Long term influence of regular intake of high dose n-3 fatty acids on CD40-ligand, pregnancy-associated plasma protein A and matrix metalloproteinase-9 following acute myocardial infarction

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Summary
Pregnancy-associated plasma protein A (PAPP-A) and matrix metalloproteinase 9 (MMP-9), both zinc-binding endopeptidases, are abundantly expressed in ruptured and eroded plaques in patients with acute coronary syndromes (ACS). The adhesion molecule CD-40 ligand (CD40L), expressed on activated platelets and T-lymphocytes, can activate metalloproteinases and thereby promote plaque rupture. N-3 fatty acids, through their anti-inflammatory and anti-thrombotic properties, might reduce the levels of these proatherosclerotic markers and thereby the development of ACS. 300 patients were randomized on day 4 to 6 following an acute myocardial infarction (MI) to receive either 4 g of n-3 fatty acids or a similar daily dose of corn oil for at least one year. We compared levels of PAPP-A, MMP-9 and sCD-40 L at baseline and 12 months in each group, and also looked for inter-group changes. In the omega-3 group, the median level of PAPP-A rose from 0.47 mU/l to 0.56 mU/l (p < 0.001). In the same group, sCD-40 L decreased from a mean baseline value of 5.19 ng/ml to 2.45 ng/ml (p < 0.001) and MMP-9 decreased non-significantly from 360.50 ng/ml to 308.00 ng/ml. Corresponding values for the corn oil group were 0.54 mU/l to 0.59 mU/l for PAPP-A (p = 0.007), 5.27 ng/ml to 2.84 ng/ml for sCD-40 L (p < 0.001) and 430.00 ng/ml to 324.00 ng/ml for MMP-9 (p = ns), respectively. In conclusion; both interventions resulted in a significant rise in PAPP-A, a significant decrease in sCD-40L and a non-significant decrease in MMP-9 after 12 months of treatment in MI survivors. No inter-group differences were noted.

Keywords
CD40-ligand, pregnancy associated plasma protein A (PAPP-A), matrix metalloproteinase 9 (MMP-9), acute myocardial infarction, N-3 fatty acids

Introduction
During the last decades, there has been a noteworthy change in our understanding of the atherosclerotic process. From being viewed as inanimate tubes, arteries are now thought of as living dynamic tissues, where intimal inflammation plays a crucial role in all steps of the pathophysiologic process leading to the development of atherosclerotic plaques. Enhanced inflammation resulting in plaque instability along with activation of intravascular thrombosis, precedes the development of acute coronary syndromes (ACS). This finding has initiated a growing interest for new therapeutic strategies aimed at retarding the atherosclerotic development.

CD40 ligand (CD40L) is one of the molecules linking inflammatory and thrombotic processes of atherosclerosis. This transmembrane protein, first identified on activated CD4+ T cells (1), has recently also been found on activated platelets, endothelial cells, smooth muscle cells and macrophages. A soluble form (sCD40L), probably fully biologically active (2), is shed from stimulated leucocytes and actively released from activated platelets, the latter contributing to more than 90% of sCD40L (3). In vivo effects are mediated by its receptor CD40, and disruption of CD40L/CD40 interactions in mice with subsequent reduced atheroma formation and development of a more stable plaque phenotype was the first evidence of a pathophysiological role of CD40L in atherosclerosis (1, 4). Later, the accumulation
of CD40L positive T-lymphocytes was demonstrated in human atheroma (5). Moreover, the same molecule is strongly upregulated on platelets in a fresh thrombus (6), mediating further platelet activation and stabilizing the arterial thrombi by binding to the glycoprotein IIb/IIIa receptors (2). Stimulation of endothelial cells and monocytes induces the release of tissue factor (TF), a potent activator of the extrinsic coagulation cascade, as well as activation of matrix metalloproteinases and synthesis of adhesion molecules, cytokines and chemokines, leading to further progression of the atherosclerotic and thrombotic process. In accordance with these findings, clinical studies have demonstrated elevated levels of sCD40L in patients with ACS (7), as well as in asymptomatic patients with angiographically verified coronary artery disease (CAD) (3, 7–9). Elevated levels have been found to predict future cardiovascular events and death in both apparently healthy women (10) and in patients admitted to hospital for chest pain or ACS (11).

Matrix metalloproteinases, a group of zinc-binding endoproteinases degrading all components of extracellular matrix, represent another type of molecules recently discovered as important contributors to the atherosclerotic process. Enhanced expression of pregnancy-associated plasma protein A (PAPP-A) and matrix metalloproteinase 9 (MMP-9) have been found in ruptured and eroded unstable coronary plaques (12), leading to the hypothesis of a predominant role of these molecules in the degradation of the fibrous cap prior to plaque rupture and ACS development. Degradation of extracellular matrix is also important for angiogenesis and for migration of smooth muscle cells into the intima, processes important for the growth of atherosclerotic plaques. For MMP-9 a proatherosclerotic effect mediated through enzymatic remodelling of LDL-particles (E-LDL) has also been proposed (13). E-LDL may induce foam-cell formation and inflammatory responses comparable to oxidized LDL, hence initiating plaque formation.

Clinical studies have confirmed elevated levels of PAPP-A and MMP-9 in patients with ACS (14–20). MMP-9 is also elevated in stable angina (20–21) as well as in otherwise healthy hypertensive patients (22, 23). For PAPP-A, the presence in stable angina is more conflicting. Raised levels have, however, been found in hypercholesterolemic (24) and diabetic patients without clinical evidence of coronary artery disease (25), and PAPP-A is associated with angiographic plaque complexity (26). The most interesting finding, however, points to both PAPP-A and MMP-9 as strong predictors of atherosclerotic progression (27) and future cardiovascular events, including death (15–16, 28).

N-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived from fish oil, have been shown to exert anti-atherothrombogenic effects (29, 30). These properties have been demonstrated in populations with a naturally high dietary intake of marine n-3 fatty acids, as well as in randomized controlled trials (31–33). Some of the effects of these fatty acids are brought about by modulation of the type of eicosanoids produced. Through replacement of arachidonic acid (AA) in phospholipids, the production of leukotrienes and prostaglandins is shifted in favour of less inflammatory and thrombotic derivatives. Anti-inflammatory and anti-thrombotic effects are also mediated at the level of gene transcription, suppressing the production of IL-1, IL-6, tumor necrosis factor α (TNFα) and TF (34).

N-3 fatty acids may affect the proatherogenic mediators CD40L, PAPP-A and MMP-9 by several mechanisms. First of all, reduced production of platelet activating factor (PAF) and tromboxane A2 (TXA2) might reduce the activation of platelets, the most important source of CD40L. Reduced production of interleukins and TNFα, potent stimulators of all three mediators, could also clearly affect their levels. Moreover, n-3 fatty acids may directly inhibit the activation of the transcription factor NF-kB (34, 35), reducing the production of cytokines, chemokines and metalloproteinases at the level of gene expression. Through the latter mechanism, n-3 fatty acids may actually antagonize the effect, and not only the production, of CD40L, a potent activator of NF-kB.

In support of these theories, Weatherill et al. (36) demonstrated that n-3 fatty acids may inhibit the CD40-system in cell cultures. Treatment with EPA and DHA in mice also reduced the levels of MMP-9 mRNA in tumor cells (37, 38). To our knowledge, no clinical trials have evaluated the effect of n-3 fatty acids on these new proatherosclerotic markers. In this setting, the aim of our study was to investigate the effect of high doses of n-3 fatty acids on sCD40L, PAPP-A and MMP-9 in patients with known CAD.

Materials and methods

Subjects
This prospective study was designed as part of the OFAMI-study (Omacor Following Acute Myocardial Infarction study) (29), a randomized, parallel, double-blind study assessing the effects on clinical outcome and serum lipids of intervention with highly concentrated n-3 fatty acids introduced early after an acute myocardial infarction (AMI).

Three hundred patients (238 men and 62 women) with an AMI verified by WHO criteria (39), were recruited at Stavanger University Hospital from September 1995 until December 1996. Written informed consent was obtained from each patient. Regular supplementation of other fish-oil products was discontinued prior to intervention. Exclusion criteria were as previously published by Nilsen et al. (29). All participants were included between the 4th and the 6th day following the acute MI. Included subjects were randomly assigned to receive gelatine capsules (OMACOR, Pronova A/S, Oslo, Norway), containing 850–882 mg EPA and DHA as concentrated ethylesters (n=150), or 1 gram of corn oil (n=150), both administered in a daily dose of 2 capsules twice a day for at least one year. This dose of PUFAs has previously been shown to be therapeutically useful for lowering triglyceride levels and blood pressure (30). Alpha-tocopherol (4 mg) was added to each capsule to protect the PUFAs against oxidation. The intervention was double-blind. Treatment was initiated immediately after inclusion and collection of baseline blood samples.

Patients grouped according to statin use
Simvastatin in a daily dose of 20–40 mg was initiated prior to discharge and continued beyond 12 months follow-up in 95 patients. HMG-CoA inhibitors in vitro have been shown to dimin-
ish the expression of CD40/CD40L on human vascular cells (4), possibly through direct gene regulatory activities. By their beneficial effect on the lipid profile, statins might also have indirect effects on CD40L (4). To account for a possible statin effect, the levels of sCD40L, PAPP-A, and MMP-9 in the 95 statin-users were compared to 79 patients completely free of statins during the entire evaluation period of 12 months. The remaining study participants were not suitable for analyses of statin effects.

**Follow-up and laboratory measurements**

Clinical follow-up, including blood tests, was performed at inclusion, 6 weeks, 6 months and after one year. All clinical events, smoking habits, ongoing medication and the average number of fish meals per week were recorded. Serum samples from 60 patients were randomly prepared to have their fatty acid profile in serum phospholipids analysed at baseline and after 12 months of treatment; fifty-six of these patients had complete data.

All blood samples were obtained following 15 minutes of rest, preceded by 12 hours of fasting. Serum for analyses of sCD40L, PAPP-A, and MMP-9 was prepared by centrifugation for 15 minutes at 2000g at 4°C after clotting of whole blood at room temperature, thereafter immediately frozen and stored at −80°C until the analysis could be performed. Analyses were performed at Stavanger University Hospital by highly qualified laboratory personnel with no knowledge of the randomization code. All samples were assayed in duplicates.

Concentrations of sCD40L in serum were measured by an enzyme-linked immunosorbent assay (ELISA) (Manufactured for BioSource International Inc., by Bender MedSystems GMBH). The limit of detection of sCD40L was 0.095 ng/ml. Overall intra-assay and inter-assay coefficient of variation (CV) was calculated to 4.0% and 6.8%, respectively.

The DSL-10–27200 ACTIVE® US PAPP-A ELISA, an enzymatically amplified “two-step” sandwich-type immun assay, was used for determination of serum concentrations of PAPP-A (Diagnostic Systems Laboratories Inc., Webster, USA). The minimum detection limit was 0.24 µU/ml. The manufacturer reported the intra- and inter-assay CV to range between 2.19–8.82%, and 3.73–4.79%, respectively.

The Quantikine MMP-9 ELISA Immunosay (R&D Systems Inc., Minneapolis, USA) was employed for the measurement of total MMP-9 (92 kDa pro- and 82 kDa active forms) in serum, with a minimum detection level of less than 0.156 ng/ml. CV was reported to be 1.9–2.9% and 6.9–7.9% for intra- and inter-assay precision, respectively.

Previous substudies had already analyzed circulating levels of some adhesion molecules, ultrasensitive-CRP (µCRP) (40) and some other markers of proinflammation and coagulation, such as activated factor XII (FXIIa) (41), at baseline and at 12 months follow-up.

Measurements of fatty acids in serum phospholipids were previously performed (40), as described by Nordøy et al. (42).

The study was approved by the Regional Board of Research Ethics and the Norwegian Health Authorities, and conducted in accordance with the Helsinki Declaration of 1975 as revised in 1983.

**Statistical methods**

Differences between groups at baseline were tested by the Mann-Whitney Rank-Sum test. Changes in parameters from baseline to 12 months follow-up were calculated for each individual patient and subjected to statistical analysis, using the Wilcoxon Signed Rank Test to evaluate whether changes in parameter values from baseline to follow-up were significantly different from zero. The Mann-Whitney Rank-Sum test was used to compare changes between treatment groups. All measurements are given as median values with interquartile ranges.

Spearman’s correlation coefficient was calculated to identify relations between different variables. In addition, the relationship between creatinine and PAPP-A was analysed by linear regression analyses.

Taking into account the Bonferroni correction, the number of patients in each group was sufficient to obtain a 30% reduction of each marker by the omega-3 intervention with a statistical power of at least 80%. Statistical analyses were performed with the statistical package SAS version 13.0. A statistically significant level of p < 0.05 was applied for all tests.

**Results**

**Compliance**

Among survivors at 12 months, 86% in the n-3 fatty acid group and 84% in the corn oil group had a mean drug compliance of >90% at that time of follow-up. As previously shown by Grundt et al. (40), the amount of EPA, DHA and total n-3 PUFAs increased significantly from baseline to 12 months follow-up in the group receiving capsules containing n-3 fatty acids. The amount of n-6 fatty acids showed a non-significant increasing trend in the corn oil group. The changes in the amount of n-3 and n-6 fatty acids during intervention differed significantly between the groups at follow-up.

**Baseline characteristics**

There were no statistically significant differences in baseline demographics and clinical characteristics between treatment groups (Table 1). In both groups there was a predominance of men (76.7% and 82%), and the median age at infarction was 65 years (range 28 to 87 years). Prior to intervention, 30.2% of patients randomized to the n-3 group had been taking fish-oil supplements, as compared to 24.7% in the corn oil group. The difference was not statistically significant, and as previously shown by Grundt et al. (40), there was no difference in the concentrations of lipid fractions or fatty acid profiles in serum phospholipids at baseline. Moreover, there was no inter-group difference in weekly fish meals throughout the study-period. Medication at baseline and during follow-up did not differ between the groups, β-blockers and aspirin being the most commonly used drugs (Table 1). Statins were mainly introduced during follow-up, but their use was similar in both groups.

**Baseline values of PAPP-A, sCD40L and MMP-9**

There were no statistically significant differences in the serum concentrations of PAPP-A, sCD40L and MMP-9 between the two randomized patient groups at baseline.
However, significant differences in baseline values were found when comparing patients under the age of 65 to those of and above 65 years of age. For PAPP-A the lowest median value was found in the youngest part of the population (0.45, 0.27–0.66 mU/L (n=145)), and this differed significantly from the level in patients ≥65 years of age (0.54, 0.33–0.79 mU/L) (n=154), p = 0.004. Contrary to PAPP-A, MMP-9 was found to be significantly higher in patients <65 years of age (440.50, 300.00–499.50 ng/mL) (n=121), p = 0.003. For sCD40L did not correlate with µCRP, TG, total-cholesterol, HDL-cholesterol and HDL/cholesterol-ratio, neither at baseline nor at 12 months.

**Changes of MMP-9 during intervention**

Serum concentrations of MMP-9 decreased non-significantly in both groups during intervention, after the Bonferroni correction, the median level of MMP-9 decreasing from 360.50 (271.50–507.50) ng/mL to 308.00 (206.50–526.50) ng/mL in the n-3 group, and from 430.00 (268.00–584.00) ng/mL to 324.00 (172.50–631.25) ng/mL in the corn oil group, respectively (Table 2). No inter-group differences were noted (p = ns). Moreover, there were no subgroup differences within or between the randomized patient groups.

**Correlations**

sCD40L did not correlate with µCRP, TG, total-cholesterol, HDL-cholesterol and HDL/cholesterol-ratio, neither at baseline nor at 12 months.
A weak negative correlation was noted between PAPP-A and TG ($r = -0.143, p = 0.015$) and $\mu$CRP ($r = -0.166, p = 0.004$), but only at baseline. A weak negative correlation at baseline was also noted between PAPP-A and peak CK-MB concentration ($r = -0.116, p = 0.04$). No significant correlation was found between this marker and total-cholesterol, HDL and HDL/cholesterol-ratio. Between PAPP-A and creatinine, there was no correlation at baseline ($r = 0.05$) or at 12 months ($r = 0.01$), and in a linear regression analysis the initial ability of creatinine to predict PAPP-A at 12 months ($p = 0.04$) disappeared after adjustment for age ($p = 0.08$).

For MMP-9 a relatively strong positive correlation with $\mu$CRP was noted at baseline ($r = 0.353, p < 0.001$), but not after 12 months ($r = 0.012$). A positive correlation at baseline was also found between MMP-9 and peak CK-MB concentration ($r = 0.230, p < 0.001$). At baseline a weak correlation was noted between this parameter and total-cholesterol ($r = -0.162, p = 0.011$), whereas no correlations were observed for the other lipid fractions. The correlation between MMP-9 and sCD40L was relatively strong both at baseline ($r = 0.271, p < 0.001$) and at 12 months ($r = 0.358, p < 0.001$), whereas no correlation was found between MMP-9 and PAPP-A.

For the 60 patients with an established fatty acid profile in serum phospholipids, no significant correlation was found between sCD40L, PAPP-A or MMP-9 and total n-3, total n-6, EPA or DHA, neither at baseline nor at 12 months.

**Discussion**

In our population of patients with a recent myocardial infarction, we observed no benefit of intervention with high doses of n-3 fatty acid as compared to corn oil on the serum concentrations of PAPP-A, sCD40L and MMP-9, three recently discovered pro-inflammatory and prothrombotic markers of ACS.

Lack of response to the omega-3 intervention has previously also been reported for other inflammatory and coagulation markers in this study (40–41). The lack of treatment effect has partly been ascribed to corn oil masking the effect of omega-3,
supported by some studies reporting anti-atherogenic properties of corn oil in both animals (43) and humans (44). Experimental studies with n-6 PUFAs also demonstrated anti-arrhythmic properties of n-6 fatty acids in rats, but not to the same degree as for n-3 PUFAs (45). The majority of studies, however, suggests a link between high dietary omega-6: omega-3 ratios and increased risk of CVD.

Moreover, our intervention consisted of 4g per day of n-3 PUFAs as compared to only 1g per day in the GISSI-prevention study (31) which demonstrated a beneficial effect of intervention on cardiovascular mortality. Furthermore, our study population had a reasonably high background level of n-3 PUFAs, with a median intake of three fish meals per week throughout the study-period. A dose-response relationship of n-3 PUFAs on lipids and hemostatic variables has previously been reported (46), but in our study we might have exceeded an optimal threshold level for n-3 fatty acids, outweighing the beneficial effects observed with lower doses.

The observed change in sCD40L, PAPP-A and MMP-9 during intervention was uninfluenced by age and statin treatment and no clinically significant correlation between these markers and any of the lipid fractions was noted. Oxidized LDL, and to a lesser extent native LDL, has previously been shown to augment the expression of CD40/CD40L on human vascular cells, an effect antagonized directly and indirectly by HMG-CoA inhibitors in vitro (4). Despite indications of pleiotropic effects of statin treatment, the influence on sCD40L, PAPP-A and MMP-9 in a clinical setting has been inconsistent, even after improvement of the lipid-status (8–9, 19, 23). Our study supports an indifferent effect of statins on the measured biomarkers.

As a striking feature of our study results, we noted a discrepancy between the behaviour of the investigated markers following an acute MI. For sCD40L and MMP-9, decreasing values were observed in both intervention groups, whereas PAPP-A increased significantly from baseline to 12 months.

Little is known about the natural behaviour of the selected inflammatory biomarkers following an acute MI, and our study was not designed to answer this question. Previous studies measuring MMP-9 concentrations following an acute MI revealed some differences during the initial phase, the main point of discrepancy being related to the actual time of maximum serum concentration. While some studies found a peak on day 1–3 (17–18), others demonstrated highly elevated levels without a further rise already at admission (19). Despite these differences, they all agree on a gradually decreasing level over time. None of these previous studies evaluated, however, whether the observed decrease is significantly different from the initial maximum level.

The same finding of a highly individual release pattern during the initial phase of an acute MI, has been noted for PAPP-A (14, 47). The time from onset of chest pain to the detection of increased levels may vary from 2 to 30 hours with PAPP-A remaining elevated for 2 hours up to more than 48 hours. Early elevations show a rapid decline, whereas later elevations seem to be more persistent. One possible explanation for these discrepancies between different studies might be related to the specificity of the antibodies and the use of different anticoagulants in the treatment of MI. The use of unfractionated heparin may create a pseudo-lowering of PAPP-A by masking the epitopes recognized by the antibodies of the immunoassays (48). However, in our study, patients were treated with low molecular weight heparin which may not exert the same masking effect as unfractionated heparin. Therefore, the measured PAPP-A values may reflect a true suppression of this marker in the early post MI period as compared to the steady state concentration at 12 months follow-up. Without knowledge of the preadmission level in our patient-population, it is not possible to know whether the supplementation with n-3 fatty acids or corn oil may have retarded the return of PAPP-A to its initial levels. Obviously, treatment with n-3 fatty acids had no advantage over corn oil on the steady state values after 12 months.

The different behaviour of the two metalloproteinases PAPP-A and MMP-9 could possibly be explained in part by a different behaviour of these markers during an acute phase reaction. MMP-9, as compared to PAPP-A, displays a relatively strong positive correlation with µCRP and reflecting the acute phase of the index MI. Although not entirely consistent (22, 26), our finding of a positive correlation between MMP-9 and µCRP has also been reported in other studies (20, 28).

A weak negative correlation between PAPP-A and µCRP and a later rise in PAPP-A, would suggest a different behaviour of this metalloproteinase as compared to MMP-9. Thus, PAPP-A may be less engaged in inflammation and may mainly participate in plaque proliferative processes (24). This possible effect of PAPP-A could be related to IGF-I, a mediator of vascular smooth muscle cell proliferation and migration. By proteolytic cleavage of IGF binding protein 4 (IGFBP-4) and IGFBP-5, PAPP-A releases free IGF-I, producing local plaque proliferation. IGF-I receptors are also present on endothelial cells and macrophages, and, when stimulated, they promote excess uptake of LDL-cholesterol as well as release of inflammatory cytokines and chemokines. Through its effect on IGF-I, PAPP-A may play a role in reparative processes after plaque rupture, rather than displaying a triggering effect on plaque formation and rupture. Minor plaque ruptures with subsequent plaque proliferation are thought of as one of the mechanisms driving the growth of atherosclerotic plaques.

No statistically significant correlation could be demonstrated between sCD40L and µCRP. As the baseline blood samples were drawn 4 – 6 days after the index MI, some degree of acute phase reaction may have influenced the sCD40L levels which demonstrated a decrease during the next 12 months. However, our study did not address the acute phase behaviour of this ligand, and there are hardly any published data on this issue.

Interestingly, PAPP-A and MMP-9 also behaved differently in relation to age. Whereas PAPP-A was significantly higher in the oldest part of the population, MMP-9 displayed its highest median level in patients below the age of 65.

A possible dependence of PAPP-A concentrations on renal function has previously been suggested (50). However, in our study we found no significant correlation between creatinine and PAPP-A, neither at baseline nor at 12 months. In a linear regression analysis, creatinine could not predict PAPP-A at 12 months after correction for age. The highest level of PAPP-A in the oldest patients could possibly be explained by its association with angiographic plaque complexity (26). The youngest patients...
would be more prone to single-vessel disease and less plaque burden as compared to the elderly. However, Zouridakis et al. (27) also demonstrated a significant association between MMP-9 and the number of complex coronary lesions, making it more difficult to explain age differences in these markers, solely by the severity of CAD.

MMP-9 is obviously involved in an acute phase reaction and may even be engaged in the remodelling process of the damaged myocardium (49). Moreover, Tayebjee et al. (21) demonstrated a trend toward lower concentrations of MMP-9 in patients well equipped with collaterals. It is well known that younger subjects may experience larger infarcts than the elderly, probably due to less collateral filling of the occluded artery, and in our study we were able to demonstrate a positive correlation between MMP-9 and CK-MB release.

In conclusion, long-term administration of a high dose of n-3 PUFAs had no effect on the levels of sCD40L, PAPP-A and MMP-9 as compared to corn oil. The variations of these markers over time may reflect different mechanistic roles of these biomarkers in atherosclerosis.

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