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Chlamydia pneumoniae is a risk factor for coronary heart disease in symptom-free elderly men, but *Helicobacter pylori* and cytomegalovirus are not

J. M. OSSEWAARDE[1]*, E. J. M. FESKENS[2], A. DE VRIES[1], C. E. VALLINGA[1], AND D. KROMHOUT[2]

[1] *Research Laboratory for Infectious Diseases, National Institute of Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands*
[2] *Department of Chronic Diseases and Environmental Epidemiology, National Institute of Public Health and the Environment, PO Box 1, 3720 BA Bilthoven, The Netherlands*

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SUMMARY

To test the hypothesis that chronic infection with *Chlamydia pneumoniae*, *Helicobacter pylori* or cytomegalovirus is associated with coronary heart disease risk in elderly men, a nested case-control study in a cohort investigated in 1985 and 1990 in the town of Zutphen, The Netherlands, was designed. Fifty-four cases with a first diagnosed coronary event between 1985 and 1990, and 108 age-matched control subjects free of coronary heart disease during follow up were included in the study. The overall prevalence of antibodies to cytomegalovirus was 74·7%, to *H. pylori* 75·9% and to *C. pneumoniae* 84·0%. A high level of antibodies to *C. pneumoniae* was associated with an increased coronary heart disease risk (OR = 2·76; 95% CI = 1·31–5·81). This association was stronger in cases developing both myocardial infarction and angina pectoris, than in cases developing only one of these. This association was independent of potential confounders. Antibodies to cytomegalovirus or *H. pylori* were not associated with coronary heart disease risk. These results support the hypothesis of a role of chronic *C. pneumoniae* infections in the immunopathogenesis of atherosclerosis.

INTRODUCTION

Chlamydia pneumoniae has been known as a respiratory tract pathogen since 1985 [1]. A few years later, an association between *C. pneumoniae* and coronary heart disease was reported in a Finnish study [2], later confirmed using a 6-months prospective design [3]. Shor and co-workers were the first to realize that the unusual particles found in foam cells in atherosclerotic lesions by electron microscopic examination could be *C. pneumoniae* [4]. This association between *C. pneumoniae* and atherosclerosis was confirmed in several seroepidemiological studies and the micro-organism itself was detected in up to 60% of the atherosclerotic lesions [5]. Recently, cross-sectional associations between infection with *C. pneumoniae* or *Helicobacter pylori* and coronary heart disease were reported [6]. Also, C reactive protein, an acute phase inflammatory protein, was shown to be involved [7]. A role for cytomegalovirus in atherogenesis has also been considered, although its impact seems to be higher in transplant patients than in healthy individuals [8].

Currently only data are available from studies with subjects having coronary heart disease or developing disease within 6 months. Long-term prospective data to rule out possible effects of a recent infection are not available. Therefore, we addressed the question whether infection with *C. pneumoniae*, *H. pylori*, or cytomegalovirus is a risk factor for developing coronary heart disease in a period of 5 years. For this

* Author for correspondence.

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purpose we used available data and specimens from a prospective cohort study, the Zutphen Elderly Study, to design a nested case-control study.

METHODS

Study population

The Zutphen Elderly Study, the Dutch contribution to the Seven Countries Study [9], is a longitudinal investigation of chronic disease risk factors in men aged 65 years or more initiated in 1960. Extensive examinations were carried out in 1985 and in 1990 [10]. For the present study all 54 cases were included who developed a first coronary heart disease event (fatal or non-fatal, myocardial infarction and/or angina pectoris) during the follow-up of the cohort (1985–90). From the remaining men free of coronary heart disease, 108 controls were randomly selected from 5-year age groups corresponding to the frequency of cases in these age-groups. Thus, the age-distribution of cases and controls was similar.

Examinations

The examinations took place between March and June 1985 by trained physicians according to a standardized protocol and included anthropometric and blood pressure measurements, non-fasting venous blood sampling, and the assessment of the medical history [9]. Hypertension was defined by the use of anti-hypertensive medication, a systolic blood pressure higher than 160 mmHg, or a diastolic blood pressure higher than 85 mmHg. Serum total and high-density lipoprotein (HDL) cholesterol were determined enzymatically [10]. The serum was stored at −20 °C until assayed for antibodies in 1994 and 1995. Information on smoking habits was assessed with a standardized questionnaire. Life-time occupation was used as an indicator of social economic status. It was obtained from a self-administered questionnaire, and divided into four categories: (i) professionals, managers and teachers; (ii) small business owners; (iii) administrative personnel; and (iv) manual workers.

Follow up

Information on the presence of coronary heart disease was obtained during the physical examination in 1985 and a similar examination in 1990 using the Dutch translation of the Rose questionnaire [11]. For men who did not participate in the 1990 examination, information on major chronic diseases was obtained from a non-response questionnaire. Coronary heart disease was considered to be present when either myocardial infarction or angina pectoris was diagnosed. The diagnoses were verified with hospital discharge data and written information from the subjects' general practitioners. For myocardial infarction the final diagnosis was based on whether two of the following three criteria were met: a specific medical history, characteristic electrocardiogram changes, and specific enzyme elevations. The diagnosis of angina pectoris was based on information obtained from the Rose questionnaire [11, 12].

Serological methods

All serological analyses were carried out without prior knowledge of case/control status. Immunoglobulin G (IgG) antibodies to cytomegalovirus and IgG and IgA to *H. pylori* were determined by commercial enzyme immunoassays (Organon Teknika, Boxtel, The Netherlands and Pyloriset EIA-G and EIA-A, Orion Diagnostica, Espoo, Finland, respectively). IgG and IgA antibodies to *C. pneumoniae* (strain TW-183), *C. psittaci* (an isolate from a lung biopsy from a psittacosis patient), and *C. trachomatis* (serovar L2, strain 434-B) were determined by in-house developed enzyme immunoassays [13–15]. Briefly, chlamydiae were propagated in six-well microtitre plates. Chlamydial elementary bodies were partly purified by centrifugation through a layer of 35% sodium diatrizoate and used to coat microtitre plates. Antibodies to native elementary bodies and to sodium periodate-treated elementary bodies were determined [13]. Under the conditions used (1% sodium periodate in PBS, pH 6·0, incubated for 10 min at room temperature), sodium periodate destroys the genus-specific lipopolysaccharide (LPS) antigens. The remaining protein antigens react more species-specific [14, 15]. Serum specimens were tested in twofold in one dilution (1:1000 for IgG and 1:500 for IgA determination). For determination of immune complex-associated antibodies, immune complexes were precipitated from 100 μl serum by 3·5% polyethyleneglycol 6000. The complexes were dissociated overnight in Tris-HCl buffer, pH 10·2 at 4 °C, and immediately tested, diluted 1:10, in the enzyme immunoassays [16]. Peroxidase-labelled rabbit anti-human IgG ad IgA (Dako, ITK Diagnostics,

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Table 1. *Descriptive variables in cases and controls*

	Control group ($n = 108$)		Coronary heart disease ($n = 54$)		Statistics	
Risk factor	Mean	S.D.	Mean	S.D.	*t* value	*P* value
Age (yr)	72·4	5·5	72·4	5·6	n.a.*	0·87†
Body mass index (kg/m²)	25·2	2·9	25·6	2·8	0·81	0·42
Total chol. (mmol/l)	6·10	1·02	6·15	1·04	0·29	0·77
HDL chol. (mmol/l)	1·13	0·28	1·07	0·32	n.a.	0·08†
Pack years of cigarette smoking	497·8‡	537·2	663·6	709·4	n.a.	0·13†
Systolic blood pressure (mmHg)	149·6	22·1	153·6	21·6	n.a.	0.20†
Diastolic blood pressure (mmHg)	83·9	10·5	84·4	11·5	0·30	0·77
	Number	Percentage	Number	Percentage	χ^2 value	*P* value
Diabetes mellitus	4/108	3·7	7/54	13·0	4·88	0·04§
Hypertension	36/108	33·3	23/54	42·6	1·33	0·25
Lower education level	44/108	42·3	28/51	54·9	2·18	0·14

* n.a., not applicable.
† *P* value from Mann–Whitney test.
‡ $n = 105$.
§ Calculated by Fisher's exact test.

Uithoorn, The Netherlands) was used to detect bound antibodies. All titres were calculated relative to a reference serum, one for each chlamydial species, that was included in a dilution series on each microtitre plate. The cut-off titre for high-level antibodies was chosen at 3200 and, for immune complex-associated antibodies, at 40. Thus, low titre antibodies (800 and 1600, and 10 and 20, respectively), possibly resulting from cross-reactions, were not included in the final analysis. Under the conditions described, these low antibody titres are equivalent to antibody titres $\leqslant 32$ in the microimmunofluorescence test (MIF test).

Statistical methods

All statistical analyses were carried out on a personal computer using SPSS/PC+ version 5.0.1, 1992 (SPSS Inc., Chicago, USA). The Chi-square test was used to analyse associations between categorical variables, except when the expected frequency was less than five. In this case Fisher's exact test was used. The *t* test was used to analyse differences between numerical variables, when reasonably normal as determined by the Kolomogorov–Smirnov test ($P > 0{\cdot}05$). When not reasonably normal the Mann–Whitney test was used. Logistic regression models were used to calculate the odds ratios for antibody levels while adjusting for the following potential confounders: age, presence of diabetes mellitus, presence of hypertension, body mass index, pack years of cigarette smoking, and total and HDL cholesterol. All tests were two-sided and *P*-values smaller than 0·05 were considered statistically significant.

RESULTS

Levels of most risk factors were higher among cases than among controls, although only the difference in the presence of diabetes mellitus reached statistical significance (Table 1). The median year of the onset of coronary heart disease was 1987. Overall, 74·7% of the subjects had IgG antibodies to cytomegalovirus, 75·9% to *H. pylori*, 84·0% to *C. pneumoniae*. Only 16 subjects had IgG antibodies to native elementary bodies of *C. psittaci* and 3 subjects to *C. trachomatis*. After sodium periodate oxidation these numbers were 6 for *C. psittaci* and 2 for *C. trachomatis*, respectively. Since these numbers were low, these results were not included in further analyses.

Univariate analysis shows that a high IgG antibody titre to native elementary bodies of *C. pneumoniae* was associated with coronary heart disease risk (Table 2). High antibody levels to the other micro-organisms were also investigated, but no significant differences were observed.

In univariate analysis a high antibody level to both native and periodate treated elementary bodies of *C. pneumoniae* was significantly associated with cases

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Table 2. *Prevalence of antibodies to* C. pneumoniae, H. pylori *and cytomegalovirus in cases and controls*

	Control group		Coronary heart disease		Statistics	
Antibody specificity	Number	Percentage	Number	Percentage	χ^2 value	*P* value
Cytomegalovirus IgG	82/108	75·9	39/54	72·2	0·26	0·61
H. pylori IgG	84/108	77·8	39/54	72·2	0·61	0·44
H. pylori IgA	33/108	30·6	21/54	38·9	1·13	0·29
C. pneumoniae IgG	89/108	82·4	47/54	87·0	0·57	0·45
C. pneumoniae IgG (1 : ⩾ 3200)	37/108	34·3	28/54	51·9	4·64	0·03
C. pneumoniae IgG*	92/108	85·2	47/54	87·0	0·10	0·75
C. pneumoniae IgG (1 : ⩾ 3200)*	45/108	41·7	29/54	53·7	2·10	0·15
C. pneumoniae IgA	47/108	43·5	24/54	44·4	0·01	0·91
C. pneumoniae IgA (1 : ⩾ 3200)	22/108	20·4	9/54	16·7	0·32	0·57
C. pneumoniae IgA*	80/108	74·1	34/54	63·0	2·13	0·14
C. pneumoniae IgA (1 : ⩾ 3200)*	33/108	30·6	22/54	40·7	1·67	0·20
C. pneumoniae IC†	93/108	86·1	46/54	85·2	0·00	0·87
C. pneumoniae IC (1 : ⩾ 40)†	26/108	24·1	11/54	20·4	0·28	0·60
C. pneumoniae IC*†	98/108	90·7	49/54	90·7	0·00	1·00
C. pneumoniae IC (1 : ⩾ 40)*†	27/108	25·0	14/54	25·9	0·02	0·90

* Antibodies determined after sodium periodate treatment of chlamydial elementary bodies.
† IgG antibodies determined from isolated immune complexes (IC).

Table 3. *Prevalence of antibodies to* C. pneumoniae *in cases categorized by type of disease and controls*

	Control group ($n = 108$)	Angina pectoris ($n = 15$)		Myocardial infarction ($n = 25$)		AP and MI ($n = 14$)	
Antibody specificity	Percentage	Percentage	χ^2 value	Percentage	χ^2 value	Percentage	χ^2 value
C. pneumoniae IgG	82·4	100·0	3·12	80·0	0·08	85·7	0·10
C. pneumoniae IgG (1 : ⩾ 3200)	34·3	40·0	0·19	52·0	2·72	64·3	4·76*
C. pneumoniae IgG‡	85·2	93·3	0·73	80·0	0·41	92·9	0·61
C. pneumoniae IgG (1 : ⩾ 3200)‡	41·7	53·3	0·73	40·0	0·02	78·6	6·80†

* *P* value < 0·05.
† *P* value < 0·01.
‡ Antibodies determined after sodium periodate treatment of chlamydial elementary bodies.

Table 4. *Odds ratios for the association between antibodies to* C. pneumoniae *and coronary heart disease adjusted for age, the presence of diabetes mellitus, the presence of hypertension, the body mass index, number of cigarette years, and the total and HDL cholesterol levels*

Antibody specificity	Odds ratio	95% confidence interval
C. pneumoniae IgG	1·38	0·52–3·61
C. pneumoniae IgG (1 : ⩾ 3200)	2·76	1·31–5·81
C. pneumoniae IgG*	1·20	0·44–3·28
C. pneumoniae IgG (1 : ⩾ 3200)*	1·71	0·85–3·45

* Antibodies determined after sodium periodate treatment of chlamydial elementary bodies.

who developed both angina pectoris and myocardial infarction, but not with cases who developed either angina pectoris or myocardial infarction (Table 3).

The odds ratios for the association between IgG antibodies to *C. pneumoniae* and coronary heart disease are listed in Table 4. Statistically significant

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odds ratios were obtained for a high level of antibodies to native elementary bodies of *C. pneumoniae* for developing any type of coronary heart disease (OR = 2·76; 95% CI = 1·31–5·81), for developing myocardial infarction alone (OR = 2·95; 95% CI = 1·06–8·22), or for developing both angina pectoris and myocardial infarction (OR = 3·84; 95% CI = 1·04–13·76). Using periodate-treated elementary bodies only the odds ratio for developing both angina pectoris and myocardial infarction (OR = 5·87; 95% CI = 1·42–24·29) reached statistical significance.

DISCUSSION

In this study we showed that *C. pneumoniae* is significantly associated with coronary heart disease risk in elderly men in a 5-year prospective study. Since the first report on *C. pneumoniae* and coronary heart disease in 1988 [2], several other cross-sectional studies and one 6-months prospective study or direct detection of the micro-organism in vessel walls have established this association [5, 17].

C. pneumoniae can cause either an acute or a chronic infection. Although acute infections may constitute a risk for a vascular event, possibly via changes in the blood coagulation system [18], chronic infection with *C. pneumoniae* seems to be more important in the immunopathogenesis of atherosclerosis [19]. The detection of *C. pneumoniae* in vessel wall specimens from young adults and our findings are in agreement with this hypothesis [20]. Our findings do not prove a causal relationship between *C. pneumoniae* and coronary heart disease. However, since we established this association in a prospective study, it strongly indicates the importance of *C. pneumoniae* infections in the immunopathogenesis of atherosclerosis.

Multiple aetiologies of the conditions studied might obscure associations. However, given the age of the study group and the validity of the Rose questionnaire [12], we feel that atherosclerosis is the main aetiology of coronary heart disease in our study group. The prevalence of antibodies to *C. pneumoniae* we observed (overall 84%) is higher than that reported from adult populations (50%). Antibodies to *C. pneumoniae* are related to gender and age [5, 21]. Persistently high IgG titres are especially observed in elderly patients caused by either repeated or chronic infections. In spite of this high prevalence, we showed that a high level of antibodies was associated with coronary heart disease, indicating the relevance of reinfections and/or chronic infections. The chosen cut-off level is comparable to a titre of 64–128 in the microimmunofluorescence test. Since similar results were observed in previous studies in Helsinki [2, 3], our conclusion is that atherosclerotic disease progression is associated with higher levels of antibodies due to chronic infection, repeated infections, enhanced antigen presentation, or any combination.

Recent research shows that antibodies to oxidized LDL (ox-LDL) are implicated in atherogenesis. Methodology, however, is not standardized and results are difficult to compare [22]. Although sodium periodate oxidation of the antigen might have induced additional aldehyde epitopes immunologically resembling those of ox-LDL, using elementary bodies of other chlamydial species did not yield any additional positives. Thus, the main effect of this treatment is enhanced specificity of the *C. pneumoniae* antibody assay by excluding antibodies to the genus-specific LPS antigens [13–15]. Excluding these antibodies could, however, also be a disadvantage, since chronic chlamydial infection of atherosclerotic lesions might release LPS. This LPS could induce or sustain inflammatory reactions and gamma-interferon production, and thus promote oxidation of LDL. In this way, chronic chlamydial infection leads to enhanced production of ox-LDL and enhanced atherogenesis [23]. This might explain the differences we observed between the results obtained with the two types of antigen, but further studies using specific anti-LPS assays are required to answer these questions. The observation that higher levels of C reactive protein are associated with coronary heart disease [7] supports the inflammatory hypothesis, but not restricted to the release of chlamydial LPS alone. Other immune reactions, especially against heat shock proteins, have been demonstrated to be involved in atherosclerosis [24].

We could not observe an association between *H. pylori* infection and coronary heart disease risk. An association between *H. pylori* and coronary heart disease was previously reported in younger patient populations than ours [6, 25]. In elderly patients the association was less clear [26]. However, these studies were cross-sectional. Our study was prospective: none of the subjects had any symptoms of coronary heart disease at the start of the study. The prevalence of antibodies to *H. pylori* rises with age and seems to be poorly correlated with active disease in elderly patients [27].

We also could not observe an association between cytomegalovirus infection and coronary heart disease

risk. The prevalence of antibodies to cytomegalovirus also increases with age. The association between cytomegalovirus and atherosclerosis is not firmly established, although in immunosuppressed patients the association is more pronounced [8].

An association of coronary heart disease with classical risk factors such as cholesterol and cigarette smoking could not be clearly demonstrated in this study. This might be caused by the number of cardiovascular accidents in our study period. Indeed, when the whole cohort was investigated, the association of classical risk factors reached statistical significance [10, 28].

In conclusion, our results support the hypothesis of a role of chronic *C. pneumoniae* infections in the immunopathogenesis of atherogenesis. Important questions now are if causal evidence can be obtained in animal models or if progression of atherosclerosis can be modified by treatment or prevention of these chronic infections.

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