

Antibody levels to single bacteria or in combination evaluated against myocardial infarction

Lise Lund Håheim^{1,2}, Ingar Olsen¹, Per Nafstad^{3,4}, Per Schwarze⁴ and Kjersti S. Rønningen⁴

¹Institute of Oral Biology, University of Oslo, Oslo, Norway; ²Norwegian Knowledge Centre for the Health Service, Oslo, Norway; ³Institute of Community Medicine, University of Oslo, Oslo, Norway; ⁴Norwegian Institute of Public Health, Oslo, Norway

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Abstract

Background: Evidence is accumulating that oral bacteria are associated with myocardial infarctions (MI). We were interested in studying the differences in the association between single bacteria or bacteria in combination and the relation to C-reactive protein (CRP).

Material and Methods: We examined the levels of antibodies against four major periodontal pathogens *Porphyromonas gingivalis* (PG), *Aggregatibacter actinomycetemcomitans* (AA), *Tannerella forsythia* (TF) and *Treponema denticola* (TD) and CRP in 548 men with a self-reported history of MI to 625 controls who took part in the Oslo II study in 2000.

Results: The mean levels of bacterial antibodies were higher for the cases than the controls, but not significant as standard deviations were large. The level of CRP was higher in the cases than the controls ($p = 0.010$). Logistic regression analyses comparing the upper quartile value with the lower value of one of either four antibodies (anti-AA, anti-TF, anti-TD and anti-PG) were significantly associated ($p = 0.032$) with MI. Equivalent analyses of either three bacteria showed significant associations for anti-AA, anti-TD and anti-PG ($p = 0.036$) and anti-AA, anti-PG and anti-TF ($p = 0.040$). CRP showed an increased relative risk with increasing quartile value; trend, $p = 0.016$, but not in multivariate analysis including the oral antigens.

Conclusions: No single bacterium but rather combinations were related to increasing relative risk for MI independent of known cardiovascular risk factors.

Key words: antibodies; marginal periodontitis; myocardial infarction; oral bacteria

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Marginal periodontitis is a common oral infection worldwide and is generally in a chronic phase with acute exacerbations. Evidence is accumulating that oral bacteria are associated with an increased risk of cardiovascular disease (CVD)

(Mattila et al. 1998, Seymore & Steele 1998, Fives-Taylor et al. 1999, Li et al. 2000), but also the absence of an association based on self-reported periodontal disease as is being used in this study has been reported (Howell et al. 2001). The systemic antibody response has been found to be associated with CVD, but not with the clinical signs of periodontal disease (Beck et al. 2005).

This study examined four major pathogens of marginal periodontitis, *Porphyromonas gingivalis* (PG), *Aggregatibacter actinomycetemcomitans* (AA), *Tannerella forsythia* (TF) and *Treponema denticola* (TD), for association between their serum antibody level

(IgG) and myocardial infarction (MI). PG appears to be involved in the progression of adult chronic periodontitis (Chiu 1999, Qi et al. 2003). AA is more prevalent in aggressive forms particularly in juveniles than in chronic periodontitis (Fives-Taylor et al. 1999, Pussinen et al. 2005). TD increases to large numbers in adult periodontitis but is almost undetectable in oral health. TF is associated with severe periodontitis (Holt & Ebersole 2005). PG, TD and TF are found to aggregate and comprise what is termed as the 'red complex' that is clearly associated with acute adult periodontitis.

In addition, the acute-phase inflammation reactant C-reactive protein

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(CRP) was measured in order to study its association with these oral bacteria and its predictivity for MI as CRP is elevated in infections. Several publications have focused on CRP as a predictor for coronary heart disease, thromboembolic stroke, metabolic syndrome and type-2 diabetes (Keaney & Vita 2002, Edward et al. 2003, Ridker et al. 2003, Pfützner & Forst 2006). The CRP level has been found to be significantly increased in patients diagnosed with periodontitis compared with controls (Noack et al. 2001).

This study aimed at acquiring more knowledge about the relationship between antibodies of periodontal pathogens in serum and CRP as risk factors for MI. Of interest was to study whether single or multiple bacteria are involved. The ELISA technique was chosen to provide information on antibody levels in serum.

Material and Methods

Study population

The participants were all men taking part in the Oslo II study in 2000 (Lund Håheim et al. 2006). They were participants of The Oslo study – cohort of men first screened in 1972/1973 (Leren et al. 1975). The screening in 1972/1973 included a health examination for recording height and weight and taking a blood sample to measure the level of total cholesterol, triglycerides and glucose. Questionnaire information included anamnestic information on previous CVD or diabetes, symptoms of CVD, smoking habits, physical activity at leisure and work and mental stress. The 2000 health screening included further anthropometric measurements. Non-fasting serum total and HDL cholesterol, glucose and triglycerides were measured directly by an enzymatic method using a Hitachi 917 auto analyzer (Roche Diagnostic, Switzerland).

Questionnaire information was more extensive than in 1972/1973 and included anamnestic information on a wider range of diseases, education, diet, drinking, smoking, medication, dental information, physical activity, slimming and other social parameters. The dental information was on past extractions and current infections. Serum samples and EDTA-blood taken at the 2000 health screening were frozen at 80°C for later analyses such as those reported here.

Included as cases were all men with a history of MI in 2000 ($N = 548$) and

controls ($N = 625$). The controls were selected at random within the selected age strata from the total cohort of 5323 men who took part in both the first and the second screening using the SPSS (SPSS Inc., Chicago, IL, USA) computer program for random sampling among men without a history of MI. Among men aged 48–67 years (who were drawn as a 7% sample of the respective age groups in 1972/1973), 5-year strata were used and four controls were drawn as the number of attending men from this age group was low. For the group of men aged 68–77 years, 2-year strata were used. Men attending parallel studies were not invited. The study went through an acceptance process before the study started and was accepted by the Norwegian National Data Directorate and the Regional Ethical Committee for Medicine in Oslo. A letter of consent was signed by all participants at the second screening in the year 2000.

Serum analyses

To detect differences in antibody levels (IgG) against the bacteria PG, TD, AA and TF, an ELISA was performed (Steinsvoll et al. 1997) with minor modifications at the Norwegian Institute of Public Health, Oslo, in the stored serum collected at the 2000 health screening.

Bacterial antigens were cultivated anaerobically at the Institute of Oral Biology, University of Oslo. The protein contents of the bacterial antigens of PG ATCC 33277, AA ATCC 33384, TD ATCC 35405 and TF ATCC 43037 were determined by the DC Protein Assay (BIO RAD, Hercules, CA, USA) after 3–5 min. of ultrasonic treatment. To capture serum antibodies to the different bacterial antigens, the ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with the bacterial antigens at a concentration of 5 µg protein/ml from one of the four bacteria. Each well was incubated with 100 µl solution overnight and the coated wells were stored up to 14 days at 4°C. Before addition to the plates, the human serum calibrator (X 0908, DakoCytomation, Glostrup, Denmark) was diluted to 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280 and 1/2560 and the samples to 1/160, 1/320 and 1/640 (the 170 first also to 1/80). Samples or serum calibrator were distributed to the washed plates and incubated for 2 h at room temperature. After washing, the conjugated secondary antibody (Polyclonal Rabbit Anti-

Human IgG/AP, D0336, 1:1000 dilution DakoCytomation) was added and the plates were incubated at room temperature for another 2 h. One hundred microlitre substrate (pNPP Tablets, N-2765, Sigma, St. Louis, MO, USA) were pipetted into each well and colour development, optical density (OD), was measured using a Sunrise plate reader and Magellan software (v 1.11, Tecan, Austria). The results were obtained as a percentage of the serum calibrator. Sample dilution curves deviating in parallelism from the serum calibrator were reassessed in other dilutions. Mean values were used except when large differences occurred between the different dilutions where the median was used. Quartile cut points for the upper quartile of OD readings were AA 111.00, TF 103.83, TD 73.17, PG 212.33 and CRP 3.92.

CRP analyses were performed at the Clinical Chemical Laboratory, Ullevål University Hospital, using the Tinaquant CRP (latex) high-sensitivity assay from Roche (Basel, Switzerland) adapted to the Hitachi 917 autoanalyser.

Statistical analyses

Descriptive values of risk factors are mean and standard deviation (SD) or percentage and median and interquartile range for antibody levels and CRP. Non-parametric tests were used due to non-normal distribution of the antibody and CRP data. The Mann–Whitney *U*-test was used to compare two independent samples and the χ^2 test for categorical data. Pearson's correlations were run for antibody levels, CRP and time since MI. Log transformation was attempted, but did not alter the conclusions. Interquartile analyses were chosen using the upper quartile *versus* lower. Logistic regression was the main analysis on associations between antibodies against bacteria, CRP level and MI presented as crude and adjusted odds ratio (OR) with a 95% confidence interval and the corresponding *p*-value. *p*-values <0.05 were considered to be significant. The analyses were performed using SPSS version 15.0.1. (SPSS Inc.).

The study had the power to detect an OR of 2.0 at a significance level of 0.05 and a power of 0.80 in a two-sided test. The number needed in each group was 230 for an estimated double increase of the antibody level of oral bacteria in serum based on a population estimate of

Table 1. Baseline characteristics of cases of myocardial infarction and age-matched controls at the screening of the Oslo study in 1972/1973 and 2000*

Risk factors	1972/1973			2000		
	cases N = 548	controls N = 625	p-value	cases N = 548	controls N = 625	p-value
Systolic blood pressure (mmHg)	136.76 ± 16.55	131.59 ± 13.39	<0.001	141.32 ± 21.50	144.3 ± 19.16	0.012
Diastolic blood pressure (mmHg)	87.77 ± 10.64	83.95 ± 9.66	<0.001	80.40 ± 11.86	83.53 ± 10.23	<0.001
Triglycerides (mmol/l)	2.53 ± 2.75	2.01 ± 1.06	<0.001	2.10 ± 1.16	1.87 ± 0.93	<0.001
Height (cm)	177.57 ± 6.22	178.37 ± 6.58	0.035	175.35 ± 6.25	176.28 ± 6.61	0.014
Weight (kg)	78.49 ± 10.13	77.21 ± 9.34	0.024	83.29 ± 12.32	81.89 ± 11.45	0.045
Body mass index (kg/cm ²)	24.88 ± 2.83	24.26 ± 3.53	<0.001	27.06 ± 3.53	26.34 ± 3.26	<0.001
Total serum cholesterol (mmol/l)	7.06 ± 1.34	6.49 ± 1.09	<0.001	5.27 ± 1.08	6.02 ± 1.04	<0.001
Glucose (mmol/l)	5.73 ± 0.96	5.57 ± 0.84	0.002	6.31 ± 2.15	5.7 ± 1.79	<0.001
Daily smoking (% yes)	52.0	39.7	<0.001	15.8	19.6	0.092
Cholesterol reducing drugs				63.8%	12.7%	<0.001
Antihypertensiva				59.5%	28.4%	<0.001

*Values are means ± SD.

a 14.6% prevalence of periodontal disease (Ebersole et al. 1982).

Results

Baseline characteristics

The baseline levels of known risk factors for MI from the first screening in 1972/1973 between cases and controls were all significantly different, confirming their status as cases and controls (Table 1). The factors compared were blood pressure, serum lipids, glucose, height, weight, BMI and daily smoking (none daily smokers included ex-smokers and never smokers). Treatment with cholesterol-reducing drugs in 63.8% of the cases versus 12.7% in the controls in part explains the lower total cholesterol level in cases than controls in year 2000. Similarly, medication for hypertension was more common among cases than controls (59.5% versus 28.4%). The time since infarction ranged from 0 to 57 years. The mean time was 10.8 years and the median 9 years. Time since infarction was not correlated to any of the bacterial antibody levels or CRP.

Serology

The ELISA results presented in Table 2 are the mean OD values. The mean OD results for each bacterium were not significantly different between cases and controls due to the SDs being very large. For AA, PG and TD, the mean values were the highest among the cases. The mean CRP level was significantly higher among cases than controls. The bacteria antibody level varied in cases of MI. TD and TF had the highest

Table 2. Optical density (OD) readings of antibody levels from ELISA analyses in percent of serum calibrator (ELISA) and level of C-reactive protein for cases of myocardial infarction and controls*

Risk factors*	Cases N = 548	Controls N = 625	p-value
PG (OD)	94.4 (54.0, 213.4)	85.7 (51.0, 209.7)	NS
TF [†] (OD)	54.7 (34.0, 102.5)	57.0 (36.0, 104.4)	NS
TD (OD)	43.0 (26.4, 72.3)	44.7 (28.3, 73.5)	NS
AA (OD)	61.0 (31.7, 123.0)	55.3 (32.7, 103.0)	NS
CRP (mg/l)	2.2 (1.0, 4.6)	1.8 (0.8, 3.5)	0.010

*Results are median and interquartile range.

[†]TF: cases – 13 missing, controls – 15 missing.

NS, no significance, PG, *Porphyromonas gingivalis*; TF, *Tannerella forsythia*; TD, *Treponema denticola*; AA, *Aggregatibacter actinomycetemcomitans*; CRP, C-reactive protein.

mean level recorded for the first quartile, PG for the third quartile and AA for the fourth quartile of CRP.

Both antibody and CRP results were analysed as continuous variables and by interquartile analyses. Only the quartile value of the antibody against AA showed a trend ($p = 0.07$; χ^2 test). The quartile values were used in further analyses (Table 3). The statistical models expanded from testing antibody against one bacterium, and then any of the two, three and four bacteria. The first analyses gave crude results, the second set of analyses were adjusted for CRP and the third set was adjusted for the known cardiovascular risk factors measured in 1972/1973 of daily smoking, total serum cholesterol, triglycerides, BMI, height, non-fasting glucose and systolic blood pressure.

First, the bacteria, single or dual, were subjected to crude analyses and analyses were adjusted for CRP, but they did not show a significant association (results not shown). Further, the risk of MI increased with increasing quartile value of CRP (Table 3). CRP level trends were clearly

opposite between cases and controls, with cases having a positive correlation to MI (Fig. 1).

Combinations of any one of three of the antibody measurements resulted in four combinations of bacteria. The two models, anti-PG, anti-TD, anti-AA and anti-PG, anti-TF, anti-AA, had a crude OR of equal size (Table 4). The models anti-TD, anti-TF, anti-AA and anti-PG, anti-TF, anti-TD had an OR of the same or a slightly reduced strength. These models were run in analyses adjusted for CRP only and for CRP and known cardiovascular risk factors. The model anti-PG, anti-TD, anti-AA was the only model that retained its significance in adjusted analyses. The combination of anti-AA, anti-TF and anti-PG changed from significant to borderline in adjusted analyses.

The OR for the model of four antibody measurements did not change appreciably from that in crude analysis, adjusted for CRP and adjusted for known risk factors for MI and CRP. The analysis of CRP (quartile values) significantly increased the crude risk estimate. However, after

Table 3. Frequency of participants across quartiles of antibody levels by optical density (OD) readings from ELISA analyses and CRP (mg/l) comparing cases to controls

Bacteria and CRP	25, 50, 75 percentile	Group allocation	First quartile	Second quartile	Third quartile	Fourth quartile	Total
PG	52, 90, 212	Case	126 (22%)	127 (23%)	154 (28%)	141 (25%)	548
		Control	157 (25%)	156 (25%)	156 (25%)	156 (25%)	625
TF	35, 56, 104	Case	151 (28%)	125 (23%)	128 (23%)	131 (24%)	535
		Control	153 (25%)	152 (25%)	151 (25%)	154 (25%)	610
TD	27, 44, 73	Case	154 (28%)	131 (23%)	128 (23%)	135 (24%)	548
		Control	153 (24%)	162 (25%)	158 (25%)	152 (24%)	625
AA	32, 57, 111	Case	145 (26%)	108 (19%)	128 (23%)	167 (30%)	548
		Control	158 (25%)	153 (24%)	158 (25%)	156 (24%)	625
CRP	0.9, 1.9, 3.9	Case	97 (17%)	126 (23%)	151 (27%)	171 (31%)	545
		Control	151 (24%)	150 (24%)	170 (27%)	154 (24%)	625

PG, *Porphyromonas gingivalis*; TF, *Tannerella forsythia*; TD, *Treponema denticola*; AA, *Aggregatibacter actinomycetemcomitans*; CRP, C-reactive protein.

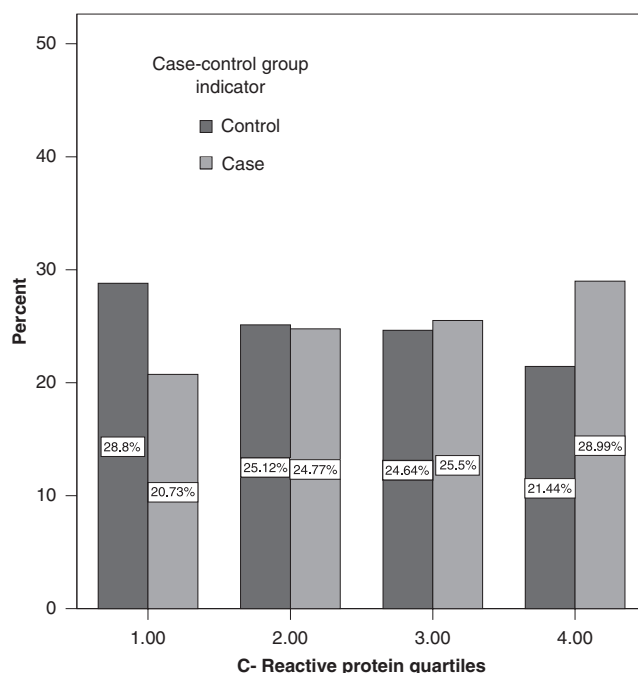


Fig. 1. Comparison of the frequency in percent of cases of myocardial infarction and age-matched controls by quartile distribution of C-reactive protein.

adjusting for any one of the four bacterial variables and known risk factors, the added effect was no longer significant.

Discussion

These results showed elevated antibody level against four putative periodontal pathogens to be associated with a 30% increased relative risk of MI. A combination of antibodies against three bacteria AA, PG and TD gave the same relative risk estimate. Other combinations of three antibodies gave slightly

weaker associations. Adjusting for CRP in the analyses did not alter the conclusions. The relative risk estimates changed marginally when known risk factors for MI were included in multivariate analyses and significance levels were about the same. This showed that the antibody level and thus oral infections were independent of the well-known risk factors in predicting MI. However, the cases had a significantly higher level of CRP than the controls and crude analyses showed a clear trend in the risk of MI with increasing level of CRP. Using information on antibodies of the specific infection, a stronger prediction than that

of CRP alone was observed. The results suggest the involvement of infections in the aetiology of MI (Keaney & Vita 2002, Beck et al. 2005).

The serum was collected in the 2000 screening at varying lengths of time since the MI occurred. No correlation between the antibody level and CRP with time since infarction was observed. The ELISA method gave the advantage of having measurements of antibodies on a continuous scale rather than on a dichotomous one. This allowed a greater scrutiny of the effect of antibodies against the different bacteria and their disease association.

The current study design does not allow us to certify that the oral infections started in time before the MI events and excludes the possibility of reverse causality. However, as marginal periodontitis is a chronic infection with acute manifestations, the chronic phase is the dominant state. The oral bacterial load in a patient is not influenced by an MI event as such and MI does not cause marginal periodontitis. Thus, the infection is seen here as a contributor to increase the risk for MI and not as a consequence of having an MI.

The participants were members of a well-described cohort of men, and the information from the 1972/1973 screening used in the analyses confirmed the distinction between the cases of MI and the controls of this study. The disease status and history of MI in 2000 were self-reported information of the intervening period. Self-reported information is expected to be less precise, but has been found to be valid concerning the number of remaining teeth and use of removable dentures (Buhlin et al. 2002). They found self-reported information to be less reliable concerning specific

Table 4. Logistic regression analyses for relative risk of myocardial infarction by combinations of antibodies of bacteria, which cause marginal periodontitis

Analytic models: upper quartile level of antibodies (OD reading) versus lower* or CRP	Univariate (crude) analysis			Adjusted analysis I [†]			Adjusted analysis II [‡]		
	odds ratio	95% CI	p-value	odds ratio	95% CI	p-value	odds ratio	95% CI	p-value
Model 1									
One of any four bacteria PG, TF, TD, AA	1.30	1.02–1.65	0.032	1.29	1.02–1.65	0.037	1.31	1.01–1.69	0.039
Model 2									
One of any three bacteria PG, TD, AA not TF	1.29	1.02–1.62	0.036	1.29	1.02–1.64	0.033	1.29	1.01–1.66	0.042
Model 3									
One of any three bacteria TF, TD, AA not PG	1.19	0.94–1.50	0.148	1.18	0.93–1.49	0.164	1.19	0.93–1.52	0.175
Model 4									
One of any three bacteria PG, TF, AA not TD	1.28	1.01–1.62	0.040	1.26	0.997–1.60	0.053	1.23	0.96–1.58	0.101
Model 5									
One of any three bacteria PG, TF, TD not AA	1.13	0.90–1.43	0.304	1.13	0.89–1.42	0.322	1.12	0.88–1.43	0.361
Model 6									
CRP – trend			0.018						0.68
Quartile 1	1.00			–	–	–	1.00		
Quartile 2 versus 1	1.31	0.92–1.85	0.131				1.12	0.78–1.63	0.54
Quartile 3 versus 1	1.38	0.99–1.94	0.059				1.08	0.75–1.55	0.70
Quartile 4 versus 1	1.73	1.24–2.42	0.001				1.24	0.86–1.79	0.24
One of any four bacteria PG, TF, TD, AA	1.33	1.04–1.68	0.021				1.30	1.01–1.68	0.041

Bold type indicates that 95% CI does not include 1.0.

*Analytic model of number of bacteria (and CRP in last model) with upper quartile antibody level versus lower value. The 75 percentile of OD values of antibodies were: PG = 212, TF = 103, TD = 73, AA = 111 and CRP = 3.9 mg/l.

[†]Analyses adjusted for C-reactive protein (CRP) in 2000 (quartile values).

[‡]Analyses adjusted for known risk factors for acute myocardial infarction; CRP in 2000 and daily smoking, total serum cholesterol, systolic blood pressure, body mass index, triglycerides and non-fasting glucose adjusted for time since last meal measured in 1972/1973. In the last model, the combined variable of CRP and any one of the four bacteria was used.

PG, *Porphyromonas gingivalis*; TF, *Tannerella forsythia*; AA, *Aggregatibacter actinomycetemcomitans*; OD, optical density.

periodontal variables, but still valuable for epidemiological studies. Balancing the self-reported information was the use of baseline values of known risk factors for CVD to control for confounders in the logistic regression analyses.

Study results indicated that an increasing bacterial load rather than single bacteria were associated with MI. The results show that single or dual combinations of bacteria were not significantly associated. This indicates that not any one of the bacteria examined was causal but that the presence of any three or any four of them contributed and, as a consequence, combinations of the bacteria influence disease occurrence.

In the Dental ARIC study (Beck et al. 2005), the risk for CHD of antibodies to single bacteria was shown to vary between smokers and non-smokers. This risk was not associated with the current periodontal status of the patient. Thus, the immune response of each individual most likely modulated the risk seen in prevalent cases of CHD. In

the Physicians Health Study I (Howell et al. 2001), self-reported periodontal disease was not found to be an independent predictor of CVD. These two prospective cohort studies did not find the clinical status of periodontal disease, whether self-reported or by clinical examination, to be independent predictors of subsequent CVD.

The antibody level of the bacteria varied according to the quartile levels of CRP. This may indicate past and present infections with variation in the persistence of elevated levels of antibodies after acute or chronic infections (Ximenez-Fyvie et al. 2000). Antibody levels may vary within disease categories as has been observed for AA in gingival crevicular fluid, irrespective of aggressive or chronic periodontitis (Ebersole 2000).

There is good evidence behind the knowledge of oral infections leading to the release of inflammatory products into the blood stream and for oral bacteria and their toxins entering the circulation during the course of infections

(Herzberg & Weyer 1998, Seymore & Steele 1998, Li et al. 2000, Pussinen et al. 2005). Oral bacteria have been isolated from atherosclerotic lesions (Chiu 1999) and are known to be thrombogenic (Herzberg & Weyer 1998, Lamont & Jenkinson 1998, Fives-Taylor et al. 1999, Sharma et al. 2000). A meta-analysis of prospective and retrospective cohort studies found the risk of CVD in periodontitis to be 1.20 and that for stroke to be 2.85. Also, the risk of peripheral vascular disease risk was elevated (Meurman et al. 2004). Our risk estimates were found to be lower than in some other studies. However, the results are expected to differ between a cross-sectional study compared with a prospective study, the reason being that fatal cases are not included as in a prospective cohort study and thus an underestimate is observed.

Conclusions

The results add more evidence to the hypothesis of an association between

oral infections and risk of MI. No single bacterium among those studied appeared to be associated but rather the risk increased with multiple bacteria independent of known cardiovascular risk factors. The results indicate that the actual infection measured by antibody levels is a stronger predictor of MI than the general inflammation reactant CRP.

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Address:
Lise Lund Håheim
Norwegian Knowledge Centre for the Health Services
PO Box 7004 St. Olavs Plass
N – 0130 Oslo
Norway
E-mail: Lise.Lund.Haheim@nokc.no

Clinical Relevance

Scientific rationale for the study: One factor not explored sufficiently for the risk of myocardial infarction is oral infections. Research on a causal relation and biological mechanisms is ongoing. We examined whether there were differences in strength of

association for single bacteria or combination of bacteria with MI. The levels of antibodies to the four known periodontal pathogens PG, AA, TF and TD and CRP were studied.

Principal findings: This study indicates that the bacterial burden rather

than single bacteria are associated with MI. CRP was associated with MI in unadjusted analysis.

Practical implications: The study increases our awareness that oral infections may have systemic implications.