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Chlamydia pneumoniae IgG seropositivity and clinical history of ischemic heart disease in Singapore

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There is still substantial uncertainty concerning the association between *Chlamydia pneumoniae* and ischemic heart disease. This may partly be explained by the adjustment for potential confounders in different population studies. This is the first study in Singapore to look at the association of *C. pneumoniae* seropositivity with ischemic heart disease in a multivariate analysis. A random sample of 714 persons aged between 35 and 69 years was selected from the participants of the Singapore National Health Survey conducted in 1998. Data on clinical measurements and conditions were collected using biochemical tests and interviewer-based questionnaires. Ischemic heart disease was defined by the Rose questionnaire and included history suggestive of angina and/or myocardial infarction. Immunoglobulin G antibodies for *C. pneumoniae* were detected using an indirect microimmunofluorescence test, and seropositivity was defined as IgG titers ≥1:16. There were no statistically significant differences in the prevalence rates of seropositivity to *C. pneumoniae* among the three ethnic groups, that is, Chinese (80.4%), Malays (74.0%), and Asian Indians (73.2%). There was no association between seropositivity and ischemic heart disease after adjustment for age alone (OR 1.00, 95% CI 0.54-1.83) or for age, sex, and other risk factors of atherosclerosis (OR 0.99, 95% CI 0.53-1.84). *C. pneumoniae* Immunoglobulin G seropositivity was not associated with an increased risk of ischemic heart disease as defined by the Rose angina questionnaire in Singapore.

Key words: *Chlamydia pneumoniae*, ischemic heart disease, Rose angina questionnaire, seroepidemiology, Singapore

Coronary artery disease constitutes one of the leading causes of mortality in Singapore and in many developed countries. Although there are established risk factors for coronary heart disease such as hypertension, diabetes mellitus, and obesity [1], about 50% of cases of cardiovascular disease are not associated with these known risk factors [2]. Consequently, despite advances in the pharmacological control of hypertension and diabetes, as well as efforts to discourage smoking, the decrease in the clinical complications of atherosclerosis has been modest, indicating that there are yet unknown contributory factors in the pathogenesis of atherosclerosis. This has spurred an ardent search for new risk factors such as coagulation factors, inflammatory factors, and infectious agents.

Since the first case-control study demonstrated a seroepidemiologic association between seropositivity of *Chlamydia pneumoniae* infection and coronary artery disease in 1988 [3], several studies have reported similar findings. Subsequent detection of *C. pneumoniae* microorganisms in the walls of atherosclerotic coronary [4], as well as other major arteries [5,6], fueled the interest and flurry of scientific activity on this link. Certainly, the prospect of being able to alleviate complications of atherosclerosis by antimicrobial therapy against a pathogen will revolutionize the way we prevent and treat coronary, cerebrovascular, and peripheral arterial disease. Nevertheless, there remains much uncertainty regarding the association between *C. pneumoniae* seropositivity and ischemic heart disease. Conflicting with earlier case-control studies such as the Helsinki Heart Study [7], several large, well-conducted prospective studies have subsequently failed to find such an association [8,9]. Possible reasons for these conflicting results may be differences in the definition of the titer level for *C. pneumoniae* seropositivity. Other investigators have also described geographic [10]

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and ethnic [11] variations in the prevalence of *C. pneumoniae* infection, which may further compound the complexity of such an association with coronary artery disease.

We have shown that there is a very high prevalence of seropositivity for *C. pneumoniae* infection among Chinese, Malays, and Indians in Singapore without any statistically significant difference between ethnic groups [12]. This present study was conducted to look for any association between *C. pneumoniae* seropositivity and ischemic heart disease after adjustment for ethnicity and other coronary risk factors in Singapore.

Patients and Methods

Study population

The study population was assembled from the participants of the National Health Survey, which was conducted in Singapore in 1998 [13]. The National Health Survey population was randomly assembled through a 2-phase sampling process from the 2.16 million Chinese, Malay, and Indian residents aged between 18 to 69 years in Singapore. In the first phase, a sample of household addresses was selected after stratification by house-type. In the second phase, 7500 persons within the specified age group of 18 to 69 years from these addresses were randomly selected after stratification by age and ethnic group. A total of 4723 persons eventually responded and participated in the survey. After stratification by ethnic group to ensure approximately equal numbers in the 3 ethnic groups, a random sample of 1068 persons was selected from the 4723 persons for the antibody assay. We included only the 714 subjects who were between the ages of 35 to 69 years for the purpose of this study.

Information collected by questionnaire includes data on cigarette smoking, history of diabetes mellitus, and hypertension. Blood pressure was measured using a standard mercury sphygmomanometer for all subjects without a prior history of hypertension. The subjects were rested adequately before taking the measurements in a quiet room. Blood pressure was measured with the subject seated and the right arm supported by the table at heart level. The mean of the 2 closest readings taken 30 sec apart was used to compute the systolic and diastolic pressures. In addition, non-diabetic subjects were given 75 g of glucose after an overnight fast for an oral glucose tolerance test. A sample of venous blood was also collected from each subject in the study after an overnight fast of at least 10 h for the determination of their fasting total cholesterol using the enzymatic colorimetric method with a BM/Hitachi 747 analyzer. For smoking status, a non-smoker was defined as one who never smoked before, smoked cigarettes but not every day, or smoked too little in the past to be regarded as an ex-smoker. A smoker included someone who had a present history of smoking cigarettes daily or one who was formerly smoking daily, but had since given up the habit completely. A case of diabetes mellitus was a subject with a known history or with a 2-h plasma glucose concentration ≥11.1 mmol/L by the oral glucose tolerance test. A subject with hypertension was either a known case on medication or one with a systolic blood pressure ≥140 mm Hg or a diastolic blood pressure ≥90 mm Hg. Hypercholesterolemia was defined as a total cholesterol level of ≥6.2 mmol/L, according to the recommendation by the US Department of Health and Human Services.

Clinical history suggestive of ischemic heart disease

The Rose questionnaire for cardiovascular disease was administered by trained interviewers for the purpose of this cross-sectional population study [14]. Based on each subject's answers to the set of questions and the diagnostic criteria of the questionnaire, angina and/or "pain of possible infarction" were scored as present or absent for each subject. The presence of either symptom was used as a marker of ischemic heart disease for the purpose of this study.

Antibody assay

The sera were stored at –20°C for the analysis of *C. pneumoniae* seropositivity by microimmunofluorescence (MIF) as described previously [12,15]. The *C. pneumoniae* Immunoglobulin (Ig) G antibodies were determined by means of an indirect MIF test kit (MRL Diagnostics, Cypress, CA, US), according to the manufacturer's recommendations. This test kit uses *C. pneumoniae* elementary bodies (strain TW183) as antigen, with the inclusion of *C. trachomatis* (8 serotypes, D-K) and *C. psittaci* (strains 6BC, DD34) antigens as controls. All the 3 antigens and a yolk sac control were mounted onto wells on slides, treated to remove interfering genus-reactive lipopolysaccharide and suspended in 3% yolk sac matrix. Twenty-five µL each of the positive control, negative control, and serum samples (diluted 1:16 in PBS) were applied to the wells and incubated in a humid chamber at 37°C for 30 min. After washing with PBS to remove unbound serum antibodies, each slide was overlaid with fluorescein-labeled goat anti-human antibody to IgG and incubated. After repeated washing, drying, and mounting, the slides were examined by 2 experienced technicians using a

Microphot FXA fluorescence microscope (Nikon, Tokyo, Japan) at 400x magnification within 24 h, and graded as 0, 1+, 2+, 3+, or 4+. A sample was defined as seropositive if it reacted with 2 or all 3 chlamydial species but gave the highest grade of reaction with *C. pneumoniae* antigen, or if it reacted only with *C. pneumoniae* antigen.

Statistical and analytical methods

The statistical significance of the differences in seroprevalence between the 2 sexes and among the 3 ethnic groups was calculated using the likelihood ratio chi-square test. Odds ratio for relative risk and their 95% confidence intervals for association of study variables with positive Rose angina questionnaire were calculated using logistic regression methods. All *p* values reported were 2-sided, and *p* values of <0.05 were considered statistically significant. All analyses were performed using the SPSS version 10.0 statistical package (SPSS, Chicago, IL, US).

Results

Sex, ethnic distribution, and associated factors for *C. pneumoniae* seropositivity

The study population consisted of 714 subjects in the following distribution: 240 Chinese (120 men and 120 women), 235 Malays (116 men and 119 women), and 239 Indians (119 men and 120 women). The age distributions were very similar in all the 3 groups, with a mean age of 49.6 ± 9.8 for Chinese, 49.6 ± 10.11 for Malays, and 49.5 ± 9.88 for Indians. Our data show a high seroprevalence of 81.4% for men and 70.5% for women in the age group of 35 to 69 years, with the difference being statistically significant ($p<0.001$). The seroprevalence rates among the different ethnic groups were 80.4% for Chinese, 74% for Malays, and 73.2% for Indians, but this difference did not reach statistical significance ($p=0.125$). Table 1 shows the distribution of study variables such as ethnic group, smoking, hypertension, diabetes mellitus, and hypercholesterolemia among *C. pneumoniae* seropositive and seronegative subjects in this study.

Table 1. Distribution of ethnic group and coronary risk factors among seropositive and seronegative subjects

Factor	Seropositive n = 542 (%)	Seronegative n = 172 (%)
Ethnic group		
Chinese	193 (35.6)	47 (27.3)
Malay	174 (32.1)	61 (35.5)
Indian	175 (32.3)	64 (37.2)
Smoking		
Non-smoker	382 (70.5)	130 (75.6)
Smoker	160 (29.5)	42 (24.4)
Hypertension		
Absent	332 (61.3)	122 (70.9)
Present	210 (38.7)	50 (29.1)
Diabetes mellitus		
Absent	428 (79.0)	144 (83.7)
Present	114 (21.0)	28 (16.3)
Hypercholesterolemia		
Absent	328 (60.5)	101 (58.7)
Present	214 (39.5)	71 (39.5)

Risk factors for a positive history of ischemic heart disease

Using the Rose questionnaire, 2.9% (21/714) gave a positive history of angina, 1.5% (11/714) gave a positive history of previous myocardial infarction, 0.3% (2/714) gave a history of both angina and infarction, while 95.2% (680/714) were negative for history suggestive of both conditions. Thus, the prevalence of ischemic heart disease as defined by a positive Rose questionnaire response for angina or myocardial infarction in our study population was 4.8%. The prevalence was higher among men (5.1%) compared with women (4.5%). Among the 3 ethnic groups, Indians had the highest rate (6.7%), followed by Malays (5.1%), and Chinese (2.5%). Table 2 shows the distribution of sex, ethnic group, and *C. pneumoniae* seropositivity, as well as classical coronary risk factors such as smoking, hypertension, diabetes mellitus, and hypercholesterolemia. Ethnicity was found to be an important risk factor, with Indians having a significantly increased risk when compared with the Chinese (OR 2.11, 95% CI 1.05-4.24). The seroprevalence of *C. pneumoniae* was 73.5% among those with a positive history of ischemic heart disease, and 76% for those with a negative history. Our data therefore did not show an increased risk of ischemic heart disease with *C. pneumoniae* seropositivity either after adjustment for age alone (OR 1.00, 95% CI 0.54-1.83) or for age, sex, and other risk factors (OR 0.99, 95% CI 0.53-1.84). Among the other classical risk factors for cardiovascular disease, our data showed a significantly increased risk among smokers and subjects with hypertension. After adjustment for the other risk factors, diabetes mellitus and hypercholesterolemia did not contribute any statistically significant risk to ischemic heart disease.

Discussion

This study is the first to study the association between *C. pneumoniae* seropositivity and ischemic heart disease in Singapore, with adjustment for sex, ethnicity, and

Table 2. Distribution of sex, ethnic group, *C. pneumoniae* seropositivity, and coronary risk factors associated with positive Rose angina questionnaire

Factor	Rose questionnaire		OR (95% CI)[a]	OR (95% CI)[b]
	Positive n = 34 (%)	Negative n = 680 (%)		
Sex				
Male	18 (52.9)	337 (49.6)	1.0	1.0
Female	16 (47.1)	343 (50.4)	0.88 (0.51-1.52)	1.36 (0.68-2.69)
Ethnic group				
Chinese	6 (17.6)	234 (34.4)	1.0	1.0
Malay	12 (35.3)	223 (32.8)	1.36 (0.65-2.84)	1.25 (0.59-2.64)
Indian	16 (47.1)	223 (32.8)	2.00 (1.01-3.99)	2.11 (1.05-4.24)
C. pneumoniae IgG				
Negative	9 (26.5)	163 (24.0)	1.0	1.0
Positive	25 (73.5)	517 (76.0)	1.00 (0.54-1.83)	0.99 (0.53-1.84)
Smoking				
Non-smoker	19 (55.9)	493 (72.5)	1.0	1.0
Smoker	14 (44.1)	187 (27.5)	1.76 (1.00-3.09)	2.30 (1.13-4.68)
Hypertension				
Absent	16 (47.1)	438 (64.4)	1.0	1.0
Present	18 (52.9)	242 (35.6)	1.73 (0.88-3.39)	2.02 (0.99-4.09)
Diabetes mellitus				
Absent	25 (73.5)	547 (80.4)	1.0	1.0
Present	9 (26.5)	133 (19.6)	1.26 (0.56-2.83)	0.96 (0.42-2.19)
Hypercholesterolemia				
Absent	21 (61.8)	408 (60.0)	1.0	1.0
Present	13 (38.2)	272 (40.0)	0.96 (0.52-1.79)	0.88 (0.46-1.67)

[a]Adjusted for age.
[b]Adjusted for age (continuous), gender, and other factors (overall adjusted).

other risk factors. While there is a high prevalence of *C. pneumoniae* seropositivity in Singapore [12], this study does not provide support for the hypothesis that *C. pneumoniae* infection is a substantial risk factor for clinical ischemic heart disease. In this study involving subjects aged 35 to 69 years, the seroprevalence rates were 81.4% for men and 70.5% for women. Consistent with our previous report, sex, but not ethnic, difference in this age group was statistically significant. We have also previously reported that the classical risk factors of atherosclerosis such as smoking, hypertension, and diabetes mellitus were not associated with *C. pneumoniae* seropositivity [12]. Once again, we see that these factors were not similar in their distribution among the seropositive and seronegative subjects in this study.

In a multivariate analysis of ethnicity and other risk factors of ischemic heart disease in this study, the findings were comparable to previous longitudinal and cross-sectional studies in Singapore [16,17]. Indians have the highest relative risk compared with Chinese and Malays, and the estimated increased risk of twice that of Chinese was similar to previous findings [18]. Indians in Singapore are relatively more obese, have greater insulin resistance, and higher prevalence of dyslipidemias, for example hypertriglyceridemia and low high-density lipoprotein cholesterol syndrome [19]. However, dietary factors cannot explain the differences in serum cholesterol levels between ethnic groups in Singapore independently of other risk factors [20]. Consistent with a previous longitudinal study in Singapore, this study also found that hypertension increased the risk by about 2-fold while hyperlipidemia was not associated with increased risk [18]. However, unlike the previous study, our study found that smoking was a very important risk factor while diabetes was not associated with an increased risk. The lack of association with diabetes mellitus may be attributed to the frequent absence of symptoms of ischemic heart disease in diabetic subjects [21].

Our study employed the Rose angina questionnaire for the determination of ischemic heart disease. The Rose questionnaire has been found to be moderately associated with disease and risk factors, and to be appropriate for use in epidemiological studies [22]. In industrialized countries such as Singapore, which have high morbidity and mortality rates of myocardial infarction, the questionnaire has been documented as a reliable tool for assessing angina prevalence [23]. To increase the sensitivity and specificity of the questionnaire in assessing prevalence of ischemic heart

disease, we have applied it only to the population aged 35 to 69 years. Studies performed in different populations with different criteria for definition of cases and controls would no doubt lead to conflicting results. Hence, further studies are justified to confirm the results of this study in angiographically demonstrated coronary artery disease or ischemic heart disease proven by electrocardiography and functional testing with imaging.

Our study selected a single titer of 1:16 as the definition of seropositivity for *C. pneumoniae*. While the relatively low titer of 1:16 is generally used for the definition of any past infection with *C. pneumoniae*, higher titers of 1:64 and above are often cited to define chronic, persistent infections. It would therefore be interesting to examine if the associations between chronic, persistent infection and past infection with ischemic heart disease could be different. We recently reported that a surrogate marker for antibody titer using the MIF technique is the grade of seropositivity [15]. However, in this study we did not find any significant correlation between grades of seropositivity and a positive history of ischemic heart disease (data not shown). Other case-control studies have found that high antibody titers have a stronger relationship compared to low titers [3,24]. The lack of consistency of findings from seroepidemiological surveys may simply relate to differences in the antibody titers considered in the studies.

IgA antibodies to *C. pneumoniae* have been reported to be a putative marker of chronic infection [7]. It is possible that it could be seropositivity for IgA but not IgG antibodies that is associated with increased risk of ischemic heart disease. Studies examining IgG and IgA have hitherto produced conflicting results [9, 25].

We have previously reported that *C. pneumoniae* seropositivity is not associated with the classical risk factors of ischemic heart disease [12]. Other studies have found an increased association of *C. pneumoniae* infection with smoking [26,27], hypertension [28], or hyperlipidemia [29]. *C. pneumoniae* seropositivity and other coronary risk factors may therefore act as confounders or as effect modifiers on one another [30, 31]. In support of this hypothesis, several studies have shown that the association with *C. pneumoniae* seropositivity weakens or disappears in multivariate analysis after adjustment for other risk factors of atherosclerosis [32] or socioeconomic status [8]. Others have shown that the association with *C. pneumoniae* seropositivity is only observed in subjects without a high risk of coronary artery disease, such as in non-diabetics [33] or in non-smokers [32]. Hence, it remains possible that in patients without other risk factors of atherosclerosis, *C. pneumoniae* seropositivity may still have a strong association with heart disease. However, this association weakens or disappears in the presence of the more important risk factors.

In conclusion, this study confirmed that *C. pneumoniae* IgG seropositivity reflective of previous infection was not associated with an increased risk of ischemic heart disease diagnosed by the Rose angina questionnaire in Singapore. Future studies should concentrate on the association of ischemic heart disease with high titers of IgG or IgA antibodies, which denote chronic, persistent infection.

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