helse, sykdom og risiko for
           sykdom i to nord-norske kystsamfunn.
           **Av Jorid Andersen, 1998.**

47.  D.    The Tromsø Study: Risk factors for non-vertebral fractures
           in a middle-aged population.
           **Av Ragnar Martin Joakimsen, 1999.**

48.  D.    The potential for reducing inappropriate hospital
           admissions: A study of health benefits and costs in a
           department of internal medicine.
           **Av Bjørn Odvar Eriksen, 1999.**

49.  D.    Echocardiographic screening in a general population.
           Normal distribution of echocardiographic measurements and
           their relation to cardiovascular risk factors and disease.
           The Tromsø Study.
           **Av Henrik Schirmer, 2000.**

50. D.  Environmental and occupational exposure, life-style
        factors and pregnancy outcome in artic and subartic
        populations of Norway and Russia.
        **Av Jon Øyvind Odland, 2000.**

50B     Окружающая и профессиональная экспозиция, факторы
        стиля жизни и исход беременности у населения
        арктической и субарктической частей Норвегии и России
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51. D.  A population based study on coronary heart disease in
        families. The Finnmark Study 1974–1989.
        **Av Tormod Brenn, 2000.**

52  D.  Ultrasound assessed carotid atherosclerosis in a general
        population. The Tromsø Study.
        **Av Oddmund Joakimsen, 2000.**

53. D.  Risk factors for carotid intima-media thickness in a
        general population. The Tromsø Study 1979–1994.
        **Av Eva Stensland-Bugge, 2000.**

54. D.  The South Asian cataract management study.
        **Av Torkel Snellingen, 2000.**

55. D.  Air pollution and health in the Norwegian-Russian border
        area.
        **Av Tone Smith-Sivertsen, 2000.**

56. D.  Interpretation of forearm bone mineral density. The
        Tromsø Study.
        **Av Gro K. Rosvold Berntsen, 2000.**

57. D.  Individual fatty acids and cardiovascular risk factors.
        **Av Sameline Grimsgaard, 2001.**

58.     Finnmarkundersøkelsene
        **Av Anders Forsdahl, Fylkesnes K, Hermansen R, Lund E,
        Lupton B, Selmer R, Straume E, 2001.**

59. D.  Dietary data in the Norwegian women and cancer study.
        Validation and analyses of health related aspects.
        **Av Anette Hjartåker, 2001.**

60. D.  The stenotic carotid artery plaque. Prevalence, risk
        factors and relations to clinical disease. The Tromsø
        Study.
        **Av Ellisiv B. Mathiesen, 2001.**

61. D.  Studies in perinatal care from a sparsely populated area.
        **Av Jan Holt, 2001.**

62. D.  Fragile bones in patients with stroke? Bone mineral
        density in acute stroke patients and changes during one
        year of follow up.
        **Av Lone Jørgensen, 2001.**

63. D.   Psychiatric morbidity and mortality in northern
         Norway in the era of deinstitutionalisation. A
         psyhiatric case register study.
         **Av Vidje Hansen, 2001.**

64. D.   Ill health in two contrasting countries.
         **Av Tom Andersen, 1978/2002.**

65. D.   Longitudinal analyses of cardiovascular risk factors.
         **Av Tom Wilsgaard, 2002.**

66.      Helseundersøkelsen i Arkangelsk 2000.
         **Av Odd Nilssen, Alexei Kalinin, Tormod Brenn, Maria
         Averina et al.,2003.**

67. D.   Bio-psycho-social aspects of severe multiple trauma.
         **Av Audny G. W. Anke, 2003.**

68. D.   Persistent organic pollutants in human plasma from
         inhabitants of the artic.
         **Av Torkjel Manning Sandanger, 2003.**

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         hormonal contraceptives and pattern of child
         bearing.
         **Av Merethe Kunmle, 2003.**

70.      Pasienterfaringer i primærlegetjenesten før og etter
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         **Av Olaug Lian, 2003.**

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         marine food traditions.
         **Av Magritt Brustad, 2004.**

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         lokalsamfunns basert hjerte- og kar forebygging i
         kystkommunene Båtsfjord og Nordkapp.
         **Av Beate Lupton, 2004.**

73. D.   Environmental factors, metabolic profile, hormones
         and breast and endiometrial cancer risk.
         **Av Anne-Sofie Furberg, 2004.**

74. D.   Det skapende mellomrommet i møtet mellom pasient og
         lege.
         **Av Eli Berg, 2004.**

75.      Kreftregisteret i Arkhangelsk oblast i nordvest
         Russland. Med en sammenligning av kreftforekomst i
         Arkhangelsk oblast og Norge 1993 - 2001.
         **Av Vaktskjold Arild, Lebedintseva Jelena, Korotov
         Dmitrij, Tkatsjov Anatolij, Podjakova Tatjana, Lund
         Eiliv, 2004**

76. D. Characteristics and prognosis of long-term stroke survivors. The Tromsø Study.
**Av Torgeir Engstad, 2004**

77. D. Withdrawal and exclusion. A study of the spoken word as means of understanding schizophrenic patients.
**Av Geir Fagerjord Lorem, 2005.**

78. "Søkelys på safunnsmedisinene." Evaluering av kommunal samfunnsmedisinsk legetjeneste, offentlig legearbeid og de forebyggende oppgaver i Fastlegeordningen.
**Av Betty Pettersen og Roar Johnsen, 2005.**

79. **Prosjekt egenmelding Kristiansand kommune.** Evaluering av kontrollert intervensjonsforsøk i stor skala, med utvidet rett til egenmelding i kombinasjon med økt og formalisert samhandling mellom arbeidstaker og arbeidsplassen ved sykefravær.
**Av Nils Fleten og Roar Johnsen, 2005.**

80. D. Abdominal aortic aneurysms:Diagnosis and epidemiology. The Tromsø study.
**Av Kulbir Singh, 2005.**

81. D. A population based study on cardiovascular diseases in Northwest Russia.The Arkhangelsk study 2000.
**Av Maria Averina, 2005.**

82. D. Exposure to exogenous hormones in women: risk factors for breast cancer and molecular signature.
**Av Vanessa Dumeaux, 2005.**

83. D. Repeated ultrasound measurements of carotid artery plaques in a general population. The Tromsø Study 1994-2001.
**Av Stein Harald Johnsen, 2005.**

84. D. Risk Factors For Fractures In Tromsø. The Tromsø Study.
**Av Luai Awad Ahmed, 2005.**

85. D. The quality and use of two health registries in Russia. The Arkhangelsk Cancer Registry and the Kola Birth Registry
Качество и использование двух медицинских регистров в России. Архангельск регистр рака и Кольский регистр родов
**Av Arild Vaktskjold, 2005.**

86. D. Haemoglobin, anaemia and haematological malignancies.
**Av Tove Skjelbakken, 2006**

De som er merket med D er doktorgradsarbeid.
De som er merket med * har vi dessverre ikke flere eksemplar av.

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