Chlamydia Pneumoniae and Coronary Artery Disease

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Dr. Yuk-ki Wong, BSc(Hons), MB ChB, MRCP.
Wessex Cardiothoracic Unit and Department of Molecular Microbiology, University of Southampton.

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Abstract

It has been proposed that *Chlamydia pneumoniae* (*C. pneumoniae*), a gram negative, obligate intracellular organism, is a cause of atherosclerosis and coronary artery disease (CAD). If confirmed, this would be of great importance to public health since coronary artery disease is the major cause of death in the developed world and infection by *C. pneumoniae* is potentially treatable by antibiotics.

In a study of deaths from myocardial infarction that occurred in a ten year period from 1988 to 1997 in England and Wales, we found that standardised mortality rates (taking into account deprivation) were higher in populations where household size was large. This is indirect evidence that infections in general may be responsible for CAD since transmission of infections is more common where contact between susceptible subjects is high.

To look for specific evidence that *C. pneumoniae* is associated with CAD, we collaborated with investigators at the Clinical Trials Service Unit, University of Oxford and the Woolfson Institute of Preventative Medicine, in a cross sectional and prospective serological study respectively. These studies were characterised by the fact that they were the largest of their type and that they were well controlled. Although we confirmed known associations between *C. pneumoniae* and age and male sex, we failed, like some other recently published studies, to show an association between *C. pneumoniae* and CAD. However, serological criteria for chronic infection are controversial and in a large study, we confirmed that serology is not specific. We therefore developed a method for detecting *C. pneumoniae* DNA in the white cells of circulating blood and used this test in a study of 1205 subjects attending for coronary arteriography. We found that the prevalence of circulating *C. pneumoniae* DNA, indicating current infection, was significantly higher in men with CAD than in normal male controls (8.8 vs. 2.9%). However, this association was not seen in women.

Evidence that *C. pneumoniae* localises to blood vessels has come from studies where its presence has been shown by techniques such as PCR and immunocytochemistry. Such studies have been hampered by the fact that histological evidence of atherosclerosis is ubiquitous and control blood vessels are difficult to find. Taking a different approach, we found in a post mortem study, that in individuals, the presence of *C. pneumoniae* did not correlate with the presence or extent of CAD. Also, in a study of patients undergoing first time and redo coronary artery bypass graft surgery, the organism was just as common in atherosclerotic vessels as in new grafts.

In all, the evidence from this thesis does not support a causative role for *C. pneumoniae* in CAD. It is still possible however, that infection of a susceptible atherosclerotic plaque makes it unstable, thus precipitating acute coronary syndromes such as myocardial infarction. Recent secondary prevention trials have suggested that antibiotics may prevent further coronary events. It is likely that only large scale intervention trials will show whether chronic *C. pneumoniae* infection is clinically important in CAD.

Declarations and Copyright

Data in Chapter 7 was from a collaborative study with John Danesh at the Clinical Trials Service Unit, University of Oxford, who will be submitting this work for his PhD thesis. Other than this, no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Contributors

Chapter 5	Serum samples were kindly provided by Dr Caroline Fall, University of Southampton MRC epidemiology unit. MIF was performed by Dr Jean Marie Sueur and Prof. Jean Orfilla at the Biobanque De Picardie, Amiens, France.
Chapter 7	John Danesh facilitated access to serum samples from the OXCHECK study and analysed the results.
Chapter 8	Prof. Nick Wald gave us access to serum samples from the British United Provident Association prospective study. His statistical team analysed the results.
Chapter 9	Drs Keith Dawkins, Huon Gray, Iain Simpson and John Morgan supervised the reporting of coronary angiograms.
Chapter 10	Damion Thomas collected coronary artery samples from the post mortem room which were then graded according to their histological severity by Dr Patrick Gallagher. Damion Thomas, Martine Thomas and myself tested the samples by PCR in triplicate.
Chapter 11	Mr Victor Tsang, Robert Lamb, Jim Monroe and Steven Livesey supplied us with surgical specimens.

List of Abbreviations

AAA	Abdominal aortic aneurysm
BP	Blood pressure
BSA	Bovine serum albumin
CABG	Coronary artery bypass graft surgery
CAD	Coronary artery disease
CI	Confidence interval
CMV	Cytomegalovirus
CRP	C-reactive protein
dATP	2 deoxyadenosine triphosphate
dCTP	2 deoxycytidine triphosphate
dGTP	2 deoxyguanosine triphosphate
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
dNTP	2 deoxyribonucleoside
dTTP	2 deoxythymidine triphosphate
EB	Elementary body
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoassay
HCl	Hydrochloric acid
HDL	High density lipoprotein
ICC	Immunocytochemistry
IMA	Internal mammary artery
LDL	Low density lipoprotein
LGV	Lymphogranuloma venereum
MI	Myocardial infarction
MIDAS	Manchester Information, Datasets and Associated Services
MIF	Microimmunofluorescence test
MOMP	Major outer membrane protein
MONICA	Monitoring of trends and determinants in cardiovascular disease
NIDDM	Non-insulin dependent diabetes mellitus
ONS	Office of National Statistics
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTCA	Percutaneous transluminal coronary angioplasty
RB	Reticulate body
SAA	Serum amyloid A
SDS	Sodium deoxycholate
SV	Saphenous vein
TRIA	Time resolved fluoroscopic immunoassay
TRIS	Tris (hydroxymethy) aminomethane
Tween	Polyoxyethylenesorbitane monolaurate
UAP	Unstable angina
UHQ	Ultra-high quality water
WHO	World Health Organisation

Single letter nucleic acid code

A	Adenosine
C	Cytidine
G	Guanosine
T	Thymidine

Introduction

Coronary artery disease secondary to atherosclerosis is the major cause of death in the developed world and although several important risk factors have been identified, these cannot fully explain the prevalence and incidence of this important disease. The histological features of atherosclerosis are well documented and it was Sir William Osler who first commented that the inflammatory changes seen may be due to infection[1]. However, evidence that bacterial or other infective agents may be important in human atherosclerosis did not appear until the 1980's. At first, evidence was based on serological associations between coronary artery disease and various viruses such as Coxsackie B[2] and Herpes virus but reports were conflicting[3]. Subsequently, serological associations were described for two bacterial agents, namely *Chlamydia pneumoniae* (*C. pneumoniae*)[4] and *Helicobacter pylori* (*H. pylori*)[5]. Recently, doubts have been cast on the role of *H. pylori* in atherosclerosis[6;7] but evidence that *C. pneumoniae* may be important has been steadily accumulating. Over twenty serological studies have reported an association between this intracellular organism and coronary artery disease while pathological studies using the polymerase chain reaction and immunocytochemistry have demonstrated the organism more frequently in atheromatous compared with normal blood vessels. Furthermore, animal studies with New Zealand White Rabbits suggest that *C. pneumoniae* may at least exacerbate atherosclerosis[8] while preliminary secondary prevention trials have reported that macrolide antibiotics reduce further coronary events following myocardial infarction[9;10]. This background provides the basis for the work carried out in the Department of Molecular Microbiology, University of Southampton over the past two years.

In this thesis, the relatively recent story of how *C. pneumoniae* was first isolated and characterised is described in Chapter 1 followed by a summary of processes thought to be involved in the pathogenesis of atherosclerosis in Chapter 2. The evidence associating *C. pneumoniae* and atherosclerosis is then critically reviewed (Chapter 3).

The reduction in mortality in the British Isles since the nineteenth century is almost entirely due to the elimination of the major endemic infectious diseases such as cholera and tuberculosis. This reduction owed more to improvements in the general quality of

life and to public and personal hygiene than to any specific medical measure. The spread of infection is facilitated by close contact amongst subjects and there is evidence to suggest that chronic infection with *C. pneumoniae* may be related to overcrowding and to the number of children at home[11]. Using mortality data from the Office of National Statistics and data from the 1991 UK National Census (Manchester Information Datasets and Associated services), we sought evidence that deaths due to myocardial infarction over the past decade in England and Wales were associated with markers of overcrowding (Chapter 4).

Most serological studies of *C. pneumoniae* and atherosclerosis to date have been small, cross-sectional and have not always taken into account possible confounding factors such as smoking and social class. In collaboration with the Clinical Trials Service Unit at the University of Oxford, we performed a large cross-sectional serological study of just under one thousand subjects which controlled well for possible confounding factors (Chapter 7). In an even larger study with twice the number of subjects, we collaborated with the Woolfson Institute of Medicine in a study that was prospective in design. This study was based on a unique serum bank collected from healthy, professional men attending for routine medical check-ups with an average of fifteen years of follow up (Chapter 8). Serological tests are currently the best and most widely available method for detecting *C. pneumoniae* infection in populations. However, they are hampered by the fact that it is difficult to distinguish accurately between current and past *C. pneumoniae* infection and also, such tests may not be specific for *C. pneumoniae* (Chapter 5). We therefore investigated whether it was possible to detect for *C. pneumoniae* DNA in the circulating white cells of patients using the polymerase chain reaction since a positive result would indicate current infection. This test was used in a study of 1200 subjects attending for diagnostic and coronary angiography at the Wessex Cardiothoracic Unit (Chapter 9).

Pathological studies have provided direct evidence that *C. pneumoniae* localises to atherosclerotic tissue. The question is whether the organism is genuinely more prevalent in atherosclerotic compared with normal tissue since atherosclerosis is ubiquitous and studies have experienced difficulty in obtaining age-matched control tissue. Also, such studies cannot determine whether infection preceded or followed the development of atherosclerosis. In two studies in collaboration with the Department of

Pathology and the Wessex Cardiothoracic Unit, Southampton General Hospital, we took different approaches compared with existing pathological studies. In a post mortem study, we asked whether the distribution of *C. pneumoniae* in the coronary circulation matched that of the distribution and severity of disease (Chapter 10). In a second study of patients attending for first-time and redo coronary artery bypass graft surgery, we compared the prevalence of *C. pneumoniae* in failed grafts and atherosclerotic native vessels compared with that in new saphenous vein and internal mammary artery grafts (Chapter 11).

Chapter 1 *Chlamydia pneumoniae*.

History.

Chlamydia are obligate intracellular bacteria of which there are 4 known species: *C. trachomatis, C. psittaci, C. pneumoniae and C. pecorum*. They are characterised by a unique dimorphic life cycle within eukaryotic host cells in which the infective form, known as the elementary body (EB) develops into the replicative form, the reticulate body (RB). The infective EB (also known as inclusion body), which is tough, "spore like" and relatively small (200-300nm in diameter), can survive outside the host cell while the more fragile RB (1μm in diameter) divides by binary fission. The intracellular environment provides a habitat free of competition from other bacteria, a rich supply of nutrients and protection from the host immune system. The classification of chlamydia into 4 species is based on phenotypic characteristics (Table 1-1) and this was how *C. pneumoniae* first came to be recognised as a distinct species. The first strain, TW-183, was isolated in 1965 from a child's conjunctiva during a trachoma vaccine trial in TaiWan[12]. However, it was recognised not to be *C. trachomatis* because it failed to kill mice following intravenous injection in the mouse toxicity prevention test[13] and it also failed to cause follicular conjunctivitis in monkeys. When cell culture techniques for *C. trachomatis* became available in 1971, TW-183 inclusion bodies were noted to be pear shaped rather than round and they did not stain for glycogen. Nowadays, it is known that *C. pneumoniae* inclusions can be round as well as pear shaped[14] and antigenic and DNA criteria are used to differentiate between *C. pneumoniae* and other species. In fact, *C. pneumoniae* cannot be differentiated from *C. psittaci* on phenotype alone and initially, TW-183 was thought to be a *C. psittaci* strain. Although TW-183 was an ocular isolate as was another strain, IOL-127, which was obtained from Iran around the same time and which was subsequently shown to be *C. pneumoniae*[15], *C. pneumoniae* is now recognised as a cause of respiratory rather than ocular infections. *C. pneumoniae* was first isolated in relation to respiratory tract disease in 1983 when it was obtained from a University of Washington student presenting with pharyngitis. This isolate was initially called AR-39 (Acute Respiratory) but when it was recognised to be the same as TW-183, *C. pneumoniae* obtained its first name, the "TWAR" organism. In a seminal study published in 1986[16], 13 of 386

students presenting with acute respiratory tract infections were found to have serological evidence of infection by TWAR and the organism was isolated from 8 students. TWAR was formally recognised as a new chlamydial species in 1989 and renamed *C. pneumoniae*[17]. Its entire genome was sequenced and described in 1998[18] and the genetic sequence is available on the Internet (ftp://ncbi.nlm.nih.gov/genbank/genomes/bacteria/Cpneu/cpneu.gbk).

Laboratory diagnosis of *C. pneumoniae* infections.

As chlamydia are intracellular bacteria, they cannot be grown by conventional techniques. *C. trachomatis* was first grown in chick embryos in 1957 and in HeLa 229 cell cultures in 1971[19]. Initially, *C. pneumoniae* grew poorly in these preparations but techniques such as centrifugation of the organism onto cell culture monolayers[20] and other refinements as regards the culture medium[21] have improved results. However, isolation of *C. pneumoniae* from clinical samples presents additional problems. Special transport medium is required and if inoculation onto cell cultures is to be delayed, samples must also be cooled and then frozen at -70°C. Certain clinical samples such as sputum are toxic towards cell cultures. Due to these problems and the fact that specialist expertise and facilities are required, culture is not routinely available. The availability of monoclonal antibodies to *C. pneumoniae* has allowed for its detection in clinical specimens by immuno-staining but this technique too, tends to be restricted to specialist laboratories.

There are two serological tests that are used for the detection of *C. pneumoniae* antibodies. The complement-fixation (CF) test detects antibodies to all chlamydial species and is not specific for *C. pneumoniae*. Also, re-infections by *C. pneumoniae* are not always associated with a CF antibody response and less than one third of infections in the elderly result in a positive CF test[22]. The microimmunofluorescence (MIF) test was devised in 1970 for the detection of *C. trachomatis* antibodies[23]. It depends on fluorescence microscopy to distinguish between the diffuse binding pattern as seen with genus-specific antibody and the sharp, homogenous, ring-like pattern seen with species-specific antibody. In expert hands, this test can distinguish between antibodies to the four chlamydial species and was originally used to type the different *C. trachomatis* serovars. Unfortunately, MIF is subjective, tedious to perform and not suited for mass

screening. However, it is the gold standard test for detection of chlamydial antibodies and is widely used in research although not routinely available.

In view of the difficulties with culture, the polymerase chain reaction (PCR) has become an important research tool which has allowed for the detection of *C. pneumoniae* DNA in clinical specimens. Primers have been designed that are targeted against the *C. pneumoniae* specific regions of the 16 sRNA gene and other DNA sequences that are known to be specific for *C. pneumoniae*[24]. In Southampton, where the DNA sequence for the major outer membrane protein (MOMP) gene was first sequenced[14], the primers used are directed against this gene. Although PCR is a sensitive technique, specimens such as atherosclerotic tissue can contain inhibitors of the PCR reaction and efforts must be made to eliminate them.

Epidemiology of *C. pneumoniae*.

The respiratory illnesses caused by *C. pneumoniae* (pneumonia, bronchitis, pharyngitis and upper respiratory tract infections) are generally indistinguishable from those caused by other organisms in terms of clinical presentation and radiological appearance. As it cannot be detected by blood culture and as *C. pneumoniae* serology is not a routine investigation, data on its prevalence and incidence are relatively sparse. A study which was unique in design and which gives the most useful information on incidence in the general population was that by Aldous and Grayston[25]. In this prospective study, serum from 343 subjects was collected up to three or more times a year for several years and was analysed for a four-fold titre rise in *C. pneumoniae* IgG antibody. Acute infections were not seen in children under the age of 5 and incidence was at a peak in those aged between 5 to 10 with an annual rate of 9.2% which dropped to 1.5% in those over 20. Transmission of infection within families was seen in one third of cases and one third of subjects appeared to be asymptomatic.

In patients presenting with acute respiratory infection, serological evidence of recent *C. pneumoniae* infection has been found in between 3.5[26] to 18.7%[27]. Epidemics have also been reported. In some cases, these have involved young people living or working in institutions[28]. In one instance, 43 of 86 military conscripts had evidence of infection[29]. In other cases, epidemics have affected the population as a whole[30-32] and these have almost all been reported from Scandinavia.

In the few studies where *C. pneumoniae* infection has been confirmed by culture or by DNA and antigen detection techniques, an increase in specific *C. pneumoniae* antibodies has not always been seen. In one study, only 3 of 8 patients with cultured confirmed infection had a rise in antibody titres[27]. This observation has lead to suggestions that some subjects may be chronically infected which explains the lack of a rising antibody response. Chronic infection with *C. trachomatis* and *C. psittaci* is well recognised and there have been reports where *C. pneumoniae* has been persistently isolated from the throats of subjects for periods of up to 12 months[33;34]. The possibility of chronic infection by *C. pneumoniae* is important because this is the basis for supposing that *C. pneumoniae* may be a cause of diseases such as coronary artery disease and asthma. It is not clear how prevalent chronic *C. pneumoniae* infection is but in a survey of 234 apparently healthy and asymptomatic Swedish subjects, 4.7% had *C. pneumoniae* isolated from throat swabs[35]. Although cultures were obtained only once for each subject, this prevalence is as high as that in some populations presenting with acute respiratory illness and suggests that chronic and asymptomatic infection is common. Recently, another study from Sweden found that 46% of 52 blood donors (mean age 49) had circulating *C. pneumoniae* DNA in their white blood cells[36]. It is possible that there are geographical and population differences since only 2 of 104 subjectively healthy individuals in Brooklyn, New York were found to have *C. pneumoniae* from naso-pharyngeal swabs by culture or PCR[37].

If *C. pneumoniae* is eventually shown to be an important cause of diseases such as coronary artery disease, then it would be important to know at what age chronic infection becomes established so that preventative treatment can be directed at the right group. Prevalence studies show that from childhood to age 20, IgG seropositivity increases quickly to 50% and persists or even increases with increasing age[38-40]. This high seroprevalence has been found in several regions around the world except in remote populations such as that in the Soloman Islands (Table 1-2). However, as the incidence of infection in adults is only 1.5% per year and as antibodies are widely quoted as disappearing about 3 years following acute infection[41], the expected seroprevalence in adults should only be 4.5%. It is possible that the high observed seropositivity is an indication that a large proportion of the population has chronic infection driving a persistent antibody response. In such a model, the best time to

eradicate the organism would be around the age of 20 when half the population is infected and after which, the incidence rate is relatively low. However, epidemics are known to occur[32;42] and IgG titres likely decay more slowly than thought. Of 90 students followed up for up to 5 years, only those with an initial low titre of 16 had a geometric mean titre of less than this at 5 years[41]. In this case, the high general seroprevalence might indicate a population characterised not by chronic infection needing treatment but by a high past exposure. The problem is how to prove persisting infection against a background of persisting antibody and what is required are more specific methods for diagnosing both acute but especially chronic infection.

Diseases associated with *C. pneumoniae* apart from acute respiratory illness.

C. pneumoniae has been linked to a remarkable number of diseases. The evidence is strongest for coronary artery disease where numerous serological studies have reported a positive association[7] and the organism itself has been found in atherosclerotic tissue[43]. Similarly, *C. pneumoniae* has been cultured from brains of patients with Alzheimer's Disease but not from controls[44] and associations have been described with sarcoidosis[45], bronchial carcinoma[46], asthma[47] and rheumatoid arthritis[48]. If *C. pneumoniae* is a cause of all these diseases, it would not be a precedent since the tubercle bacillus and syphilis are also known to cause multi-systemic diseases. However, the diseases associated with *C. pneumoniae* do not appear to share many common features in terms of prevalence, age and sex distribution and clinical course (Table 1-3). Nevertheless, the finding of viable organism in damaged tissue cannot be ignored. An intracellular organism which is actively replicating and killing its host cell in the process can be expected to at least exacerbate disease.

Table 1-1. Characteristics of the four chlamydial species.

Characteristic	*C. trachomatis*	*C. pneumoniae*	*C. psittaci*	*C. pecorum*
Natural Hosts.	Humans, pigs, mice.	Humans, horses.	Birds, occasionally humans.	Cattle and sheep.
Main infections in humans.	Trachoma, genital tract infection.	Respiratory tract infection.	Respiratory tract infection. Frequently systemic.	
EB morphology.	Round.	Round or pear shaped.	Round.	Round.
Iodine staining for glycogen.	Yes.	No.	No.	No.
Number of serovars.	At least 15.	1 (possibly more).	Unknown (numerous).	3.
DNA homology relative to *C. pneumoniae*[49].	10%.	100%.	10%*.	10%.

*The genome of *C. psittaci* is extremely diverse and intra-species homology varies from 14 to 95% indicating that new species will be eventually derived from *C. psittaci*.

Table 1-2. Seroprevalence of *C. pneumoniae* in normal subjects.

	London[11] (n=196).	Jerusalem[50] (n=581).	Rhode Island[51] (n=147).	SW Finland[40] (n=1154).	E Finland[40] (n=1188).	Hiroshima[38] (n=600).	Soloman Islands[52] (n=2293)
Recruitment of subjects.	General Practice health screening clinic	Randomly selected from population registry	Fireman and policeman	Random sampling from population stratified according to sex, age, area and degree of urbanisation		"Healthy Individuals"	Melanesian groups with little contact with outsiders
Diagnostic IgG titre.	≥16	≥32	≥16	≥16	≥16	≥32	≥16 and ≤256
Age range of subjects (years).	18 to 79	25 to 64	Mean age 35.5 ± 3.3.	25 to 59	25 to 59	16 to 60+	
Seropositive males.	28 (38.8%)	326 (84.5%)	120 (81.6%)	(65%)	(55%)		177 (20%)
Seropositive females.	53 (42.7%)	134 (68.7%)	-	(53%)	(41%)		167 (10%)
Total.	81 (41.3%)	460 (79.2%)	120 (81.6%)	(59%)	(50%)	310 (51.6%)	344 (15%)
Increasing prevalence with age.	No	No	No	Yes	Yes	No*	
Prevalence significantly greater in males.	No	No	-	Yes	Yes	No	Yes

* This study also looked at 60 neonates and 322 children under the age of 16. 51% of neonates were seropositive suggesting placental transfer of maternal antibodies. The percentage of children who were seropositive was low until the age of 8.

Table 1-3. Epidemiology of some diseases associated with *Chlamydia pneumoniae*.

	Coronary artery disease	Asthma	Rheumatoid arthritis	Sarcoidosis	Alzheimer's dementia[53;54]
Main age(s) of onset.	Rises steeply from 35 yrs onwards.	Early (childhood) and late onset types recognised.	30-40 yrs.	20-30 yrs.	Above 50 yrs.
UK Prevalence.	3-4% (40-49yrs). 6-7% (60-64yrs).*	5%. 15% in second decade.	1-2%. 3% in women over 65yrs.	19 in 100 000.	4-7% in those over 65yrs. Increases with age.
Male:Female prevalence.	5.5:1 (35-44yrs). Ratio reduces with increasing age.	Equal.	1:3.	Men<Women.	1:1.6
Acute exacerbations.	Myocardial infarction and unstable angina can occur.	Yes.	Yes.	No. Usually subacute or chronic.	No.
Course.	Variable but increased risk of mortality.	Childhood asthma can improve in teens but frequently returns. Adult asthma can improve with age.	Variable. Mortality not increased.	Variable.	Progressive.
Geography/Race.	Common in countries where fat consumption is high.	Uncommon in Far East and third world.	Global although it can be uncommon in black Africans.	All racial groups but 10 times more common in Afro-Caribbeans than Caucasians.	Little geographical or racial variation.
Change in prevalence in recent times.	Reducing in most developed countries.	Increasing in industrial countries.	A "modern" disease? Little archaeological evidence before 15th Century.	No change.	No change.

*Male Prevalence.

Chapter 2 Atherosclerosis.

Atherosclerosis (Greek: athere = porridge, sclerosis = hard) affects the arterial circulation and is most commonly found in the lower descending aorta, the coronary arteries and arteries of the lower extremities. Atherosclerotic plaques tend to develop at areas of haemodynamic stress such as branching points but distribution can be extremely variable. Fatty streaks are the earliest visible lesion and by the age of 10, cover approximately 10% of the aortic intimal surface. Monoclonal staining techniques have demonstrated that important cellular constituent of fatty streaks are macrophages. It is thought that fatty streaks progress to fibrous plaques especially in the coronary arteries but in other locations, they may disappear or remain harmless. The raised fibrous plaques are the lesions which cause narrowing of the arterial lumen. The histology of plaques vary but typically, consist of a fibrous cap, a cellular area beneath and to the side of this (macropahges, smooth muscle cells and T lymphocytes) and a deeper necrotic core (hence porridge) consisting of cellular debris, lipid droplets, cholesterol crystals and calcium deposits. Progressive narrowing of arterial lumen by atherosclerotic plaque results in symptoms associated with insufficient blood flow such as angina and intermittent claudication. However, plaque rupture, erosion or haemorrhage may cause acute occlusion resulting in acute syndromes such as myocardial infarction.

Atherosclerosis is ubiquitous throughout the world and its cardiovascular complications account for 50% of all deaths in the age group 35-64 years in industrialised countries[55]. In England and Wales, myocardial infarction alone results in approximately 80 000 deaths a year. Although its pathogenesis is incompletely understood, atherosclerosis appears to be a multi-factorial disease. In a study of more than 20000 Swedish twins[56], male monozygote twins were at higher risk of dying from coronary heart disease (CHD) if their twin had also died from CHD than dizygote twins (Table 2-1). However, this risk was reduced with increasing age at which the twin died. These findings suggest that there is a genetic influence which becomes relatively less important with time. Environmental and other risk factors have also been recognised. Smoking, hypertension and hypercholesterolaemia are major independent risk factors as has been shown by

cohort studies. However, the WHO MONICA project found that these three major risk factors accounted for less that 25% of the variance in cardiovascular mortality in men from 35 populations[55]. It is likely that there are other risk factors which are important.

Infections as risk factors for acute coronary syndromes

Coronary risk factors can be considered in terms of those which contribute to atherogenesis and those which can trigger acute complications such as myocardial infarction. The incidence of myocardial infarction shows daily, weekly and seasonal variations. Generally, it has been reported that myocardial infarction is more frequent in the mornings, at the beginning of the week and in winter[57;58]. Such temporal variation suggests that factors external to the atherosclerotic plaque are important in causing plaque rupture and ambient temperature[59], lack of ultraviolet radiation, work stress[60] and vitamin C levels[61] have all been implicated. Respiratory infections are generally more frequent in winter and in cross-sectional studies, a significant number of subjects presenting with myocardial infarction had preceding respiratory symptoms[62]. Also, excess cardiovascular deaths have been reported during influenza epidemics[63].

Infections as risk factors for atherogenesis

The cellular and molecular events that lead up to atherosclerosis are incompletely understood. The current hypothesis which has stimulated the most research is the "response to injury" hypothesis as formulated by Ross[64;65]. This hypothesis was based on observations that lesions resembling atheromatous plaques could be induced in animals by endothelial denudation and that factors derived from platelets (platelet derived growth factor or PDGF) could induce smooth muscle growth. It was postulated that arterial endothelial damage resulted in platelet adhesion and aggregation with subsequent release of PDGF and migration and proliferation of smooth muscle cells. However, no specific cause of injury was stated and it became apparent that endothelial denudation and platelet adhesion were not consistent features. As more knowledge was acquired, the hypothesis was modified and currently, it is thought that endothelial dysfunction rather than denudation is important and that cells other than platelets, such as monocytes and macrophages are important[66]. Nevertheless, the stimulus for atherogenesis remains unclear although hypercholesterolaemia is thought to have a central role. Seminal work by Brown and Goldstein for which they were to receive the Nobel prize showed that atherosclerosis is induced in multiple species by mutations that

involve a single gene, the low-density lipoprotein (LDL) receptor[67]. This provides strong evidence that elevations in LDL are sufficient to induce all the components of the atherosclerotic reaction. It is thought that LDL becomes trapped in the extracellular matrix of the subendothelial space and that oxidation of LDL lipids (ox-LDL) results in an inflammatory response which causes monocyte infilteration, smooth muscle migration and proliferation and the development of atheroma[68].

There is no direct evidence that bacteria can cause atherosclerosis. Bacteria such as *C. pneumoniae*[4], *H. pylori*[69] and periodontal bacteria[70] have been linked to heart disease because of studies that have shown a serological association. The case of *C. pneumoniae* is considered further in the next chapter. Indirect evidence that bacteria may be involved in atherosclerosis has come from studies which have shown that bacterial lipopolysaccharide is transported across the endothelium by LDL in a form which may induce inflammation[71;72] and that infections can alter lipoprotein levels[73], although not always in an adverse manner[74]. The idea that chronic bacterial vascular infection can result in inflammation and atherosclerosis is a plausible one and important since it is potentially treatable with antibiotics.

Table 2-1. Relative risk of coronary heart disease in men according to the age at which their twins died from coronary heart disease (Data from Marenberg, M.E et al[56]).

Age at which twin died from coronary heart disease (yrs)	Relative risk of death from coronary heart disease	
	Monozygote twins	Dizygote Twins
36-55	8	4
66-75	4	2

Chapter 3 Serological and pathological studies of *Chlamydia pneumoniae* infection and atherosclerosis.

The initial studies of Chlamydia and atherosclerosis.

Lymphogranuloma venereum (LGV) occurs mainly in tropical and subtropical countries and is caused by infection with *C. trachomatis* serotypes L1-3. It is almost always acquired by coitus and may result in progressive fibrosis and destruction of the inguinal lymph nodes and lymphatics with subsequent elephantiasis of the genital region. In the 1940's, South American researchers thought that there were similarities between the pathological processes of atherosclerosis and the lymphatic destruction that occurred in LGV. Subsequently, they reported that subjects with atherosclerosis were significantly more likely to have a positive result with the intra-dermal skin test using Frei's LGV antigen[75-77]. It was not until the 1980's that further work was done in this field. Finnish researchers discovered that 70% of paired sera collected from subjects presenting with acute myocardial infarction had a three-fold or greater rise in antibody titres against genus specific chlamydial lipopolysaccharide. The only known chlamydial species that was common enough to be the candidate was the then recently described *C. pneumoniae*[4;78]. Initially, this study was criticised because no attempt had been made to control for smoking, a possible confounding factor[79] since smokers are more likely to suffer from respiratory infection and to have coronary artery disease[80]. However, numerous serological studies from different populations around the world have since reported a positive association between seropositivity for *C. pneumoniae* and atherosclerosis (Table 3-1). This association has been reported in subjects with chronic stable disease as well as in those presenting with acute myocardial infarction and stroke leading to the hypothesis that chronic infection with *C. pneumoniae* is a cause of atherosclerosis. Unfortunately, it is not easy to diagnose chronic *C. pneumoniae* infection. Whereas accurate detection of acute infection requires paired serum samples taken 4 to 6 weeks apart and the demonstration of three-fold or more rising titre, serological criteria for chronic infection are more controversial. It is generally accepted that persistently raised specific IgA and IgG is an indicator of chronic infection since IgA is thought to have a much shorter half-life than IgG in the absence of persistent infection. However, titres at which antibody levels are considered significantly raised

are not standardised and different authors have used different criteria for seropositivity (Table 3-1). Also, not all studies have measured IgA and it is impossible to distinguish accurately between past and current infection with a single IgG measurement. Compounding these problems, serology uses crude, whole *C. pneumoniae* antigens, containing epitopes potentially cross reactive with antibodies to other chlamydial species[51], to other Gram negative bacteria[81], or even to human heat shock protein as has been described for *C. trachomatis*[82]. Atherosclerosis is known to be associated with the presence of antibody to human heat shock protein[83] and the possible confounding effects of such antibody has not been determined in any serological study of *C. pneumoniae* and heart disease.

Serological studies of *C. pneumoniae* and atherosclerosis.

Table 3-1 shows 27 serological studies that have investigated *C. pneumoniae* and atherosclerosis between 1988 and up to the end of October 1998. Studies were found by searching the Medline and Institute of Science and Information Inc. bibliographic databases using the following indexing terms - chlamydi* and heart, chlamydi* and coronary and chlamydi* and atherosclerosis. Twenty one of these studies reported some sort of positive serological association. However, although all studies measured IgG, only 3 prospective and 15 cross sectional studies measured IgA. Of the cross sectional studies, 5 found an association with both immunoglobulins, 3 with IgG alone and 2 with IgA alone. Five studies found no association with either immunoglobulin, although 3 of these did show an association with circulating chlamydial immune complexes[84,85;86] Therefore, the serological evidence that chronic rather than past *C. pneumoniae* infection is associated with atherosclerosis is not compelling. In fact, the number of cases in studies which found a positive association with both immunoglobulins[4;87-92] is similar to that in studies which found no association with either[36;84-86;88;93], although controls were numerically far greater in the former group (642 and 3069 vs. 767 and 1382). People with chronic debilitating diseases including heart failure are predisposed to respiratory tract infections and this may be one reason why people with coronary artery disease are more likely to have experienced *C. pneumoniae* infection. Of the 5 prospective studies, 3 reported a positive association but 2 of these depended on subset analysis. In one study, seropositivity was associated

with coronary events 6 months but not 5 years before an event[87] while in the other, an association was seen in non-diabetic men in East but not West Finland and not in diabetic men[88].

Acute infections are easier to diagnose and 4 studies investigated whether antibody titre rises were associated with acute vascular events such as myocardial infarction (MI) and cerebrovascular accident (CVA). Cook et al[91] found an association with CVAs while Saikku et al[4] found an association between rising IgM titres to chlamydial group lipopolysaccharide and MI. Blasi et al[94] found that 20% of patients with MI had a rise in IgG but the proportion in the control group was unknown. No association was seen in the fourth study[84]. Therefore, there is some evidence that acute infection is associated with acute vascular events. If this is true, the incidence of such events should increase during epidemics. East Finland has one of the highest coronary mortality rates in the world but there has been a gradual decrease since the 1970's due to a primary prevention programme. East Finland has also contributed to the WHO MONICA project (monitoring of trends and determinants in cardiovascular disease)[95] and serum samples from this project have been tested to estimate the population prevalence of *C. pneumoniae*[40]. Figure 3-1 shows the attack rates of coronary events in two regions of East Finland and the seroprevalence of *C. pneumoniae*. It can be seen that in the epidemic year of 1987, acute coronary events did not increase.

Pathological studies of *C. pneumoniae* in blood vessels.

Following on from serological studies, attempts were made to see whether atherosclerotic blood vessels were actually infected by *C. pneumoniae*. In 1992, Alan Shor in South Africa demonstrated that structures with the appearances of *C. pneumoniae* could be detected in atherosclerotic tissue by electron microscopy[96]. Subsequently, techniques such as immunocytochemistry (ICC), the polymerase chain reaction (PCR) and culture have provided direct evidence that *C. pneumoniae* localises to blood vessels (Table 3-2). Generally, *C. pneumoniae* has been found more often in atherosclerotic than control blood vessels. However such studies do not show whether infection preceded or followed the development of atherosclerosis and it has been proposed that *C. pneumoniae* may merely be an "innocent bystander" or in other words,

an organism of no pathological significance but one which merely colonises damaged tissue.

Table 3-2 shows twenty-five pathological studies which have looked for *C. pneumoniae* in atherosclerotic tissue. Histological evidence of atherosclerosis is ubiquitous [97] and 11 of 25 studies did not have control vessels. Only 3 studies had controls completely matched for age, origin of tissue and tests used to detect for *C. pneumoniae*. Even then, control arteries either had histological evidence of early atherosclerosis[98] or came from arteries where adjacent segments had disease[99]. For the other studies, control vessels tended to be numerically smaller and obtained at autopsy from younger subjects.

Although 3 studies failed to find evidence for *C. pneumoniae* in atherosclerotic vessels[86,100;101] most have found it in 15 to 100% of cases. In contrast, it appears to be uncommon in control vessels and only in two studies was *C. pneumoniae* as prevalent in control as in diseased vessels[102;103]. In reality, it is likely that most control vessels, although macroscopically normal, did have early histological disease, which would suggest that *C. pneumoniae* is commoner in people with severe rather than mild atherosclerosis. There were four studies in which the severity of atherosclerosis was formally graded on a histological basis. In one, *C. pneumoniae* was detected in 86% of severe compared with 6% of mild lesions using ICC[104]. However, when a subset of samples was tested by PCR, discordant results were obtained and the prevalence in non-atherosclerotic or minimally atherosclerotic lesions was said to be quite high[105]. In an autopsy study of 60 Alaskan natives who died mainly from non-cardiovascular causes (mean age 34.1), the Stary classification was used to grade the severity of a segment of the right coronary artery obtained from each subject[106]. Twenty two subjects were found to have *C. pneumoniae* but there was no difference in the severity of their coronary artery segments compared with that of subjects without *C. pneumoniae*. In total, 14 of 40 specimens with raised lesions (35%) were positive for *C. pneumoniae* compared with 7 of 18 specimens with flat lesions (39%). However, in a study of young adults aged 15 to 34, *C. pneumoniae* was more common in arteries with atheroma than in arteries with intimal thickening but was absent in arteries that appeared normal[99]. Similarly, *C. pneumoniae* was found in diseased segments of carotid artery but not in adjacent segments that were macroscopically normal but had early disease[98]. It would be expected that if *C. pneumoniae* were an important cause of

atherosclerosis, then the extent and distribution of the organism in any individual should match that of atherosclerosis. However, this has not been systematically investigated although in one study, contiguous segments from diseased coronary arteries or different coronary arteries from the same subject were not all positive for *C. pneumoniae*[107].

In summary, pathological studies have shown that *C. pneumoniae* is common in atherosclerotic vessels from a wide variety of sources including those from young subjects[99 108]. This would suggest that chronic infection occurs. *C. pneumoniae* may be more prevalent in severe compared with mild lesions but its distribution in an individual does not necessarily match that of atherosclerosis. These findings suggest that *C. pneumoniae* exacerbates rather than causes atherosclerosis. Thus, one study using ICC and electron microscopy reported that it was more frequently found in atherectomy samples from patients presenting with acute coronary syndrome than from patients with stable angina.[109]

Conclusion

In summary, serological studies have not provided convincing evidence that atherosclerosis is associated with chronic rather than past *C. pneumoniae* infection and the distribution of *C. pneumoniae* in the vascular tree in relation to the severity and extent of atherosclerosis remains unclear. In the case of serological studies, what is needed are adequately powered case control studies which take into account potential confounding factors.

In the case of pathological studies, one of the main problems has been the fact that it is almost impossible to find age matched control tissue. We therefore took a different approach. In a study of subjects attending for first time and redo coronary artery bypass graft surgery (CABG), we compared the prevalence of *C. pneumoniae* in new saphenous vein and internal mammary artery grafts with the prevalence in failed grafts and diseased native coronary arteries. In a separate study, we collected all three coronary arteries from subjects at post mortem. The aim was to see whether the distribution of *C. pneumoniae* correlated with the extent and severity of atherosclerosis.

Table 3-1. Studies investigating the serological association between *C. pneumoniae* and .atherosclerosis (Key to table is on next page).

Prospective Studies

Study	Cases/Controls[#]	Association with*			Diagnostic Antibody titre[¶]
		IgG	IgA	ICs	
Saikku (1992)[87]	103 vs. 103 Cardiac death or MI	2.2 (1.1 - 4.5) 2.6 (1.2 - 5.2) 6 months before event but not 5 years before			IgG ≥ 128 &/or IC OR IgA ≥ 64 &/or IC
Miettinen (1996)[88]	162 vs. 636 (NIDDM) 40 vs. 1155 (No DM) Cardiac death or MI	32% vs. 15%. 2.44 (0.98 - 6.08) Only in non diabetic men in East Finland			IgG ≥ 128 & IgA ≥ 40
Ossewaarde. (1998)[110]	54 vs. 108 Cardiac death or MI or angina	52 vs. 34% 2.8 (1.3-5.8)	No	No	ELISA
Nieto (1997)[111]	256 vs. 550 Cardiac death, MI or revascularisation.	No			IgG ≥ 64
Siscovick. (1998)[112]	100 vs. 183 Cardiac death or MI	No			Not stated (abstract)

Cross sectional Studies

Diedrichs (1997)[89]	131 vs. 63 CA	66 vs. 48%	44 vs. 22%		
Halme. (1997)[90]	93 vs. 115 CA	100 vs. 61% (in men only)		No	IgG ≥ 128 &/or IgA ≥ 40
Saikku (1988)[4]	70 vs. 41 MI or stable angina	49 vs. 15% 5.5 (2.1-14.7)	41 vs. 10%		IgG ≥ 128 IgA ≥ 32
Cook. (1998)[91]	176 vs. 1518 CVA	Acute infection 13.6 vs. 5.7% 4.2 (2.5 - 7.1)			IgG ≥ 512 or 4 fold IgG rise or IgM ≥ 8
		Chronic infection 32.4 vs. 12.7% 4.4 (3 - 6.5)			IgG ≥ 64 & ≤ 256 or IgA ≥ 8
Mazzoli. (1998)[92]	29 vs. 74 MI	82 vs. 34%	71 vs. 14.9%		
Dahlen (1995)[113]	60 vs. 60	93.3% vs. 78.3% 3.56 (1 - 16.1)	No		IgG ≥ 32 IgA ≥ 16
Mendall (1995)[11]	100 vs. 64 CA	21 vs. 9.4% 2.6 (1.0 - 6.8)	No		IgG ≥ 64 IgA not stated
Blasi. (1997)[94]	61 vs. 61 MI	57 vs. 30% 3.2 (1.5 - 6.8)	No		IgG ≥ 16 IgA ≥ 16
Thom (1991)[114]	461 vs. 95 CA	22 vs. 13% 2 (1-4)			IgG ≥ 64
Thom (1992)[115]	171 vs. 120 CA	67 vs 56% 2.6 (1.4-4.8)			IgG ≥ 8
Melnick (1993)[116]	326 matched pairs. Carotid doppler	73 vs. 63% 2 (1.2 - 3.4)			IgG ≥ 8
Patel (1995)[69]	83 vs. 305 Rose questionaire, ecg	30 vs. 18% 2.25 (1.1-4.6)			IgG ≥ 64
Thomas (1997)[117]	83 vs. 93 MI/IHD	71.1 vs. 31.2% 5.4 (2.7-10.9)			IgG ≥ 16
Toss (1998)[118]	256 vs. 190 Unstable angina	Seroprevalence not reported.	36% vs. 19%.		IgG ≥ 16 IgA ≥ 64
Wimmer. (1996)[119]	58 vs. 52 CVA	No	47 vs. 23% 1.7 (1.1-2.7)	24 vs. 8% 2 (1.1-3.8)	IgG ≥ 32 IgA ≥ 16
Leinonen (1990)[84]	42 vs. 41 MI	No	No	57 vs. 12% 10 (3 - 29)	IgG ≥ 128 IgA ≥ 32
Leinonen (1994)[85]	95 vs. 139 MI	No	No	58 vs. 26% 4 (2 - 7)	IgG ≥ 32 & ≤ 128 IgA ≥ 8 & ≤ 32
Weiss (1996)[86]	65 vs. 28 CA	No	No		All titres considered
Kark (1997)[93]	302 vs. 486 MI	No	No	No	All titres considered.
Boman (1998)[36]	101 vs. 52	No	No		
Linnanmaki (1993)[120]	46 vs 46 CA	No		41 vs. 15% 4 (1.4 - 11)	IgG ≥ 32
Anderson[121] (1998)	124 vs. 97 CA	No			IgG ≥ 16

Key to Table 3-1

[#]Number of cases and controls in final analysis and diagnosis of cases.

CA=Coronary arteriograms. CVA=Cerebrovascular accident. MI=Myocardial infarction. IHD = Ischaemic heart disease. ECG =Electrocardiogram. NIDDM=non-insulin dependent diabetes.

*Percentage of cases and controls who are seropositive or who have immune complexes (ICs). (Odd's ratio and 95% confidence interval. Adjusted figures are shown where reported by the authors.)

[¶]Microimmunofluorescence test unless indicated

Table 3-2. Studies investigating the presence of *C. pneumoniae* in Blood vessels (Key to table is on next page).

Controlled Studies

(a) Age, tissue and diagnostic method matched

Study	Number of Cases/Controls[†] (Description of case tissue)	Cases and controls positive for *C. pneumoniae.*[†]		
		PCR	ICC	Other
Kuo. (1995)[99]	18 vs. 31. (Coronary artery).	17 vs. 0%	39 vs. 0%	
Maass. (1997)[98]	61 vs. 39. (Carotid endarterectomy).	15 vs. 0%		
Petersen. (1998)[122]	40 vs. 40. (AAA)	35 vs. 5%		

(b) Other controls

Grayston[#]. (1995)[123]	5 vs. 0. (Carotid endarterectomy).	60%	100%	
	56 vs. 6 (archival or autopsy carotid specimens).		57 vs. 0%	
Ong[#]. (1995)[102]	32 AAA repairs vs. 6 patients with normal vascular tissue.	44 vs. 50%	3/8 (38%) vs. Not done	
Jackson. (1997)[124]	38 vs. 38. (Vascular vs. non-vascular tissue from autopsy cases).	16% vs. 0 to 8%	24% vs. 5 to 11%	
Juvonen (1997)[125]	12 vs. 9 (Abdominal aortic aneurysm)	6/6 vs. 0/9	100 vs. 0%	
Kuo[#]. (1997)[126]	23 vs. 8 (Diseased femoral & popliteal arteries)	48 vs. 0%		0%* vs. not done
Maass[#]. (1998)[43]	70 vs. 17. (Coronary atherectomy, failed grafts and other vessels).	30 vs. 0%		16%* vs. Not done
Wong. (1998)[103]	58 vs. 58 (Coronary atherectomy and failed grafts)	39 vs. 12 to 30%		
Shor. (1992)[96]	10 vs. 5 (coronary artery). Cases were highly selected from 1000 arteries.		5/7 (71%) vs. 0/5 (0%)	10/10[‡] vs. Not done.
Kuo. (1993)[127]	20 vs. 4. (Aortic atheroma).		30 vs. 0%	
Chiu[#]. (1997)[128]	76 vs. 20 (carotid and aortic tissue).		71 vs. 0%	
Muhlestein[#]. (1996)[129]	90 coronary atherectomies. 24 other controls.			79 vs. 4.2%[¶]

Studies without controls

Kuo. (1993)[130]	36. (Coronary artery).	13/30 (43%)	15/36 (42%)	6/21[‡] (29%)
Campbell. (1995)[131]	37. (Coronary atherectomy).	32%	45%	2/2[‡]
Ramirez. (1996)[107]	12. (Coronary artery)	41.7%	41.7%	8%* 25%[‡]
Jackson. (1997)[132]	25. (Carotid endarterectomy).	24%	8/16 (50%)	4%*
Blasi. (1996)[133]	51. (AAA)	51%		
Weiss. (1996)[86]	72 (Coronary atherectomies).	1/50 (2%)		0/22 *[‡]
Davidson. (1998)[106]	60 (Coronary artery)	14/60 (23.3%)	20/60 (33.3%)	
Bauriedel. (1998)[109]	32 (Carotid & coronary arteries)		47%	
Lindholt. (1998)[100]	20. (AAA)	0%		
Paterson (1998)[101]	30 (carotid and coronary arteries)	0%		
Saldeen (1998)[104]	60 (coronary arteries)		62%	

Key to Table 3-2.

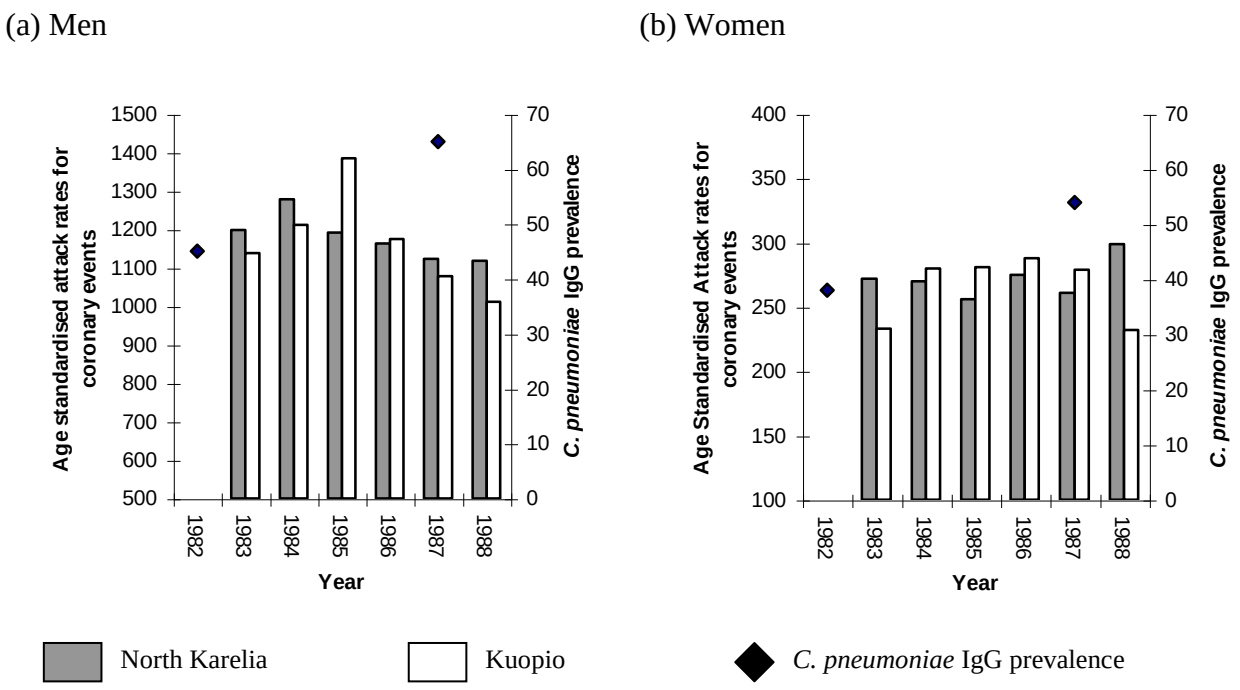
† In some cases, more than 1 specimen was obtained from each patient. In these cases and where it was possible to determine from the papers, results are expressed as positive patients per number of patients.

PCR = Polymerase Chain Reaction. ICC = Immunocytochemistry. AAA = abdominal aortic aneurysm. IMA = Internal mammary artery.

#Studies where control vessels did not come from age matched subjects or where control subjects were poorly described.

* Culture ‡ Electron microscopy ¶ Direct immunofluorescence

Figure 3-1. Age standardised attack rates for coronary events (definite and probable myocardial infarction and coronary deaths) in 2 regions of East Finland and prevalence of *C. pneumoniae* IgG.



Chapter 4 The association of mortality due to acute myocardial infarction and the percentage of households with four or more persons in electoral wards in England and Wales.

The classical coronary risk factors of hypercholesterolaemia, smoking and hypertension do not adequately explain the differences in incidence and prevalence of coronary artery disease throughout the world. It has been proposed that chronic infection may be an additional important risk factor. Situations that facilitate the transmission of infection in a community include poor personal and public hygiene and where contact between subjects is high. We hypothesise that if chronic infections are important in coronary artery disease, then coronary mortality rates should be higher in communities where measures of interpersonal contact are high.

Method

Population data for England and Wales was obtained from the 1991 UK national census (MIDAS - Manchester Information Datasets and Associated Services, University of Manchester). For each electoral ward, area size, Carstairs score and number of persons according to age range and sex was recorded. The Carstairs score is a measure of social deprivation for a particular population. It is a composite score based on four variables - unemployment (unemployed male residents over 16 as a proportion of all economically active male residents aged over 16), overcrowding (persons in households with 1 and more persons per room as a proportion of all residents in households), non-car ownership (residents in households with no car as a proportion of all residents in households) and low social class (residents in households with an economically active head of household in social class IV or V as a proportion of all residents in households). The greater the Carstairs score, the greater the degree of deprivation and we categorised electoral wards into three groups according to their score so that the size of each group was approximately the same (less than -1.5, between -1.5 and 1.7 and greater than 1.7). Categorical data on the number of persons per household in each ward was available and the percentage of households with four or more persons was used as an arbitrary measure of household size at ward level. The number of persons per hectare was used as a measure of population density. Information on deaths due to myocardial infarction

(ICD9 code 410) in England and Wales between 1988 and 1997 was obtained from the Office of National Statistics (ONS). For each death, sex, age, date of death and electoral ward of residence was recorded. Data from non-residents was excluded. Electoral wards were coded differently by MIDAS and ONS but were matched using tables supplied by the census division of ONS. Ward boundary changes from 1993 onwards meant that not all wards could be reconciled and for this reason and as census data becomes less accurate with time, we concentrated on mortality data from 1991 and the adjacent years although the presence of significant findings in other years was checked for. For each category of household size and population density, we calculated mortality rate ratios with 95% confidence intervals[134].

Results

In 1991, there were 9509 electoral wards for which requisite data was available with an average of 5246.7 persons each and a total population of 49890834. In the same year, there were 87314 deaths and ward data was available for 87123 of these (99.8%). Mortality rates per thousand persons by age, sex, deprivation and household size are shown in Table 4-1. It can be seen that in subjects over 45 years, mortality rates tended to increase with household size. In younger subjects, the number of deaths was small (1.1% of total) and no trend was seen. For all deaths, mortality rate ratios standardised for age, sex and deprivation are shown in Figure 4-1. This increase in the standardised mortality rate with overcrowding was seen in all years and was logarithmic (Table 4-2). The magnitude of the increase was inversely related to age but independent of sex. Electoral wards with the most deprivation tended to show the greatest increase but this was not statistically significant.

There was no increase in mortality with population density and in fact, mortality was lower in wards with seventy or more persons per hectare (6.4% of the population, Table 4-3). However, wards with seventy or more persons per hectare were less likely to have households with four or more persons than wards with smaller population densities (18.9 vs. 24.0%, $P<10^{-33}$, 2 tail independent t test, Figure 4-2).

Mortality from myocardial infarction dropped gradually from 1988 to 1997. In each year, mortality was lowest around August and rose to a peak in December or January.

Following a second peak in March, mortality then declined (Figure 4-3). This pattern was seen in both men and women. In all years, the number of deaths occurring in the first or last quarter of the year was independent of household size or population density. In 1989 (year of a large influenza epidemic), the mortality rate for December was associated with overcrowding but not with population density (Figure 4-4). Similar findings were also seen in 1991.

Discussion

The main finding of this study was that in electoral wards in England and Wales, standardised mortality rate ratios for myocardial infarction were positively associated with the percentage of households with four or more persons but not with population density. One possible explanation for this finding is that a risk factor for coronary artery disease (CAD) is chronic infection by an organism whose spread is facilitated by close contact of susceptible subjects. If this explanation is correct, it would seem that it is contact within the home which is important rather than contact with the population as a whole. This could reflect increased duration of contact in the home as well as the sharing of toilet and cooking facilities. Another potential marker of interpersonal contact is the number of persons per room. We did not use this marker because the number of wards with significant amounts of such overcrowding was small. Epidemics of respiratory infections such as influenza are known to cause excess mortality from CAD[135] and it would be important to distinguish between the effects of acute and chronic infection. This is because chronic infections have the theoretical possibility of causing CAD and are thus modifiable risk factors whereas acute infections can cause death in those with CAD but have no causal role in atherosclerosis. We found that generally, household size and population density was not associated with the seasonal variation in mortality even though an association between household size and annual mortality was present every year. In 1989, when there was a large influenza epidemic that began in mid November and peaked in December[136], a trend of increasing mortality with household size was seen in December but not during the smaller influenza epidemics of late 1993 and early 1995[137]. Therefore, our results are more likely to reflect the effects of chronic, endemic infection rather than acute, seasonal epidemics.

As expected, we found that mortality from myocardial infarction was greatest in the elderly, in males and in deprived areas (Table 4-1). However, even though no consistent association of mortality with household size was seen in subjects younger than 45 years, the effect of household size on mortality was otherwise inversely related to age. Compared to subjects aged 45 to 54 in wards with the least number of large households, subjects of the same age in wards with the most number of large households had a standardised mortality ratio of 2.71. The same ratio for subjects older than 85 years was only 1.1 (Table 4-2). Atherosclerosis is a multifactorial disease and multiple risk factors are likely to be present with increasing age. Therefore, the effects of individual risk factors are relatively more important in the young. Diseases associated with infection have fallen in industrialised countries because of improvements in sanitation and housing[138]. However, we found that the effect of household size on mortality was independent of deprivation as well as sex although there was a non-significant trend for overcrowding to be more important in the most deprived areas. Infections can affect both sexes equally but *Chlamydia pneumoniae*, the organism most strongly associated with atherosclerosis, is more prevalent in males. Apart from *Chlamydia pneumoniae*, other organisms such as *Helicobacter pylori*, periodontal bacteria and CMV have also been implicated in atherosclerosis[7].

There are other possible explanations for the association between household size and coronary mortality. With regards to the conventional coronary risk factors, the affects of passive smoking may be increased in wards where household sizes are large. A family history of premature CAD is a coronary risk factor[56] and populations with large households may increase the chance of relations with such a history living together. It has been proposed that air pollution can increase coronary mortality[139] but in this study, we found that mortality was not positively associated with population density even though densely populated areas can be expected to suffer the most from pollution. In countries undergoing industrialisation, it has been noted that the prevalence of some diseases, such as CAD, are greater in urban compared with rural areas[140;141]. This has been attributed to environmental, dietary and lifestyle changes. Smoking, sedentary lifestyle and obesity have all been implicated. This situation is likely to be different in countries that are already industrialised. Although we did not look specifically at urban and rural areas, we found that wards with the greatest population density actually had

the smallest standardised mortality rate ratio. A possible explanation for this is that the number of large households in these wards is smaller.

In this study, data used was available at different levels of spatial aggregation. Date of death, sex and age were available at the individual level whereas deprivation, household size and population density were available at the electoral ward level. Therefore, we can not be certain that individual deaths were more likely to have occurred in large households. Other sources of error include those associated with the use of routinely collected data such as miscoding of deaths and the fact that data on smoking and other coronary risk factors were absent. Nevertheless, the finding that the standardised mortality rate ratio was highest in wards with the greatest percentage of large households was a consistent finding in all years studied. It would seem important to identify the factor for which large household size is a surrogate marker. Although there is no compelling evidence to implicate any particular organism in atherosclerosis, it is possible that large household size is a marker for chronic infection. A recent retrospective analysis found that subjects who had received either tetracyclines or quinolones were less likely to have had a heart attack than controls[142]. It would be important to investigate whether chronic bacterial infections are important in atherosclerosis since they are potentially treatable.

Table 4-1. Mortality rates for myocardial infarction per thousand persons in 1991 by age, sex, deprivation and the percentage of households with four or more persons.

Sex	Age	Deprivation (Carstairs Score)	Percentage of households with 4 or more persons					
			<10	10 to 14.9	15 to 19.9	20 to 24.9	25 to 29.9	>= 30
Male	45-54.9	Bottom third	0 (0/1586)	0.61 (10/16398)	0.84 (65/77278)	0.74 (291/391294)	0.67 (313/465354)	0.59 (95/161510)
		Middle third	0.62 (5/8048)	0.70 (29/41491)	0.93 (159/170405)	0.84 (367/436757)	0.90 (227/251399)	1.04 (55/52852)
		Top third	1.10 (15/13657)	1.22 (60/49291)	1.18 (210/177726)	1.23 (405/328945)	1.40 (268/191100)	1.69 (107/63216)
	55-64.9	Bottom third	2.81 (5/1780)	2.76 (47/17045)	2.25 (164/72932)	2.51 (839/334079)	2.39 (863/361245)	2.31 (253/109652)
		Middle third	2.06 (15/7295)	2.81 (111/39465)	2.95 (467/158315)	3.15 (1209/383799)	3.01 (618/205368)	3.14 (114/36250)
		Top third	2.84 (35/12312)	3.75 (178/47436)	3.65 (638/174834)	3.87 (1225/316534)	3.85 (675/175521)	4.56 (257/56412)
	65-74.9	Bottom third	3.51 (8/2278)	6.44 (129/20046)	6.39 (437/68369)	6.65 (1768/265940)	7.08 (1840/259817)	6.75 (480/71088)
		Middle third	6.58 (53/8058)	6.52 (261/40047)	7.41 (1095/147837)	8.02 (2582/322111)	7.75 (1199/154724)	7.76 (186/23955)
		Top third	6.61 (75/11343)	8.69 (345/39712)	8.73 (1313/150451)	9.23 (2454/265965)	8.76 (1170/133604)	9.58 (360/37591)
	75-84.9	Bottom third	12.70 (22/1732)	13.28 (176/13255)	14.10 (571/40494)	15.63 (2221/142101)	15.58 (2029/130203)	15.72 (523/33266)
		Middle third	15.24 (100/6560)	14.84 (385/25943)	15.49 (1325/85518)	16.35 (2680/163866)	16.49 (1206/73135)	17.77 (188/10579)
		Top third	14.22 (106/7454)	16.43 (352/21420)	16.32 (1211/74201)	17.22 (2108/122446)	17.90 (1028/57441)	17.56 (289/16455)
	85+	Bottom third	34.40 (14/407)	21.66 (63/2908)	25.17 (213/8462)	24.94 (685/27471)	25.14 (621/24699)	26.15 (158/6041)
		Middle third	22.22 (43/1935)	23.99 (139/5795)	24.26 (406/16859)	25.38 (742/29230)	25.52 (318/12461)	20.00 (35/1750)
		Top third	20.11 (33/1641)	18.74 (76/4054)	24.55 (309/12589)	26.71 (529/19809)	23.64 (222/9389)	26.17 (78/2980)

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Sex	Age	Deprivation (Carstairs Score)	Percentage of households with 4 or more persons					
			<10	10 to 14.9	15 to 19.9	20 to 24.9	25 to 29.9	>= 30
Female	45-54.9	Bottom third	0 (0/1681)	0.28 (5/17892)	0.12 (10/81278)	0.11 (44/394554)	0.08 (37/458085)	0.08 (12/154598)
		Middle third	0 (0/8245)	0.07 (3/43386)	0.17 (30/174415)	0.18 (78/437853)	0.13 (33/248239)	0.16 (8/50740)
		Top third	0.16 (2/12300)	0.21 (10/47162)	0.26 (46/178021)	0.26 (87/330915)	0.24 (47/192590)	0.44 (28/63246)
	55-64.9	Bottom third	0.47 (1/2131)	0.43 (9/20879)	0.78 (63/80714)	0.63 (220/348340)	0.70 (254/361573)	0.45 (47/104590)
		Middle third	0.24 (2/8495)	0.78 (35/45063)	0.87 (153/174961)	1.02 (416/408223)	0.97 (204/211168)	1.09 (39/35716)
		Top third	0.48 (6/12475)	1.10 (52/47079)	1.35 (246/181566)	1.67 (559/335576)	1.56 (283/181961)	1.59 (87/54652)
	65-74.9	Bottom third	1.94 (6/3098)	2.16 (58/26906)	2.62 (228/86865)	2.92 (939/321829)	3.00 (911/304126)	3.09 (250/80784)
		Middle third	2.77 (33/11924)	2.93 (161/54916)	3.43 (659/192052)	3.38 (1354/400210)	3.72 (692/185879)	3.38 (95/28390)
		Top third	3.18 (49/15403)	4.15 (204/49165)	4.27 (796/186575)	4.69 (1542/329090)	4.60 (743/161425)	5.50 (230/43377)
	75-84.9	Bottom third	7.22 (20/2769)	6.89 (158/22932)	8.22 (563/68459)	8.56 (1985/231875)	8.81 (1833/208028)	8.73 (454/51989)
		Middle third	8.45 (112/13247)	8.65 (422/48808)	9.02 (1367/151616)	9.40 (2617/278257)	9.70 (1166/120251)	11.27 (195/17297)
		Top third	7.65 (113/14764)	8.95 (359/40118)	9.89 (1339/135403)	10.59 (2295/216813)	10.01 (1008/100682)	9.72 (268/27562)
	85+	Bottom third	11.96 (13/1087)	14.19 (129/9090)	18.63 (459/24632)	17.93 (1439/80244)	18.26 (1268/69439)	18.54 (320/17263)
		Middle third	18.88 (129/6831)	17.40 (325/18673)	17.42 (918/52710)	17.78 (1561/87778)	18.16 (674/37110)	18.31 (96/5243)
		Top third	18.68 (105/5622)	17.80 (241/13539)	18.41 (779/42317)	19.11 (1239/64456)	18.53 (542/29244)	19.32 (166/8591)

Figure 4-1. Mortality rate ratios and 95% confidence intervals for myocardial infarction deaths in 1991 standardised for age, sex and deprivation by percentage of households with four or more persons.

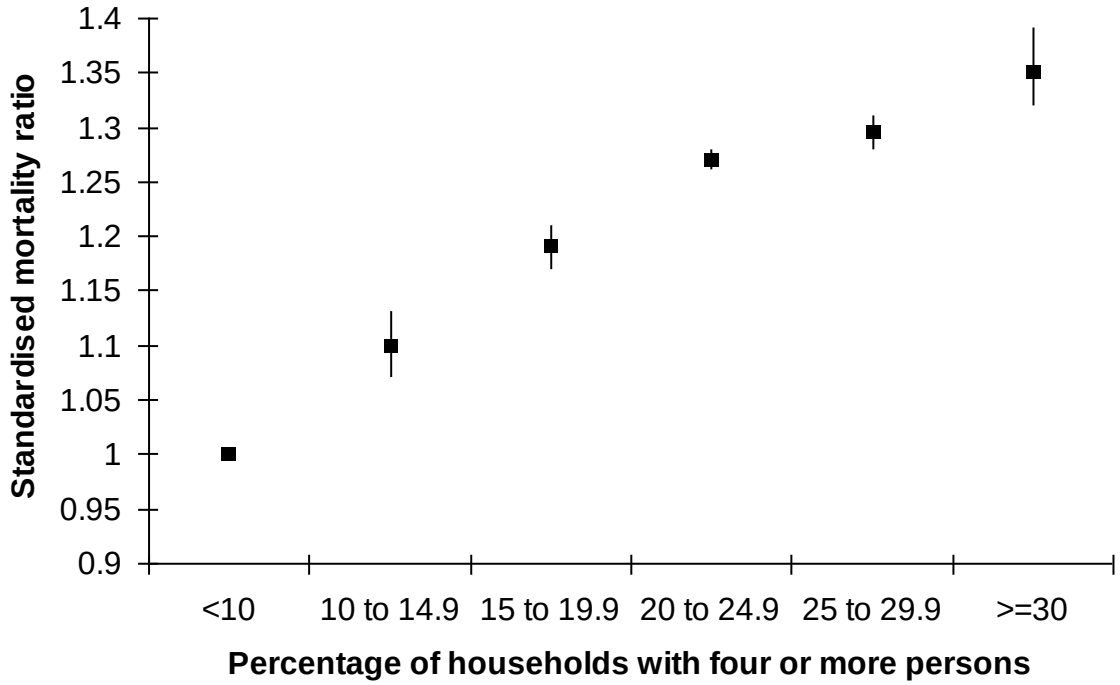


Table 4-2. Mortality rate ratios (95% confidence interval) by percentage of households with four or more persons in 1991.

		Percentage of households with four or more persons					
		<10	10 - 14.9	15 - 19.9	20 - 24.9	25 - 29.9	>=30
Age*	45 - 54.9	1.00	1.34 (1.10 - 1.59)	1.58 (1.44 - 1.71)	1.85 (1.75 - 1.96)	2.33 (2.18 - 2.48)	2.71 (2.41 - 3.03)
	55 - 64.9	1.00	1.41 (1.28 - 1.54)	1.45 (1.38 - 1.52)	1.47 (1.42 - 1.51)	1.29 (1.25 - 1.34)	1.27 (1.19 - 1.36)
	65 - 74.9	1.00	1.21 (1.14 - 1.28)	1.29 (1.26 - 1.33)	1.40 (1.38 - 1.43)	1.48 (1.44 - 1.52)	1.56 (1.48 - 1.63)
	75 - 84.9	1.00	1.06 (1.01 - 1.10)	1.12 (1.09 - 1.15)	1.19 (1.17 - 1.21)	1.20 (1.18 - 1.23)	1.24 (1.18 - 1.29)
	>= 85	1.00	0.95 (0.89 - 1.01)	1.03 (0.99 - 1.07)	1.07 (1.04 - 1.09)	1.07 (1.04 - 1.11)	1.10 (1.03 - 1.18)
	All ages	1.00	1.10 (1.07 - 1.13)	1.19 (1.17 - 1.21)	1.27 (1.26 - 1.28)	1.30 (1.28 - 1.31)	1.35 (1.32 - 1.39)
	All ages (1990)§	1.00	1.14 (1.11 - 1.17)	1.24 (1.22 - 1.25)	1.25 (1.24 - 1.27)	1.27 (1.25 - 1.29)	1.34 (1.30 - 1.37)
	All ages (1992) §	1.00	1.10 (1.06 - 1.13)	1.18 (1.16 - 1.20)	1.24 (1.22 - 1.25)	1.24 (1.22 - 1.26)	1.25 (1.22 - 1.29)
Deprivation†	Bottom third	1.00	1.09 (1.01 - 1.16)	1.24 (1.20 - 1.29)	1.31 (1.28 - 1.33)	1.34 (1.31 - 1.37)	1.33 (1.28 - 1.38)
	Middle third	1.00	1.03 (0.98 - 1.07)	1.11 (1.08 - 1.14)	1.18 (1.16 - 1.20)	1.21 (1.18 - 1.24)	1.26 (1.19 - 1.34)
	Top third	1.00	1.19 (1.14 - 1.24)	1.26 (1.23 - 1.29)	1.35 (1.32 - 1.37)	1.32 (1.29 - 1.36)	1.44 (1.37 - 1.50)
Sex‡	Male	1.00	1.12 (1.07 - 1.16)	1.18 (1.15 - 1.20)	1.26 (1.24 - 1.27)	1.27 (1.25 - 1.29)	1.33 (1.28 - 1.37)
	Female	1.00	1.08 (1.04 - 1.13)	1.20 (1.18 - 1.23)	1.29 (1.26 - 1.31)	1.33 (1.30 - 1.36)	1.39 (1.33 - 1.44)

* Standardised for sex and deprivation.

§ Standardised for age, sex and deprivation.

† Standardised for age and sex.

‡ Standardised for age and deprivation.

Table 4-3. Mortality rate ratios (95% confidence interval) standardised for age, sex and deprivation by number of persons per hectare.

Year	Number of persons per hectare							
	<10	10 - 19.9	20 - 29.9	30-39.9	40 - 49.9	50 - 59.9	60 - 69.9	>= 70
1990	1.00	1.01 (1.00 - 1.03)	0.99 (0.98 - 1.01)	0.97 (0.95 - 0.99)	0.97 (0.96 - 0.99)	0.96 (0.93 - 99)	0.96 (0.93 - 1.00)	0.86 (0.84 - 0.88)
1991	1.00	1.01 (0.99 - 1.03)	0.99 (0.97 - 1.01)	0.97 (0.94 - 0.98)	0.98 (0.96 - 1.00)	0.95 (0.92 - 0.98)	0.97 (0.93 - 1.00)	0.84 (0.82 - 0.87)
1992	1.00	1.00 (0.98 - 1.02)	0.97 (0.95 - 0.98)	0.96 (0.94 - 0.98)	0.96 (0.94 - 0.98)	0.94 (0.91 - 0.96)	0.94 (0.90 - 0.98)	0.81 (0.78 - 0.83)

Figure 4-2. The inverse relationship between the percentage of households with four or more persons and the number of persons per hectare in 9509 electoral wards in England and Wales.

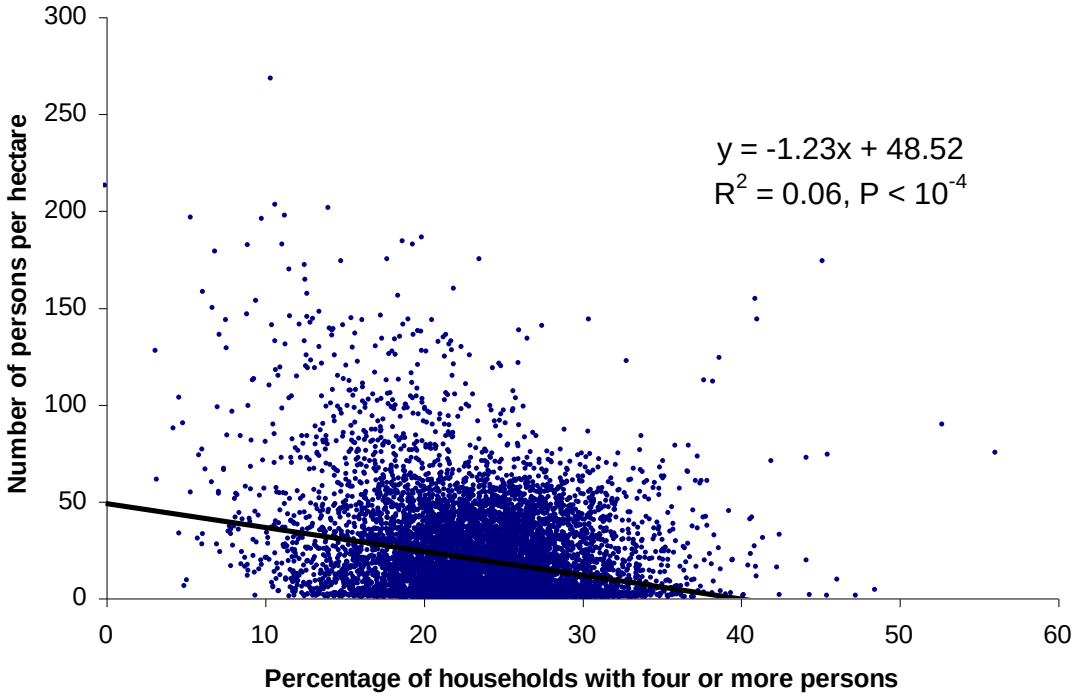


Figure 4-3. Monthly number of deaths from myocardial infarction in England and Wales.

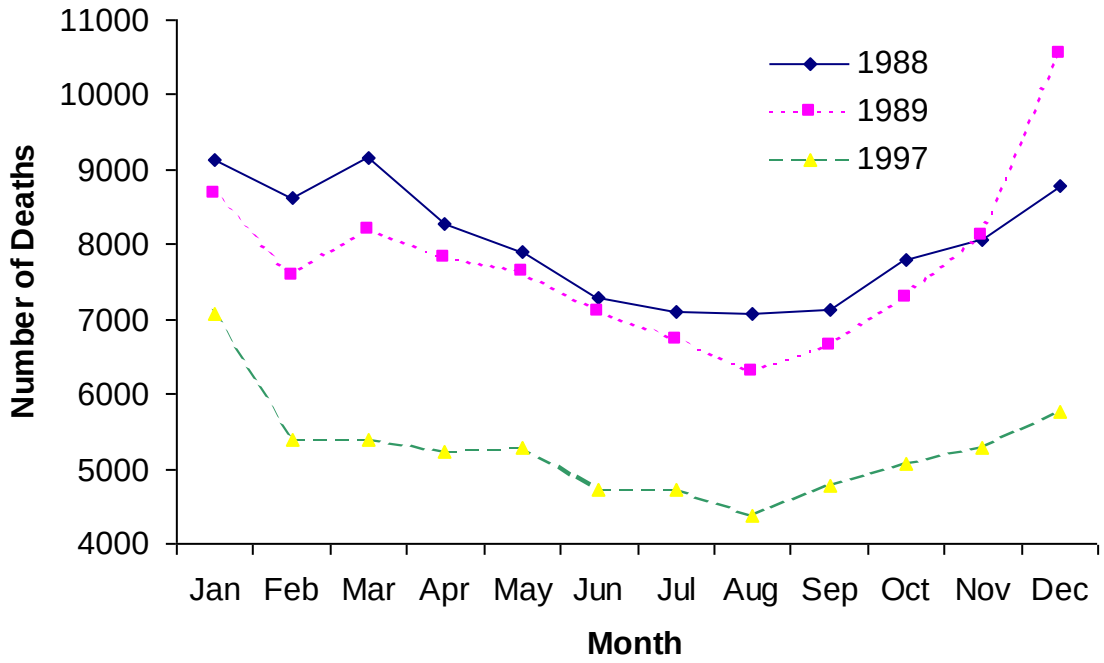
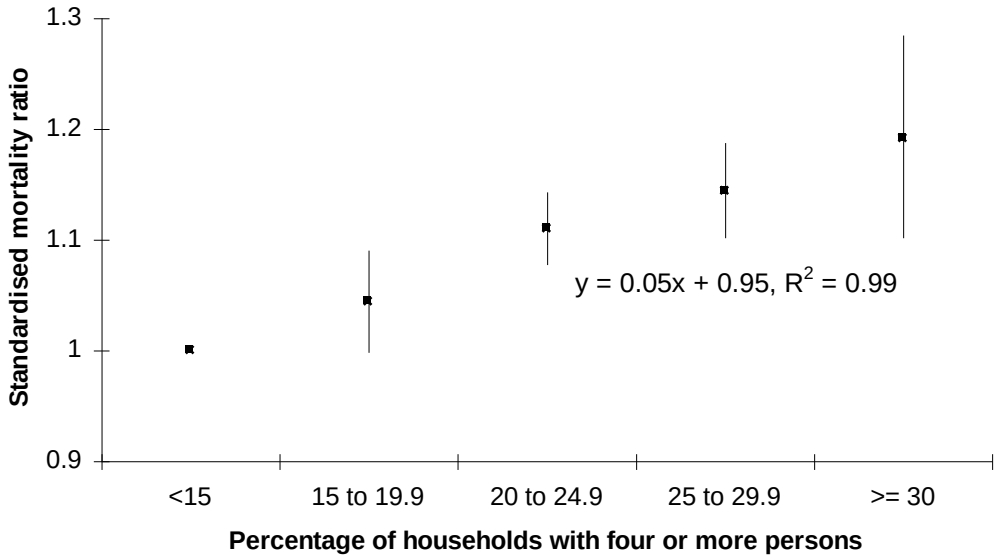


Figure 4-4. Mortality rate ratios and 95% confidence intervals for myocardial infarction deaths in December 1989 standardised for age, sex and deprivation by percentage of households with four or more persons. (Due to the relatively small number of deaths, data for wards with less than 10 and less than 15% of households with four or more persons have been aggregated together).



Chapter 5 *Chlamydia pneumoniae* Serology. Species specificity of the microimmunofluorescence test and comparisons with a time resolved fluoroscopic immunoassay for measuring IgG antibodies to *Chlamydia pneumoniae*.

As it is a difficult organism to culture, most of the evidence associating *C. pneumoniae* with various diseases has come from serological studies. The microimmunofluorescence test (MIF) of Wang and Grayston[23] was first validated for the measurement of *Chlamydia trachomatis* antibodies and is now the "gold standard" serological test for antibody to chlamydia. In expert hands, it is generally considered to be a species-specific test as it is possible to visually discriminate ring-like specific fluorescence due to antibody to outer envelope proteins from diffuse genus-specific staining of antibody to lipopolysaccharide[143]. However, some authors have questioned this specificity and have claimed that it is difficult to make such visual distinctions[51,144]. Undoubtedly, the biggest problem of MIF is that it is subjective and tedious when used to screen large numbers of sera. During its development, several chinese ladies were apparently confined to a dark basement room dabbing antigen from different strains of *C. trachomatis* onto microscope slides using calligraphy brushes (as told by Prof. P Watt, Southampton University). The darkness was to facilitate fluorescence microscopy. This story may be apocryphal but it serves to illustrate the point that MIF is labour intensive and dependent on expertise. Enzyme immunoassay is more suitable for large scale screening, being both objective and relatively simple to perform. However, it is considered not to be as species-specific as MIF and its results are linear over only a relatively narrow range of antibody concentration. Time resolved fluoroscopic immunoassay (TRIA) is similar in practice to enzyme immunoassay except that europium (a rare earth metal) is used as the labelling agent. The advantage of TRIA is that its results are linear over a greater range[145] so that for screening purposes it may be sufficient to test for antibody at a single serum dilution only.

In collaboration with researchers in France and the MRC epidemiology unit in Southampton, we examined the species-specificity of MIF and compared it with TRIA for measuring antibodies to *C. pneumoniae*.

Methods

Subjects Our study sample were 1020 healthy men (n=632) and women (n=388) aged 60 to 75 years, who took part in an earlier study of the association between foetal and infant growth and adult *Helicobacter pylori* infection[146]. Aliquots of serum taken at that time (1992-1994) were stored at -80°C until used for the current study. The subjects were selected on the basis that they had been born and still lived in East or North-west Hertfordshire and agreed to take part.

MIF Indirect MIF assay was performed by the Biobanque De Picardie Laboratory (Amiens, France) using as antigen either *C. pneumoniae* (IOL-207), *C. psittaci* (Loth) or *C. trachomatis* (LB_1 and the serovar L_2) prepared from acetone fixed preparations of infected egg yolk sacs[147]. Non-infected egg yolk sacs were used as negative controls. Serial twofold dilutions of each serum sample (1:16 - 1:2048 for IgG, 1:16 to 1:512 for IgA and 1:12 to 1:48 for IgM) were tested. Specific antibody was detected with 1:100 FITC-conjugated anti-human immunoglobulin (TAGO Inc, USA). For the detection of IgM, serum samples were pre-treated with RF (rheumatoid factor) absorbant (Behringwerke AG, Germany) according to manufacturer's instructions. Where control preparations were positive (indicating serum antibodies against egg yolk antigen), chlamydial antigen prepared from organisms grown in tissue culture (Hela 229 cells) was used instead. For IgG and IgA, we considered that species-specific antibodies were absent if titres were less than 16 but that they were present if titres for one species were at least two twofold dilutions greater than for the other species or if titres for the other species were absent. Similar criteria were used for IgM except that titres less than 12 were considered negative.

TRIA 96 well plates (Maxisorb, Nunc, Denmark) were coated with whole, purified *C. pneumoniae* (VR1310) organisms (2 µg in 100µl 1% sodium azide PBS solution) at room temperature overnight. Plates were then blocked with 200µl 10% normal goat serum and sequentially incubated with 100µl 1:100 serum, 100µl 1:1000 biotin-labelled goat anti human IgG immunoglobulin (Kirkegaard & Perry Laboratories, Maryland) and with 100µl 1:1000 Europium labelled streptavidin (Wallac Oy, Finland). All incubations were at 37°C. The first 2 incubation stages were for 2 hours and the final two for 1 hour. Sera, anti-human immunoglobulin and europium labelled streptavidin

were diluted in a solution containing 150mM NaCl, 25mM Tris, 0.005% Tween 40, 0.25% bovine serum albumin, 0.023% bovine γ globulin, 10μmol Diethylenetriaminepentaacetic acid and 0.025% sodium azide at pH 7.8. Plates were washed between each incubation with a solution containing 0.1M NaCl, 0.025M Tris and 0.05% Tween 20 at pH 8. Finally, 100μl DELFIA® enhancement agent (Wallac Oy, Finland) was added to each well and the fluorescence count was determined with a 1234 Wallac fluorometer. Eight control sera were included in each plate. Each serum sample was tested in duplicate without knowledge of the MIF result.

Results

Specificity of the MIF Test

Valid results were obtained for 947 (IgG), 985 (IgA) and 950 (IgM) subjects. For 71 (IgG), 33 (IgA) and 68 (IgM) subjects, control preparations from egg yolk were reported as positive. Antigen preparations from tissue culture grown organisms were therefore used for 54 of these subjects (IgG only) giving a total of 1001 IgG results. For two subjects, no result was available due to insufficient serum.

Table 5-1 shows that seropositivity for *C. trachomatis* and *C. psittaci* was strongly correlated with seropositivity for *C. pneumoniae* and that this correlation was highly significant. The correlation was found at all titres examined and for all immunoglobulin classes although it seemed to be particularly strong for the IgM class of immunoglobulins. For IgG, there was an almost linear association between *C. pneumoniae* titres and geometric mean titres for *C. trachomatis* and *C. psittaci* (Figure 5-1). Such an association was not seen for IgA and IgM, probably because of the small number of samples with high titre values.

Table 5-2 shows the number of samples with specificity for one chlamydial species and the number of subjects seropositive for *C. pneumoniae, C. trachomatis* and *C. psittaci*.

Comparison of MIF and TRIA in measuring IgG antibodies for *C. pneumoniae*

501 serum samples were assayed by TRIA in duplicate with intra and inter-assay coefficients of variation of 4.7 and 18.2 respectively. However, only 484 samples had IgG antibodies for *C. pneumoniae* measured by both MIF and TRIA (294 male, 190 female, average age 67) of which only 389 had species-specific MIF results. Table 5-3 shows a comparison of results for the MIF and TRIA tests for these 389 subjects. Comparison is limited by the fact that MIF titres are categorical variables whereas TRIA fluorescence counts are continuous variables. Nevertheless, for any fluorescence count, a range of possible MIF titres can be predicted within which most values will fall and which consist of the modal MIF titre ± 1 titre. Furthermore, the mean fluorescence count for a particular population has a linear relationship with the $\log_2$ geometric mean MIF titre for that population (Figure 5-2). However, some sera clearly fall outside the predicted MIF titre, 57 (14.7%) serum samples had MIF titres that were lower than predicted and 41 (10.5%) had titres that were higher. Similar findings were obtained when MIF was compared with TRIA using all 484 samples that had IgG antibodies measured by both techniques and not just those that had species-specific MIF results.

Discussion

The MIF test is the classic and time-honoured method for measuring antibody to chlamydia but it involves fluorescence microscopy which inevitably has a large subjective component.[148] Although MIF uses crude, whole chlamydial antigen which inevitably cross-reacts with antibody to other bacteria, the strength of the test is that species-specific antibody can be distinguished from other antibody by the pattern of fluorescence. Such distinctions are difficult to standardise, require considerable experience and observer errors can occur. Also, the chlamydial strains used and the method of antigen preparation vary between different laboratories and this may contribute to differences in results[149]. In this large study, MIF was performed by a national reference laboratory that achieved excellent results in an inter-laboratory comparison of MIF results[149]. However, a strong association between antibody titres to the three chlamydial species was still seen indicating that even with major experience, genus-specific antibody still influences the results. Nevertheless, the expected finding

for this relatively elderly population, that antibodies to *C. pneumoniae* were more common than antibodies to *C. trachomatis* which in turn, were more common than antibodies to *C. psittaci* was seen. One possibility is that genus-specific antibody occasionally produces a homogenous, ring fluorescence. Figure 5-1 shows the geometric mean *C. trachomatis* and *C. psittaci* IgG titres for the corresponding *C. pneumoniae* IgG titre. If MIF is species-specific then the geometric mean IgG titres should be the same no matter what the IgG titre for *C. pneumoniae*. However, there is clearly a linear increase in the $\log_2$ geometric mean titre for *C. psittaci* and *C. trachomatis* with increasing $\log_2$ *C. pneumoniae* titre with the rate of increase (gradient of the equations) about the same for both *C. trachomatis* (30%) and *C. psittaci* (34%). This may indicate that on average, 30 to 34% of the $\log_2$ IgG titre for *C. pneumoniae* is due to the effect of genus-specific antibody. The $\log_2$ IgG titre is essentially the number of twofold dilution steps. Therefore, on average, approximately one third of the dilution steps that make up the final titre may be due to genus-specific antibody. Whereas the gradient of the respective equations are the same, the intercepts are different and represent the geometric mean titres for *C. trachomatis* and *C. psittaci* when there is no *C. pneumoniae* IgG antibody and presumably no cross-reactive antibody (in this study, the starting titre was 16). One approach widely used to reduce these problems of cross-reactivity is to consider that species-specific antibodies in MIF are only present if their titre is at least 2 dilution steps greater than that for the other species. This should improve specificity but in samples where there are antibodies to more than one species, sensitivity will be reduced. Thus, in this study, 1001 IgG results were available but from the point of view of *C. pneumoniae*, it was not possible to assess 154 (15.4%) samples because titres were within 2 dilution steps of the other 2 species. For *C. psittaci*, it was not possible to assess 276 samples (27.6%). Another problem encountered with MIF was that a significant proportion of control preparations from egg yolk was positive especially for measurement of IgG (7%). This is probably due to the presence of serum antibodies against egg yolk antigen.

In this study, we also compared TRIA with MIF. In comparing a "new" test with an established method, it should be at least as good as the old test in terms of specificity and sensitivity or offer some other significant advantage. There is no doubt that TRIA is much easier to perform and does not require expert interpretation. For instance, the TRIA analysis in this study was completed in three days. Also, TRIA gives

reproducible results and is amenable to automation. In 75% of samples, we were able to predict the MIF titre ± 1 titre from the fluorescence count. Moreover, the accuracy of MIF itself is unlikely to be better than ± 1 titre. Agreement between TRIA and MIF was the best for those sera which had the lowest or highest fluorescence counts (Table 5-3) and this may reflect the fact that MIF is most accurate when antibodies are absent or are present in very high quantities. We did not use TRIA to assay for antibodies to *C. trachomatis* or *C. psittaci* and therefore cannot compare its species-specificity with that of MIF although it is unlikely to be as good. However, if TRIA's specificity were much worse than that of MIF, then we would expect it's results to overestimate MIF titres when in fact, it was just as likely to underestimate them. The specificity of TRIA will partly depend on the age of the population being screened. *C. trachomatis* antibodies are more prevalent in young adults compared with the typical subjects being investigated for coronary artery disease. Therefore, the specificity of TRIA when used to detect for *C. pneumoniae* antibodies in the elderly is likely to be better.

In conclusion, MIF cannot be considered a truly species-specific test even in expert hands and we have attempted to quantify this disparity. TRIA has the advantages of being more objective and easier to perform and it can predict MIF titres well. We believe it is a rapid, useful method for comparing populations although obviously, no test can be better than the comparative gold standard. The increasing suspicion that *C. pneumoniae* may cause chronic disease means that there is an urgent need for the development of truly species-specific tests for chlamydial antibody based on defined antigens.

Table 5-1. The association between seropositivity for *C. pneumoniae*, *C. trachomatis* and *C. psittaci*

	C. Pneumoniae +ve [¶]	*C. Pneumoniae* -ve	Odds ratio (95% CI)[†]	P[§]
Titre >= 1:16				
C. trachomatis IgG +ve	350 (46.7%)	22 (11.1%)	7.1 (4.4 - 11.3)	$<10^{-5}$
C. psittaci IgG +ve	261 (34.9%)	8 (4.0%)	12.8 (6.2 - 26.4)	$<10^{-5}$
C. trachomatis IgA +ve	28 (7.8%)	10 (1.6%)	5.1 (2.5 - 10.7)	$<10^{-5}$
C. psittaci IgA +ve	15 (4.2%)	0	*	$<10^{-5}$
C. trachomatis IgM +ve	5 (31.3%)	4 (0.3%)	141.1 (29.9 - 664.5)	$<10^{-5}$‡
C. psittaci IgM +ve	2 (12.5%)	0	*	$<10^{-3}$‡
Titre >= 1:32				
C. trachomatis IgG +ve	192 (27.8%)	20 (7.8%)	4.6 (2.8 - 7.4)	$<10^{-5}$
C. psittaci IgG +ve	104 (15.1%)	3 (1.2%)	15.0 (4.7 - 47.8)	$<10^{-5}$
C. trachomatis IgA +ve	11 (4.2%)	9 (1.2%)	3.5 (1.4 - 8.6)	$<10^{-2}$
C. psittaci IgA +ve	6 (2.3%)	0	*	$<10^{-3}$‡
C. trachomatis IgM +ve	2 (25.0%)	0	#	$<10^{-4}$‡
Titre >= 1:64				
C. trachomatis IgG +ve	110 (18.6%)	23 (6.5%)	3.3 (2.1 - 5.3)	$<10^{-5}$
C. psittaci IgG +ve	43 (7.3%)	2 (0.6%)	13.9 (3.3 - 57.7)	$<10^{-5}$
C. trachomatis IgA +ve	5 (3.3%)	2 (0.2%)	14.0 (2.7 - 72.8)	$<10^{-2}$‡
C. psittaci IgA +ve	0	0		
Titre >= 1:256				
C. trachomatis IgG +ve	15 (6.7%)	15 (2.1%)	3.4 (1.6 - 7.0)	$<10^{-3}$
C. psittaci IgG +ve	5 (2.2%)	0	*	$<10^{-3}$‡
Titre >= 1:512				
C. trachomatis IgG +ve	6 (5.8%)	4 (0.5%)	12.8 (3.6 - 46.3)	$<10^{-3}$‡

[¶]The number (percentage) of subjects who were seropositive for *C. trachomatis* and *C. psittaci* of those who were seropositive for *C. pneumoniae* (at the same titre and for the same immunoglobulin class)

[†]Odds Ratio (95% confidence interval) for seropositivity for *C. psittaci* and *C. trachomatis* when seropositive for *C. pneumoniae* compared with when seronegative for *C. pneumoniae*

*No subject uniquely seropositive for *C. psittaci*

[#]No subject uniquely seropositive for *C. trachomatis*

[§]χ^2 test unless otherwise indicated

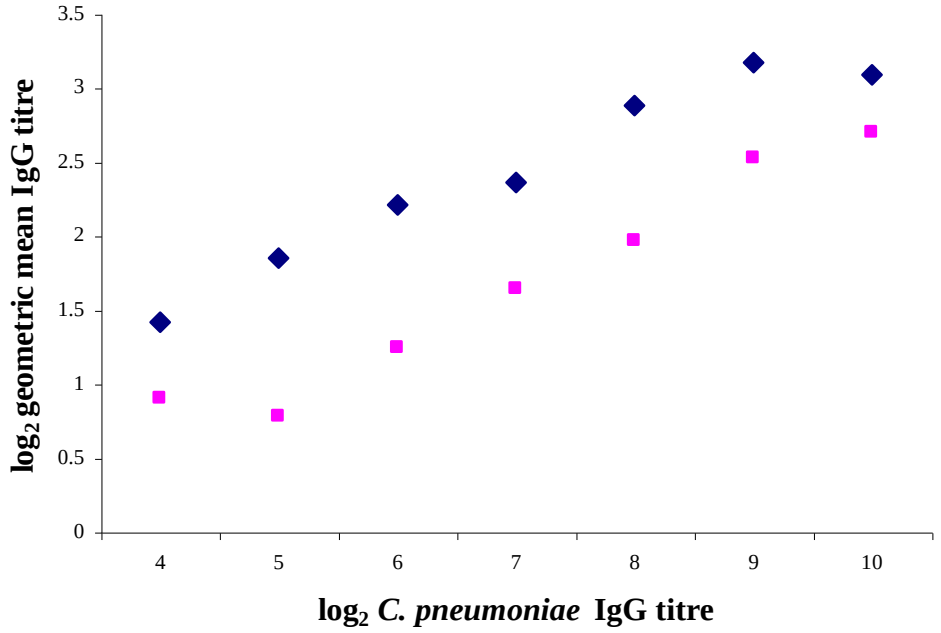
[‡]2 tail Fisher's exact text

Table 5-2. Number (percentage) of subjects with specific antibody to *C. pneumoniae*, *C. trachomatis* and *C. psittaci* by microimmunofluorescence.

	C. pneumoniae	***C. trachomatis***	***C. psittaci***
IgG Titre (n=1001*)			
Genus-specific antibody	154	346	276
<16	218 (25.7)	616 (94.0)	724 (99.9)
16	42 (5.0)	6 (0.9)	0
32	60 (7.1)	5 (0.8)	0
64	146 (17.2)	7 (1.1)	1 (0.1)
128	167 (19.7)	7 (1.1)	0
256	110 (13.0)	10 (1.5)	0
512	75 (8.9)	2 (0.3)	0
1024	22 (2.6)	2 (0.3)	0
2048	6 (0.7)	0	0
4096	1 (0.1)	0	0
IgA Titre (n=985*)			
Genus-specific antibody	20	28	15
<16	622 (64.5)	947 (99.0)	970 (100)
16	94 (9.7)	3 (0.3)	0
32	102 (10.6)	5 (0.5)	0
48	2 (0.2)	0	0
64	71 (7.4)	1 (0.1)	0
128	53 (5.5)	0	0
256	16 (1.7)	1 (0.1)	0
512	5 (0.5)	0	0
IgM Titre (n=950*)			
Genus-specific antibody	14	16	8
<12	895 (95.6)	928 (99.4)	942 (100)
12	30 (3.2)	5 (0.5)	0
24	6 (0.6)	1 (0.1)	0
48	5 (0.6)	0	0

*The denominator used in calculating the percentage of subjects with species-specific antibody was the total number of subjects with species-specific antibody for that immunoglobulin class.

Figure 5-1. The correlation between *C. pneumoniae*, *C. trachomatis* and *C. psittaci* MIF IgG titres.



◆ *C. trachomatis.* Equation of line is y = 0.30x + 1.24. $R^2 = 0.95$

■ *C. psittaci.* Equation of line is y = 0.34x + 0.31. $R^2 = 0.96$

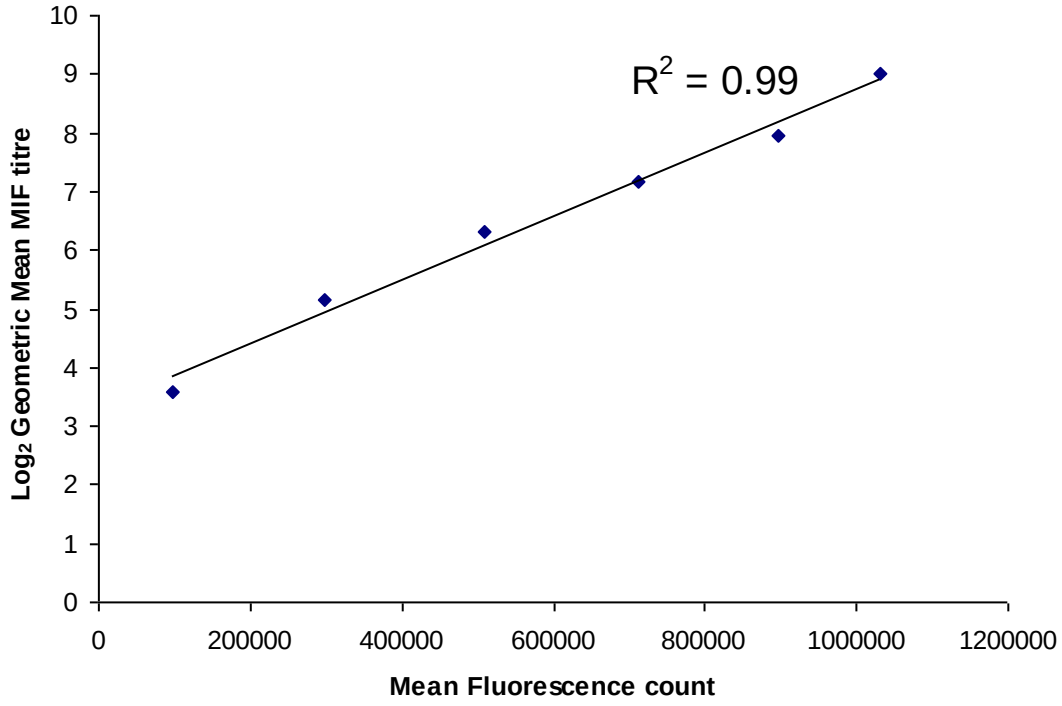
Table 5-3. The correlation between Time Resolved Fluoroscopic Immunoassay and the Microimmunofluorescence Test for measuring IgG antibodies to *C. pneumoniae.*

TRIA fluorescence count (arbitrary units)	Mean fluorescence count	MIF <16	MIF 16	MIF 32	MIF 64	MIF 128	MIF 256	MIF 512	MIF 1024	MIF 2048	Geometric Mean MIF titre*	Subjects within predicted MIF range#
<200000	96571.3	81 (89.0%)	2 (2.2%)	1 (1.1%)	5 (5.5%)	1 (1.1%)	0	1 (1.1%)	0	0	1:12.1	83 (91.2%)
200000-399999	296961.2	15 (30.6%)	4 (8.2%)	8 (16.3%)	12 (24.5%)	6 (12.2%)	4 (8.2%)	0	0	0	1:35.2	26 (53.0%)
400000-599999	507878.8	7 (9.7%)	2 (2.7%)	8 (11.1%)	20 (27.8%)	22 (30.6%)	8 (11.1%)	5 (6.9%)	0	0	1:79.3	50 (69.5%)
600000-799999	711326.5	1 (1.2%)	1 (1.2%)	6 (7.0%)	20 (23.3%)	22 (25.6%)	23 (26.7%)	10 (11.6%)	3 (3.5%)	0	1:142.5	65 (75.6%)
800000-999999	896996.2	3 (3.8%)	0	1 (1.3%)	8 (10.0%)	14 (17.5%)	23 (28.8%)	21 (26.3%)	8 (10.0%)	2 (2.5%)	1:247.2	58 (72.6 %)
1000000-1199999	1030441.4	0	0	0	0	1 (9.1%)	2 (18.2%)	5 (45.5%)	2 (18.2%)	1 (9.1%)	1:512	9 (81.9%)

*To allow calculation of the geometric mean MIF titre, samples with a titre < 16 were given a titre value of 1

#The predicted MIF range for the corresponding TRIA range is the modal MIF titre ± 1 titre (highlighted cells)

Figure 5-2. The linear correlation between mean TRIA fluorescence count and log_2 geometric mean MIF titre.



Chapter 6 Methods of detecting *Chlamydia pneumoniae* in blood vessels.

The two main techniques that have been used to detect for *C. pneumoniae* in blood vessels have been the polymerase chain reaction (PCR) and immunocytochemistry (ICC).

However, the specificity and sensitivity of these methods are unknown. Ideally, infection by *C. pneumoniae* should be confirmed by culture and the results of PCR and ICC compared against this. Unfortunately, culture of *C. pneumoniae*, an intracellular organism, from atherosclerotic vessels, is technically difficult and there have only been three reports of this. In the first study, *C. pneumoniae* was cultured from a coronary artery and this was confirmed by three of four laboratories using PCR and one of two laboratories using ICC[107]. In the second study, *C. pneumoniae* was isolated from a carotid artery but both PCR and ICC were negative[132]. In the most recent study, *C. pneumoniae* was successfully isolated from eleven of seventy coronary arteries[43]. This excellent result was achieved by multiple serial passage[150] whereby cell cultures were inoculated with homogenised coronary arteries and the cultures were then repeatedly divided and cultured using more cycles than had previously been described (at least 10 cycles). All eleven coronary arteries that were positive for *C. pneumoniae* by culture were also positive by PCR but in this study, ICC was not used. Therefore, there is insufficient data in the literature to assess whether PCR or ICC is the most sensitive or specific for detection of vascular *C. pneumoniae* infection.

Immunocytochemistry

The advantage of immunocytochemistry is that it should be possible to see which cells involved in the atherosclerotic process are infected by *C. pneumoniae*. Studies have reported that *C. pneumoniae* can be localised to macrophages[131], smooth muscle cells[127] and endothelial cells[128]. Generally, ICC finds more evidence for *C. pneumoniae* in blood vessels than PCR[99;132] although this is not always the case[107;130]. Some authors have interpreted this as indicating that ICC is more sensitive than PCR. The concern is that antibodies used in ICC may cross-react with components of atherosclerotic

tissue[151]. To control for such cross-reactivity and false positive staining, studies have employed "control tissue". Ideally, control tissue should be atherosclerotic tissue which is known not to be infected by *C. pneumoniae*. Unfortunately, there is no test which can exclude infection and control tissue which has been used, such as monkey kidney cells[129] and normal vascular tissue[128], do not give convincing evidence that false positive staining does not occur with atherosclerotic tissue. In one study, *C. pneumoniae* was detected in 86% of severely diseased compared with 6% of mildly diseased lesions using ICC[104]. However, when a subset of samples was tested by PCR, discordant results were obtained and the prevalence in non-atherosclerotic or minimally atherosclerotic lesions was said to be quite high[105]. This could indicate that ICC is not as sensitive as PCR and that cross-reactivity occurs with severely diseased tissue. In other studies, results have depended on the antibody used. Genus-specific antibodies against lipopolysaccharide have been reported to give more positive results than species-specific antibodies[125;152] and some species-specific antibodies appear to be more sensitive than others[128]. Furthermore, the results of these different antibodies do not necessarily correlate with each other. For example, a genus specific antibody found 79.8% of 114 atherectomy samples to be positive for *C. pneumoniae* compared with 53.2% using a species-specific antibody but the kappa statistic was only 0.047[152]. In Southampton, preliminary studies of ICC by a specialist cardiac pathologist (Dr Patrick J Gallagher) and a regional immunocytochemistry laboratory failed to convince them of its specificity. Other groups have also commented on the problems of non-specific staining [102]. In one study, it was impossible to evaluate 28 of 35 samples because of background staining[153]. Therefore, although other groups have been successful with ICC, we have used PCR for the detection of *C. pneumoniae* in blood vessels.

Polymerase Chain Reaction

The main advantages of PCR are that it is an exquisitely sensitive technique for the detection of DNA and that positive results can be confirmed by sequencing of PCR products or by Southern hybridisation. Unfortunately, clinical samples may contain many inhibitors of PCR. We found that coronary artery samples were more likely to demonstrate PCR inhibition (Chapter 10) if adjacent samples had histological evidence of lipid (OR 2.3, CI 1.1 to 5.0) or calcium (OR 3.8, CI 1.9 to 8.0). The magnitude of this inhibition problem in relation to atherosclerotic tissue has not been reported well by

any study but we found that between 35 to 43% of DNA samples extracted from atherosclerotic vessels contained inhibitors of PCR. This contrasts with 17.7% of 237 naso-pharyngeal swabs reported by another study[154]. PCR inhibition can lead to false negative results and attempts to eliminate it are important. Generally, when DNA is extracted from tissue, it is important to ensure that the amount of extracted DNA is high relative to the amount of inhibitors and attempts should be made to detect for inhibition and to reduce it. Our extraction process involves EDTA which chelates ions such as Ca^{2+} while phenol-chloroform removes lipid soluble inhibitors (Appendix 1). There are other methods of extracting DNA including commercially available columns (QIAamp®, Qiagen, Crawley). In a study which compared various methods, phenol chloroform was found to be amongst the best[100]. To check for inhibitors, we spiked extracted DNA with known amounts of bacterial phage λ DNA and subjected it to PCR with λ specific primers (Appendix 2). If amplification of λ DNA was not seen, inhibition was assumed to be present and the extracted DNA was diluted tenfold. Dilution reduces the amount of inhibitors and we found that PCR inhibition was eliminated in virtually all cases as has been reported by other groups[154]. Unfortunately, dilution also reduces the amount of DNA. However, we found that samples requiring dilution were just as likely to be positive for *C. pneumoniae* as samples that were not inhibited.

To detect for *C. pneumoniae* DNA, we used a nested PCR with primers against the momp-1 (major outer membrane protein) gene[155] which was first sequenced in Southampton by Carter et al[14]. This PCR can detect an amount of DNA equivalent to that from between 1 to 10 elementary bodies in a volume of 3 μl. Positive PCR products were confirmed by Southern blotting using an oligo-nucleotide probe against the VS4 region (Appendix 3). The volumes involved in PCR are small and sampling errors may result in false negative results especially if the amount of *C. pneumoniae* DNA is low. To reduce these errors, we tested each sample in triplicate. In the few studies where samples have been tested on more than one occasion, it has been reported that repeat testing does not always give consistent results[86;107] and we found this to be true as only 40% of our samples were positive on two or more occasions (Chapter 10 and Chapter 11). In some studies, authors have considered results that were not consistently positive as being false positive results arising from DNA contamination[36].

However, we took the opposite view and considered that any positive result was genuinely positive so long as we were confident that DNA contamination had not occurred. To check for contamination, we controlled for both the DNA extraction process and for the PCR reaction. We never extracted DNA from more than five blood vessels at any one time and a "mock extraction" sample was included on all occasions. For each PCR experiment, we included a negative control consisting of water every five to six samples and to control for sensitivity, a positive control consisting of DNA equivalent to that from between one to ten elementary bodies was used. When an extraction control was positive, a spare sample of the blood vessel was re-extracted and similarly, when a PCR negative control was positive, the PCR was repeated. In our studies involving blood vessel specimens (Chapter 10 and Chapter 11) where 106 of 326 specimens were positive for *C. pneumoniae* by PCR, extraction controls were positive on 2 occasions and PCR negative controls were positive on 5 occasions. In our study involving the buffy coat of blood samples from 1205 patients where 100 samples were positive for *C. pneumoniae* (Chapter 9), no extraction control and no PCR negative control were positive.

Electron Microscopy

The presence of *C. pneumoniae* in blood vessels was first reported when particles with appearances highly suggestive of elementary bodies were seen in intimal smooth muscle cells and lipid rich areas of fibrolipid plaques by electron microscopy[96]. In another study by the same group, 7 of 8 samples that were positive for *C. pneumoniae* by PCR were also positive by electron microscopy[153]. However, electron microscopy has not been used by many groups.

It can be seen that all the methods for detecting *C. pneumoniae* in blood vessels have their advantages and disadvantages. PCR is the method which has been most commonly used but the method which is chosen will often depend on local expertise and availability. None of the methods have been standardised and this has been criticised by some authors[156]. Nevertheless, we believe that a sensitive PCR which has been adequately controlled for PCR inhibition, PCR sensitivity and specificity and DNA contamination can give results that are the best currently available.

Chapter 7 The association of *Chlamydia pneumoniae* with coronary risk factors, markers of inflammation and coronary artery disease.

In serological studies, *C. pneumoniae* has been associated with coronary artery disease (CAD). This association may be causal or it may be due to confounding factors. It is therefore important to know what possible confounding factors there may be. Potential candidates are factors which predispose to both CAD and infection by *C. pneumoniae*. Seroprevalence studies have suggested that exposure to *C. pneumoniae* increases with age[25;157] and that infection is more common in men and in smokers[80]. These factors also predispose to CAD but much of these data has come from studies where subjects have been patients presenting with atypical pneumonia or other respiratory infections. Few studies have examined the general population. Other organisms apart from *C. pneumoniae* have also been linked to atherosclerosis. An association with *H. pylori*[69] has been described while CMV has been linked mainly to CAD following cardiac transplantation[158]. It would be important to know whether infection by one organism is associated with infection by another since this is another potential cause of confounding. It would also be interesting to know whether chronic infection with *C. pneumoniae* is associated with any evidence of chronic inflammation as this is increasingly thought to have a role in atherogenesis[105].

In this study, we correlated the presence of *C. pneumoniae* antibodies with vascular risk factors and biochemical markers of chronic inflammation in a large group of 704 control subjects recruited from the general population. The prevalence of seropositivity in this control group was compared to that of 288 cases with CAD.

Methods

Subjects and laboratory methods

The subjects had been recruited for an earlier study which had investigated the outcome of offering health checks systematically to a general practice adult population[159]. Five general practices in Luton and Dunstable, Bedfordshire were involved and between them, they had a population of 13817 that was aged 35 to 64 of which 8100 (58.6%)

agreed to take part. Practice nurses made measurements of participants' height, weight and blood pressure. A questionnaire which covered various aspects of lifestyle including smoking, alcohol intake and social status and the Rose questionnaire[160], designed to record symptoms suggestive of CAD were completed. Cholesterol was measured at the time of the study and non-fasting serum samples were centrifuged and stored at -80ºC within 24 hours of the study.

From the 8100 participants, we randomly selected 288 cases who had CAD and 704 age and sex-matched controls (nested case-control study). Cases were defined on the basis of incident CAD death notified by the Office of National Statistics before 1997 (n=76) or on the basis of self-reported CAD at baseline (103 cases with history of myocardial infarction and 109 with angina but no myocardial infarction).

Serology was performed at Southampton without knowledge of the case-control status of blood samples. Specific IgG antibodies to *C. pneumoniae* and CMV were measured by time-resolved fluoroscopic immunoassay (TRIA) as described in Chapter 5 and specific IgG antibodies to *H. pylori* were measured with a commercial assay (Pyloriset EIA-G, Orion, Finland). Concentrations of C-reactive protein CRP[161] and serum amyloid A (SAA)[162] were measured at the Royal Postgraduate Medical School using sensitive automated enzyme immunoassays. The median normal value for CRP is around 0.8 mg/l and 99% of normal values are less than 10 mg/l. For SAA, the median value is 3 mg/l and 96% of normal values are less than 10 mg/l.

Statistics

As there are no generally agreed cut-offs for seropositivity to *C. pneumoniae* or CMV antibodies, analysis by thirds of TRIA fluorescence count was pre-specified. For antibodies to *H. pylori*, the manufacturer's recommended cut-off point was used. The distribution of CRP and SAA concentrations were skewed but logarithmic transformation gave approximately normal distributions. Analysis by thirds of the concentrations of CRP and SAA were also pre-specified. The 2-tailed t test was used to compare continuous variables with normal distributions and the χ^2 test for categorical variables. Regression modelling was used to explore possible associations of *C. pneumoniae* with conventional coronary risk factors, infection with *H. pylori* and CMV

and with CAD (STATA Corporation, Texas, USA). The sample size was sufficient to detect an odds ratio of 1.5-fold or larger with 80% power at the 5% level of significance, assuming about 50% seropositivity.

Results

When stratified by sex, overall findings were unchanged and so findings are presented as combined results for men and women. The distribution of *C. pneumoniae* antibody levels was approximately bimodal. In control subjects, the top third of *C. pneumoniae* antibody levels was associated with male sex, smoking (OR 1.5, 95% CI 1.1 to 2.5, Table 7-1) and higher CRP (Table 7-2) and SAA concentrations (Table 7-3).

The distribution of CMV antibody levels was also bimodal and the top third of antibody levels was associated with increasing age alone (60.2 ± 5.6 vs. 58.5 ± 6.1, adjusted P<0.001). *H. pylori* serology was available for only 640 controls of which 294 (45.9%) were seropositive. Seropositivity was associated with increasing age (60.1 ± 5.8 vs. 58.0 ± 6.2, adjusted P<0.01) and smoking (35% vs. 25%, adjusted P<0.01). Compared to subjects who were seronegative for *H. pylori*, subjects who were seropositive had higher antibody levels against *C. pneumoniae* (114 ± 33.9 fluorescence counts vs. 93.4 ± 41.2, P<0.00001) but not against CMV (90.9 ± 55.3 fluorescence counts vs. 81.8 ± 59.8).

Control subjects with CRP levels in the top third were more likely to have SAA levels in the top third. There was also a strong association between both smoking and body mass index with the top third of CRP and SAA concentrations.

As expected, cases had a higher prevalence of conventional coronary risk factors compared with controls (Table 7-4). No association between top third of *C. pneumoniae* antibody levels and CAD was seen (Table 7-5) and although there were weak associations between both *H. pylori* and CMV with CAD, these disappeared after adjustment for coronary risk factors.

Discussion

Association of *C. pneumoniae* with coronary risk factors.

The finding that IgG seropositivity for *C. pneumoniae* was associated with smoking is not unexpected since smoking predisposes to respiratory tract infections. In 365 middle-age men presenting with respiratory illness, the odds ratio for a *C. pneumoniae* IgG antibody titre greater than 128 was 2.4 for smokers compared with non-smokers[80]. The magnitude of this association though, appears to be reduced in the general population. In 210 subjects attending a health-screening clinic in London, non-smokers were just as likely as smokers to be seropositive for *C. pneumoniae*[11]. However, in a much larger study involving 2400 randomly selected Finnish subjects, the odds ratio for seropositivity was 1.5 for smokers compared with non-smokers[163]. This moderate association is similar to that found in this study. We also found that *C. pneumoniae* IgG antibodies were more common in men as has been widely reported[25;50].

In contrast to previous studies, we did not find an association with either age or hyperlipidaemia. The lack of an association with age is perhaps unsurprising since the lower age limit of our patients was 35. Population studies suggest that by the age of 20, 50% of a general population will be seropositive for *C. pneumoniae* IgG and that the seroprevalence remains at this level or increases slightly with age[25]. The observation that acute bacterial infections can result in raised triglyceride and reduced HDL cholesterol levels[73] has lead to the suggestion that chronic bacterial infections may also result in an adverse lipid profile. However, there is a paucity of data on this subject. A study of 388 London men failed to confirm this[69] although a study of 1053 male Finnish subjects reported that seropositive subjects had significantly lower HDL cholesterol levels (1.24 vs. 1.32 mmol/l, $P < 0.01$) [164]. However, there were no differences in total cholesterol or triglyceride levels. Taken together with the results of our study, it would appear that any differences in lipid levels are likely to be small or non-existent.

Markers of chronic inflammation and seropositivity for *C. pneumoniae*.

CRP and SAA are acute phase proteins, raised concentrations of which are non-specific markers of systemic inflammation. All control subjects in this study had CRP and SAA concentrations that were within the normal range. However, even concentrations in the high normal range are associated with poor cardiovascular outcome in patients

presenting with acute coronary syndromes[165] and in the general population[166]. The stimulus for the increased production of acute phase proteins in such subjects is unknown but chronic infections may be responsible. In this study, there was a weak association between seropositivity for *C. pneumoniae* and CRP and SAA. However, no association was seen between the acute phase proteins and seropositivity for *H. pylori* or CMV. Therefore, we did not find consistent evidence that seropositivity for any of the three organisms was associated with low-grade inflammation. These results are consistent with 2 previous studies which found no [121] or only weak associations[167].

Association of seropositivity for *C. pneumoniae* with seropositivity for *H. pylori*.
In this study, seropositivity for *C. pneumoniae* was significantly associated with seropositivity for *H. pylori*. A possible reason for this association is that these organisms have common antigens that result in the production of cross-reactive antibodies. Of particular interest is the heat shock protein known as hsp-60. Heat shock proteins are produced under conditions of stress by both bacterial and mammalian species[168]. They are involved in the correct folding of proteins and protein transport giving rise to their alternative name of chaperonins. As they are highly conserved with a striking homology of their DNA and protein sequences and as they are major targets of both humoral and cellular immune responses directed against micro-organisms, hsps may cause an autoimmune response. Patients with carotid atherosclerosis have been found to have raised antibody titres against hsp-60[83] and it has been hypothesised that atherosclerosis is an autoimmune response triggered by bacterial infections. Recently, it was reported that chlamydial hsp-60 was found in 9 of 19 atherosclerotic carotid specimens and in 7 of these, human hsp-60 was also found[169]. However, we did not find an association of seropositivity for *C. pneumoniae* or *H. pylori* with CAD (Table 7-5).

Serological association of *C. pneumoniae* with coronary artery disease.
The number of cases and controls in this study qualifies it as the largest cross sectional study which has investigated the serological association between *C. pneumoniae* and CAD (Table 3-1). We found that cases were just as likely as controls to have *C. pneumoniae* antibody levels in the top third. Varying the cut-off point made no significant difference to this result. This would seem to contradict results from previously published studies. However, most of these studies were small and did not

adequately adjust for possible confounding factors such as smoking, age and social class. Another large study (302 cases, 486controls) also found no association between *C. pneumoniae* and CAD[93] and in fact, there was a trend towards an inverse association in both men (OR 0.74, 95% CI 0.47 to 1.17) and women (OR 0.91, 95% CI 0.43 to 1.94). It is possible though, that the association between *C. pneumoniae* and atherosclerosis is only weak or moderate and that even larger studies are required to demonstrate this.

Table 7-1. Coronary risk factors and socio-economic characteristics by thirds of *C. pneumoniae* IgG antibody levels in control subjects.

Characteristic	**Thirds of *Chlamydia pneumoniae* IgG antibody levels[¶]**			**t***	**t****
	<93.9 x 10^4	**93.4-126.9 x 10^4**	**>126.9 x 10^4**		
Coronary Risk Factors					
Age (years)	58.5 ± 6.4	58.8 ± 5.8	59.6 ± 5.9	1.6	1.3
Male (n=704)	149 (63%)	156 (66%)	170 (73%)	3.6	2.9[†]
Current Smokers (n=704)	61 (26%)	68 (29%)	74 (32%)	3.6	2.8[†]
Body mass index (kg/m^2) (n=703)	25.8 ± 3.9	25.9 ± 3.7	26.6 ± 3.6	1.5	1.4
Total cholesterol (mmol/L) (n=701)	6.47 ± 1.27	6.35 ± 1.17	6.24 ± 1.05	2.3	2.3
LDL cholesterol (mmol/L) (n=640)	4.06 ± 1.03	4.04 ± 0.97	4.02 ± 0.95	0.6	0.6
HDL cholesterol (mmol/L) (n=692)	1.35 ± 0.43	1.31 ± 0.36	1.30 ± 0.35	1.4	1.4
Triglycerides (mmol/L) (n=701)	2.17 ± 1.37	2.11 ± 1.20	2.20 ± 1.41	0.0	1.6
Systolic BP (mm Hg) (n=704)	133 ± 22	138 ± 22	136 ± 21	1.0	0.2
Diastolic BP (mm Hg) (n=704)	78 ± 13	80 ± 12	79 ± 12	0.6	0.1
Alcohol (units/week) (n=704)	8 ± 15	9 ± 16	8 ± 14	0.6	0.2
Markers of socio-economic status					
Without own car (n=699)	44 (19%)	37 (16%)	42 (18%)	0.1	0.0
In rented housing (n=694)	20 (9%)	45 (20%)	44 (19%)	2.5	2.0
Completed education by 16 yrs (n=692)	188 (81%)	206 (90%)	201 (87%)	2.7	2.6[†]
Employed (n=700)	157 (67%)	146 (63%)	154 (66%)	1.0	0.3
Manual Workers (n=655)	88 (37%)	97 (41%)	105 (46%)	0.9	0.6
Married (n=692)	188 (80%)	194 (85%)	200 (87%)	0.3	0.2

[¶] Fluorescence counts

* t tests derived from simple linear regression of *C. pneumoniae* antibody levels (fluorescence counts) on each characteristic separately.

** t tests derived from multiple linear regression of *C. pneumoniae* antibody levels adjusting for sex, age and body mass index (continuous variables), smoking and markers of social class. Adjustments for social class were omitted in the regressions involving markers of socio-economic status.

[†] $P<0.01$

Table 7-2. Coronary risk factors and socio-economic characteristics by thirds of C-reactive protein concentration in control subjects.

Characteristic	**Thirds of C-reactive protein concentration (mg/L)**			**t***	**t****
	<0.9 (median 0.4)	0.91-2.80 (median 1.6)	>2.80 (median 6.85)		
Coronary Risk Factors					
Age (years) (n=704)	58.1 ± 6.4	58.8 ± 6.1	60.0 ± 5.4	3.8	1.8
Male (n=704)	172 (69%)	160 (69%)	143 (65%)	1.0	1.2
Current Smokers (n=704)	54 (22%)	59 (25%)	90 (41%)	5.7	6.1†
Body mass index (kg/m^2) (n=703)	25.0 ± 3.3	26.2 ± 3.4	27.1 ± 4.3	6.5	7.1†
Total cholesterol (mmol/L) (n=701)	6.17 ± 1.2	6.43 ± 1.18	6.40 ± 1.33	1.4	0.0
LDL cholesterol (mmol/L) (n=640)	3.93 ± 0.99	4.11 ± 0.98	4.09 ± 0.96	0.5	0.5
HDL cholesterol (mmol/L) (n=692)	1.40 ± 0.43	1.31 ± 0.34	1.24 ± 0.34	3.9	1.9
Triglycerides (mmol/L) (n=701)	2.00 ± 1.42	2.18 ± 1.28	2.31 ± 1.26	2.0	0.1
Systolic BP (mm Hg) (n=704)	131 ± 19	136 ± 22	140 ± 22	3.9	1.6
Diastolic BP (mm Hg) (n=704)	77 ± 12	80 ± 12	80 ± 13	2.6	1.3
Alcohol (units/week) (n=704)	9.7 ± 18.1	8.5 ± 13.9	6.8 ± 11.9	1.8	2.3
Loge serum amyloid A (mg/l)	-0.25 ± 1.27	0.71 ± 0.66	1.49 ± 1.15	29	28†
Antibodies for infective organisms					
C. pneumoniae (x 10^4)¶ (n=676)	95.7 ± 41.3	105.0 ± 38.2	106.4 ± 38.9	3.7	2.8††
H. pylori seropositivity (n=641)	69 (37%)	115 (50%)	110 (50%)	2.2	0.7
CMV (x 10^4)¶ (n=676)	76.2 ± 54.1	84.0 ± 59.3	95.0 ± 58.4	3.7	1.8
Markers of socio-economic status					
Without own car (n=699)	36 (14%)	38 (17%)	50 (23%)	1.9	1.2
In rented housing (n=694)	2 (11%)	42 (18%)	41 (19%)	1.6	0.9
Completed education by 16 yrs (n=692)	199 (81%)	207 (90%)	189 (87%)	2.5	1.1
Employed (n=700)	183 (74%)	150 (65%)	124 (56%)	3.6	3.0
Manual Workers (n=655)	97 (39%)	98 (42%)	95 (43%)	1.9	0.9
Married (n=692)	205 (25%)	197 (34%)	180 (31%)	1.0	0.7

¶ Fluorescence counts.

* t tests derived from simple linear regression of $\log_e$CRP concentration on each characteristic separately.** t tests derived from multiple linear regression of $\log_e$CRP concentration adjusting for sex, age and body mass index (continuous variables), smoking and markers of social class. Adjustments for social class were omitted in the regressions involving markers of socio-economic status.

† P<0.00001. †† P<0.01

Table 7-3. Coronary risk factors and socio-economic characteristics by thirds of serum amyloid A protein concentration in control subjects.

Characteristic	Thirds of serum amyloid A concentration (mg/L)			t*	t**
	<0.99 (median 0.9)	1.0-2.99 (median 2.0)	>3.80 (median 5.0)		
Coronary Risk Factors					
Age (years) (n=704)	58.3 ± 6.5	59.1 ± 5.8	59.5 ± 5.8	1.9	1.5
Male (n=704)	176 (75%)	177 (71%)	122 (56%)	3.2	2.0
Current Smokers (n=704)	61 (26%)	78 (31%)	64 (29%)	1.5	2.8†
Body mass index (kg/m^2) (n=703)	25.1 ± 3.2	26.0 ± 3.2	27.3 ± 4.5	4.8	5.0††
Total cholesterol (mmol/L) (n=701)	6.12 ± 1.12	6.44 ± 1.12	6.52 ± 1.25	0.9	0.6
LDL cholesterol (mmol/L) (n=640)	3.88 ± 0.95	4.12 ± 0.97	4.11 ± 1.01	0.9	0.3
HDL cholesterol (mmol/L) (n=692)	1.33 ± 0.39	1.29 ± 0.36	1.34 ± 0.40	0.2	0.9
Triglycerides (mmol/L) (n=701)	1.97 ± 1.20	2.30 ± 1.43	2.19 ± 1.31	0.4	1.7
Systolic BP (mm Hg) (n=704)	133 ± 22	135 ± 20	139 ± 22	2.4	1.2
Diastolic BP (mm Hg) (n=704)	78 ± 11	79 ± 12	81 ± 13	2.0	1.1
Alcohol (units/week) (n=704)	8.8 ± 15.4	9.2 ± 14.5	7.0 ± 15.1	1.2	0.3
Log_e C-reactive protein (mg/l) (n=704)	-0.65 ± 1.32	0.38 ± 1.01	1.39 ± 1.21	29	28††
Antibodies for infective organisms					
C. pneumoniae (x 10^4)¶ (n=676)	98.4 ± 41.6	104.3 ± 37.9	104.0 ± 39.7	2.8	2.4††
H. pylori seropositivity (n=641)	76 (43%)	110 (44%)	108 (50%)	1.6	1.0
CMV (x 10^4)¶ (n=676)	85.2 ± 57.0	83.6 ± 58.2	86.5 ± 58.2	0.5	0.9
Markers of socio-economic status					
Without own car (n=699)	38 (16%)	44 (18%)	42 (19%)	0.7	0.2
In rented housing (n=694)	27 (12%)	40 (16%)	42 (20%)	1.1	0.8
Completed education by 16 yrs (n=692)	191 (83%)	212 (85%)	192 (90%)	2.0	1.2
Employed (n=700)	171 (73%)	158 (63%)	128 (59%)	4.1	2.6
Manual Workers (n=655)	98 (42%)	102 (41%)	90 (41%)	2.0	0.4
Married (n=692)	196 (85%)	206 (84%)	180 (84%)	0.4	0.1

¶ Fluorescence counts.

* t tests derived from simple linear regression of log_eSAA concentration on each characteristic separately.

** t tests derived from multiple linear regression of log_eSAA concentration adjusting for sex, age and body mass index (continuous variables), smoking and markers of social class. Adjustments for social class were omitted in the regressions involving markers of socio-economic status.

† P<0.01. †† P<0.00001

Table 7-4. Baseline characteristics of cases and controls.

Characteristic	Cases (n=288)	Controls (n=704)	P
Age (yrs)	59.3 ± 5.8	59.0 ± 6.1	Matched
Male	221 (69%)	475 (67%)	Matched
Body mass index (kg/m^2)	27.4 ± 4.3	26.1 ± 3.8	$<10^{-4}$
Smokers	261 (82%)	501 (68%)	$<10^{-4}$
Treated diabetes	25 (8%)	23 (3%)	$<10^{-2}$
Treated hypertensives	149 (47%)	123 (17%)	$<10^{-4}$
Total cholesterol (mmol/l)	6.53 ± 1.67	6.32 ± 1.23	0.03
LDL cholesterol (mmol/l)	3.63 ± 1.79	3.67 ± 1.49	NS
HDL cholesterol (mmol/l)	1.18 ± 0.44	1.29 ± 0.42	$<10^{-4}$
Triglyceride (mmol/l)	2.48 ± 1.43	2.15 ± 1.33	$<10^{-3}$

Table 7-5. Relationship between coronary artery disease and seropositivity to *C. pneumoniae*, *H. pylori* and CMV.

Organism	Seropositive		Seronegative		Odds ratio and 95% confidence interval	
	Cases	Controls	Cases	Controls	Adjusted for age and sex only	Adjusted for age, sex & other factors*
H. pylori	134 (54.5%)	294 (45.8%)	112 (45.5%)	348 (54.2%)	1.41 (1.05 to 1.90)	1.28 (0.93 to 1.75)
C. pneumoniae	94† (28.7%)	234† (71.3%)	91†† (27.8%)	236†† (72.2%)	1.04 (0.74 to 1.48)	0.95 (0.66 to 1.36)
Cytomegalovirus	114† (34.0%)	221† (66.0%)	82†† (25.1%)	245†† (74.9%)	1.57 (1.11 to 2.22)	1.40 (0.96 to 2.05)

* smoking, markers of socio-economic status (age at stopping education, occupation, housing status, car ownership, marital status, employment status), serum lipids and blood pressure.

†Subjects with antibody levels in the top third

††Subjects with antibody levels in the bottom third

Chapter 8 A prospective serological study of *Chlamydia pneumoniae* infection and mortality from coronary artery disease.

Serological studies of *C. pneumonaie* and coronary artery disease (reviewed in Chapter 3) have been criticised because they have tended to be small, case control in design and many have not taken into account confounding factors such as social class and smoking. It is recognised that large scale prospective studies are required to determine whether chronic *C. pneumoniae* infection is associated with an increased risk of coronary artery disease. So far, there have been three prospective studies that have been fully reported (Table 3-1). In none of these studies was there convincing evidence for an association between seropositivity for *C. pneumonaie* and coronary artery disease. In one study, an association was seen only in non-diabetic men living in East but not West Finland and not in diabetics[88]. In a second Finnish study, seropositivity was associated with coronary events six months but not five years before an event[87].

In collaboration with Professor Nick Wald at the Woolfson Institute of Preventive Medicine, we were given access to a valuable collection of sera. These samples were from the British United Provident Association (BUPA) prospective study[170;171]. In this study, blood was taken from 21 520 healthy professional men aged 35-64 between 1975 and 1982. At the end of 1994, after an average follow up period of 15.6 years, 648 men who had had no history of heart disease on entry had died from ischaemic heart disease (ICD 9th revision, codes 410-414). Sera from 647 of these men were available for this study and for each case, two controls matched for age and duration of storage of the serum sample were selected. The prospective nature of this study would be expected to reduce bias whilst the homogeneity of the study group would be expected to reduce the confounding effect of social class. A previous study of this same group of subjects had shown that there was no significant association between seropositivity for *H. pylori* and death due to ischaemic heart disease[6].

Method

At the time of the health check, blood was taken and serum cholesterol concentration and other risk factors for ischaemic heart disease were measured. Serum samples were then stored at -40°C. The cohort of subjects was flagged at the NHS central register in Southport, permitting automatic notification of death (with the certified cause) by the Office of Population Censuses and Surveys (now the Office for National Statistics). For this study, serum samples were retrieved and *C. pneumoniae* IgG antibodies measured by time resolved fluoroscopic immunoassay as described in Chapter 5. All assays were done in duplicate without knowledge of the case-control status.

Cox's proportional hazards models (with strata to identify each case with its two controls) were used to estimate the independent association of risk factors with ischaemic heart disease. Cox Regression is a method for modelling time-to-event data which allows the inclusion of predictor variables (co-variates) in the model.

Results

As expected, men who died of coronary artery disease had a higher prevalence of the classical coronary risk factors (Table 8-1). *C. pneumoniae* IgG antibody levels showed a bimodal distribution and the midpoint between the two peaks was taken as an arbitrary cut-off (Figure 8-1). The intra and inter-assay coefficients of variation were 8.1 and 20 respectively. The prevalence of *C. pneumoniae* seropositivity in controls increased with age (30, 40, 49 and 73 percent of men aged 35-44, 45-54,55-64 and 65 or more) and there was also an association with body mass index but not with smoking (Table 8-2). However, no association was seen between *C. pneumoniae* seropositivity and mortality due to ischaemic heart disease. Figure 8-1 shows that the distribution of IgG antibody levels in cases and controls was virtually the same. The odds of death from ischaemic heart disease in men who were seropositive relative to men who were seronegative was 1.04 (95% confidence interval 0.83 to 1.31). After adjusting for body mass index, the odds ratio was 1.003 (0.8 to 1.26). Adjusting for other risk factors did not make any significant difference.

Discussion

This is the largest serological study of *C. pneumoniae* and ischaemic heart disease to date. Not only was it prospective but all subjects came from the same socio-economic group. As expected, we found that in controls, seropositivity was associated with age although we did not find an association with smoking. In agreement with the OXCHECK study, there was no association between *C. pneumoniae* and atherosclerosis which in this study was represented by mortality due to ischaemic heart disease. Our chosen cut-off point for seropositivity was arbitrary but Figure 8-1 shows that no cut-off point could result in more cases than controls being positive.

Like all serological studies, it cannot be certain that seropositivity represents chronic rather than past infection. Ideally, repeated serology at regular intervals may have demonstrated that some subjects had persistently raised antibody levels so providing stronger evidence for chronic infection. Nevertheless, compared with previous serological studies, this is the best able to assess the serological relationship between *C. pneumoniae* and atherosclerosis. Its size and design minimised both random and systematic errors and its results provides strong evidence that *C. pneumoniae* is not an important cause of atherosclerosis.

Table 8-1. Risk factors for ischaemic heart disease in cases and controls.

Variable	Cases (n=647)*	Controls (n=1294)*	P value†
Age (years)	53.6 ± 7.1	53.5 ± 7.1	NS
Body mass index (kg/m2)	25.9 ± 3.2	25.4 ± 2.7	<0.001
Height (m)	1.75 ± 0.06	1.75 ± 0.06	NS
Weight (kg)	79.3 ± 10.7	78.1 ± 9.9	<0.05
Total cholesterol (mmol/l)	7.1 ± 1.2	6.7 ± 1.1	<0.001
Triglycerides (mmol/l) ¶	1.72 ± 1.67	1.5 ± 1.67	<0.001
Systolic BP (mm Hg)	144 ± 22	135 ± 19	<0.001
Diastolic BP (mm Hg)	89 ± 13	84 ± 12	<0.001
No (%) current cigarette smokers	329 (50.8)	503 (38.8)	<0.001
No (%) father died of heart disease	124 (19.1)	176 (13.6)	<0.001
No (%) mother died of heart disease	76 (11.7)	86 (6.6)	<0.001

* mean ± SD unless indicated otherwise

† t tests and $\chi 2$ test

¶ geometric mean (SD expressed as multiple of geometric mean)

Figure 8-1. *C. pneumoniae* IgG antibody levels in cases and controls

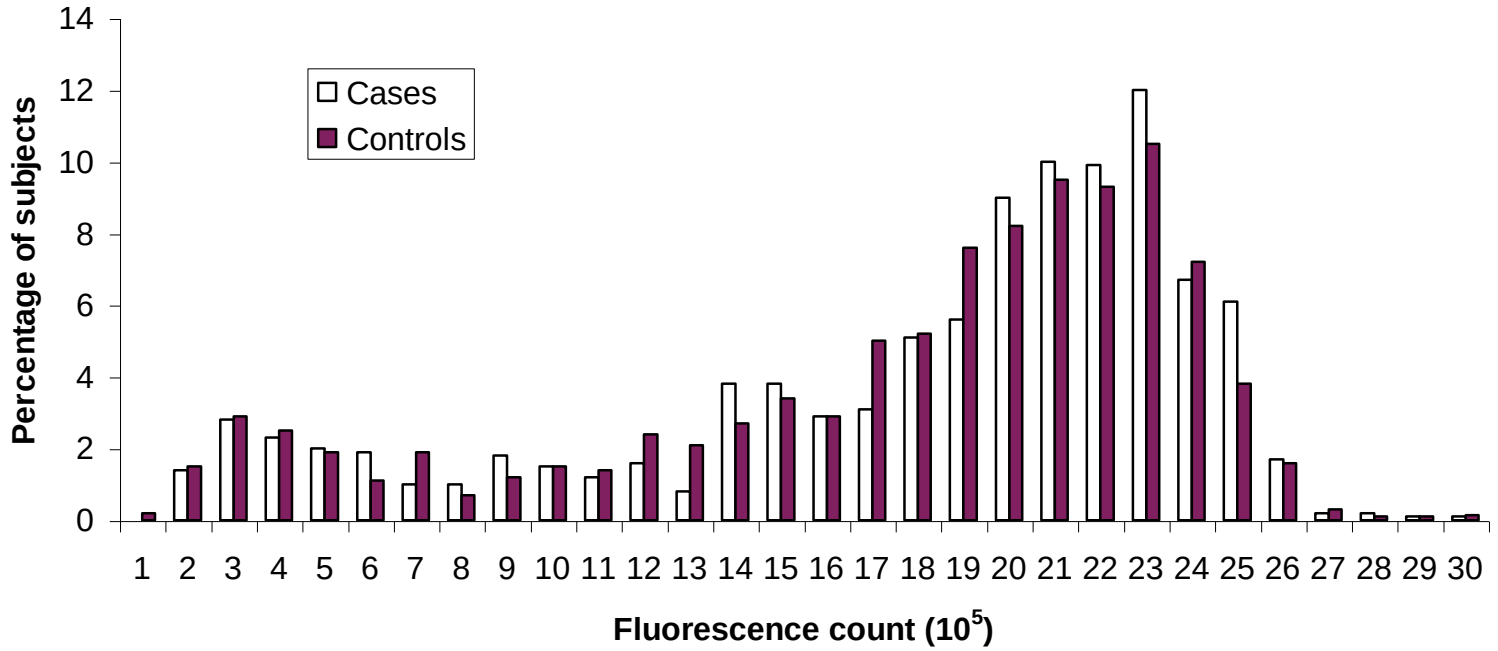


Table 8-2. Coronary risk factors according to *Chlamydia pneumoniae* antibody status in 1294 controls.

	Seropositive* (n=955)	Seronegative* (n=339)	P value†
Age	53.7 ± 7.1	52.9 ± 6.9	NS
Body mass index (kg/m^2)	25.5 ± 2.7	25.1 ± 2.7	0.02
Total cholesterol (mmol/l)	6.3 ± 1.1	6.4 ± 1.1	NS
Triglycerides (mmol/l)	1.49 ± 1.65†	1.56 ± 1.70†	NS
Systolic BP (mm Hg)	135 ± 19	134 ± 20	NS
Diastolic BP (mm Hg)	84 ± 12	83 ± 12	NS
Current cigarette smokers (%)	372 (39%)	122 (36%)	NS
Father died of heart disease (%)	131 (14%)	50 (15%)	NS
Mother died of heart disease (%)	62 (6%)	25 (7%)	NS

*mean ± SD unless indicated otherwise

† t tests and $\chi 2$ test

¶ geometric mean (SD expressed as multiple of geometric mean)

Chapter 9 The prevalence of circulating *Chlamydia pneumoniae* DNA in 1,205 subjects attending for coronary angiography.

The serological association between antibody to C*hlamydia pneumoniae* and coronary artery disease (CAD) was first reported in 1988[4] and has subsequently been confirmed by numerous serological studies[7]. These studies have used the presence of IgG and IgA antibody to *C. pneumoniae* as a proxy for chronic infection, but the validity of this approach is uncertain (Chapter 3). Essentially, the serological response to chronic vascular infection is poorly documented. What are required are simple methods for diagnosing current vascular infection with *C. pneumoniae*. Currently, it is believed that *C. pneumoniae* infection starts in the respiratory tract and disseminates systemically in the blood stream within alveolar macrophages. This theory is based on experimental models. In one experiment, mice were inoculated with intranasal *C. pneumoniae* which were subsequently recovered from alveolar and peritoneal macrophages as well as from peripheral blood mononuclear cells but not from plasma[172]. Infection could be transferred by intraperitoneal injection of infected macrophages from infected to uninfected mice. Studies have also shown that *C. pneumoniae* can persist[173] and replicate[174] in macrophages. In humans, a Malaysian group has reported that *C. pneumoniae* can be detected by the polymerase chain reaction (PCR) in the serum of patients presenting with respiratory infection[175] and myocardial infarction[176]. However, another group failed to detect the organism in the serum of patients with abdominal aortic aneurysms even though it was found in tissue from 14 of 40 aneurysms[177]. Similarly, other groups have failed to detect the organism from whole blood of patients with abdominal aortic aneurysms[178]. In this study, we have assessed the prevalence of *C. pneumoniae* DNA in purified buffy coat cells (containing macrophages, other white cells and platelets) of 1,205 patients referred for coronary arteriography and correlated the results with clinical and other parameters. We decided to focus on white cells since this would be expected to increase the probability of detecting *C. pneumoniae*.

Methods

This study was approved by the local regional ethics committee. Subjects were recruited consecutively from individuals referred for diagnostic and interventional coronary arteriography to the Wessex Cardiothoracic Unit, a tertiary referral centre, from August 1997 to March 1998. Exclusion criteria were age under 30 and those subjects not requiring blood tests as part of their routine care. Data available included a full blood count and, in most cases, indirect fibrinogen levels and fasting lipids. From each subject, 4 to 5mls of EDTA blood were available for serology and for the detection of *C. pneumoniae* DNA by the polymerase chain reaction (PCR). Cardiac risk factors were recorded and hypercholesterolaemia was diagnosed if the fasting total cholesterol was greater than 5.2 mmol/l. A family history of CAD in a sibling, parent, grandparent or sibling of a parent was recorded. The Carstairs deprivation score standardised against national data was calculated from 1991 UK census data (Manchester Information Datasets and Associated Services) using the residential postcode and census enumeration district. Coronary arteriograms were interpreted by a consultant cardiologist and classified as normal, minor irregularities or significant disease.

Buffy coat PCR

EDTA blood was left to settle for 12 to 24 hours at 4ºC. The plasma was clarified by centrifugation and the supernatant stored at -72ºC for serology. Blood cells were mixed with an equal volume of phosphate buffered saline (PBS) and layered carefully onto 3ml of Lymphoprep® (Nycomed). The purified buffy coat cells were then collected after centrifugation at 500g for 15 minutes, washed twice in PBS and stored at -72ºC. DNA was prepared from the buffy coat by conventional methods involving digestion with proteinase K, extraction with phenol-chloroform, precipitation with sodium acetate-ethanol and the addition of 50µl of distilled water (Appendix 1). The detection and elimination of PCR inhibitors and *C. pneumoniae* PCR was carried out as described in Appendix 2.

Serology

IgG antibodies to *C. pneumoniae* were measured by a time-resolved fluoroscopic immunoassay (TRIA) as described in Chapter 5. In this study, it was plasma rather than serum that was available and this is likely to have increased the background noise of our

TRIA method. However, it would be expected that results from cases and controls would be affected equally.

Analysis of data

Conditional multiple logistic regression (SPSS version 8) was used to explore possible associations of the presence of *C. pneumoniae* DNA and conventional coronary risk factors with coronary artery disease. Of those subjects who did have CAD, possible associations of the presence of *C. pneumoniae* DNA with indirect fibrinogen and various haematological variables were investigated after adjusting for age, smoking and month of recruitment. The 2-tailed t test was used to compare continuous variables with normal distributions and the χ^2 test for categorical variables. The distribution of Carstairs score was skewed and we categorised subjects into three equal groups according to their score.

Results

Subjects Recruited

1205 subjects were recruited (804 men, 401 women). Significant CAD was found by coronary arteriography in 669 (83%) men and 244 (60.6%) women while 135 men and 157 women had normal coronary arteries or coronary arteries with mild irregularities only. Of these latter subjects, 73 (54.1%) men and 81 (51.6%) women had been investigated for chest pain; the majority of the remainder had valvular heart disease (Table 9-1). As expected, the prevalence of conventional coronary risk factors, apart from deprivation as measured by the Carstairs score, was greater in those with CAD (Table 9-2). The Carstairs score takes into account unemployment, social class, overcrowding and car ownership and 62.5% of all subjects had a Carstairs score that was better than the national average while scores were unavailable for 5.1% of subjects.

***C. pneumoniae* DNA in buffy coat of blood.**

100 subjects (8.3%) had *C. pneumoniae* DNA in their buffy coat cells. Men with CAD were significantly more likely to have circulating *C. pneumoniae* DNA than men with normal coronaries (Table 9-2) but this difference was not seen in women. Among men with CAD, those who were positive for *C. pneumoniae* had higher mean platelet counts (Table 9-3). Mean monocyte counts and indirect fibrinogen levels were also higher, but

not significantly so. Similar comparisons were not made among women as the number of subjects was smaller**.** Figure 9-1 shows that the monthly prevalence of circulating *C. pneumoniae* DNA varied for both men and women with peaks in autumn and spring. However, no data was available for the summer.

Serology

Plasma samples were assayed in duplicate, with intra- and inter-assay coefficients of variation of 4 and 20% respectively. Antibody to *C. pneumoniae* showed a bimodal distribution (Figure 9-2). For analysis, the cut-off point for seropositivity was predefined as the value midway between the two peaks. Males were more likely to be seropositive than females but no association of serology with CAD was observed for either sex (Table 9-2). Although our cut-off point was arbitrary, Figure 9-2 shows that no cut-off point could result in more cases than controls being positive. Subjects who were seropositive were more likely to have *C. pneumoniae* DNA in the buffy coat but this was not statistically significant (76/834 vs. 23/346, OR = 1.4, 95% CI = 0.9 to 2.3). No monthly variation in the percentage of seropositive subjects was seen.

Discussion

Detection of current vascular infection by *C. pneumoniae*.

We have shown in a large study that *C. pneumoniae* DNA can be detected by PCR in the buffy coat cells of blood of patients referred for coronary arteriography. This is the first time that the clinical and haematological correlates of vascular chlamydial infection have been assessed in patients clearly shown to be infected because of the presence of circulating *C. pneumoniae* DNA. In both laboratory animals[172] and in human subjects[43], the persistence of vascular *C. pneumoniae* DNA has been associated with the presence of viable organism. Other studies have attempted to equate the persistence of specific IgG and IgA antibody to *C. pneumoniae* to the presence of chronic infection with the organism. However, data on the serological response associated with culture confirmed chronic *C. pneumoniae* infection are sparse and no generally agreed standards for chlamydial serology exist. Culture confirmed chronic respiratory infection with *C. pneumoniae* is not always associated with a specific antibody response[27] and it is often not possible to predict subjects with *C. pneumoniae* in their blood vessels on the basis of their serology[130;131;179]. A recent autopsy study of young Alaskan natives (mean age

34.1), did find that subjects who had *C. pneumoniae* in their blood vessels were more likely to have had a specific IgG titre of ≥ 128 an average 8.7 years before death[106]. However, that serology is a blunt predictive tool was demonstrated by the fact that 15 of 32 subjects with a prior titre of ≥ 128 were positive for *C. pneumoniae* compared with 3 of 7 subjects without measurable antibody. In this study, seropositive subjects were not significantly more likely to have circulating *C. pneumoniae* DNA. Culture of *C. pneumoniae* is an exacting and tedious procedure and is not a feasible option for the routine detection of vascular infection with this organism. Our results suggest that for *C. pneumoniae* vascular infection, as for *C. trachomatis* genital tract infection, PCR or other nucleic acid-based amplification procedures are the most appropriate method for detecting current infection[180].

Association of *C. pneumoniae* infection and coronary artery disease.

Our study population was typical in that it showed the expected correlations with known risk factors of coronary disease. In males, interestingly, circulating *C. pneumoniae* DNA was a stronger predictor of CAD than any of the known cardiac risk factors, with an adjusted odds ratio of 3.4 although the absolute risk was low. By contrast, in women, the presence of *C. pneumoniae* DNA was not a predictor of CAD. The reason for this is unknown but aortic stenosis has been associated with *C. pneumoniae* infection[181] and approximately half the subjects with normal coronary arteries had valvular heart disease. Nevertheless, exclusion of these subjects did not significantly change the results of the analysis. Among men with CAD, those with *C. pneumoniae* DNA had higher platelet counts, a feature of some infectious diseases although not previously reported for *C. pneumoniae*. Previous studies have reported that *C. pneumoniae* infection is associated with raised fibrinogen levels and that this may explain the increased cardiovascular risk[69]. However, in this study, indirect fibrinogen levels were not significantly raised. Men were more likely than women to be seropositive for *C. pneumoniae* as has been widely reported but in neither sex was there an association of specific antibody with CAD, in agreement with several recent studies[93;121] including two that were prospective [111;112].

Population differences in the prevalence of *C. pneumoniae* infection.

During the course of this study, a similar but smaller study was published with dramatically different results[36]. In Umeå, Northern Sweden, a remarkable 59.4% of 101 patients attending for coronary arteriography (mean age 64) were found to have *C. pneumoniae* DNA as were 46% of 52 blood donors (mean age 49). Surprisingly, 11 of 14 (78.6%) patients found to have normal coronary arteries were positive for *C. pneumoniae* compared with 49 of 86 (57%) patients with CAD. The much higher prevalence of *C. pneumoniae* infection reported from Umeå might reflect population or methodological differences. Epidemics of *C. pneumoniae* respiratory disease have been described throughout Scandinavia and an unusual 94% of patients and 90% of blood donors were seropositive for *C. pneumoniae*, possibly the highest seroprevalence rates ever reported. To exclude the possibility that methodological differences may account for our differing results, we re-tested specimens from our all male subjects with normal coronary arteries using the touch-down PCR method of the Swedish study, but found only one more positive result. It is likely therefore that there are genuine population differences but further studies are necessary to clarify these points.

An association between circulating *C. pneumoniae* DNA and CAD does not necessarily imply causation. In our study of patients undergoing coronary artery bypass graft surgery, *C. pneumoniae* was just as likely to be found in new internal mammary artery grafts as in failed grafts[182]. Also, the Alaskan study above found no difference in disease severity between coronary artery segments which had evidence for *C. pneumoniae* and those which didn't. The monthly variation in the prevalence of circulating *C. pneumoniae* DNA seen in this study may reflect episodes of acute infection and it is possible that subjects with CAD are more likely to be infected by *C. pneumoniae* than the converse situation. Unfortunately, the number of subjects per month was small and such variation will need to be confirmed by larger studies. Two small secondary prevention trials have reported that macrolide antibiotics reduce the incidence of further adverse coronary events[9;10] although preliminary reports from a third study found no such benefit[183]. Further trials are under way, with one trial giving antibiotics for periods of up to 1 year without evidence of prior infection[184]. Anti-microbials are only likely to benefit those who are currently infected and *C. pneumoniae* can be considered neither universal nor the only organism that might be important in CAD. In our population many CAD patients were not *C. pneumoniae* infected.

Although further research is required, the demonstration of circulating *C. pneumoniae* DNA in blood may be a useful, possibly prognostic test for selecting patients for antibiotic trials designed to clarify the role of *C. pneumoniae*.

Table 9-1. Indications for coronary arteriography and arteriographic findings.

	Male[†] (n = 804)	Female[†] (n = 401)
Indications for coronary arteriography		
Elective Investigation of chest pain*	466 (37)	206 (18)
Unstable symptoms (post MI or UAP)[#]	153 (9)	71 (4)
Elective PTCA	116 (12)	41 (4)
Cardiomyopathy	5 (0)	4 (0)
Cardiac valvular disease	56 (3)	67 (9)
Cardiac valvular disease and coronary artery disease	6 (2)	6 (1)
Miscellaneous	2 (0)	6 (1)
Coronary Artery Status		
Normal/Mild irregularities	135 (4)	157 (17)
One vessel disease	199 (12)	90 (10)
Two vessel disease	190 (23)	55 (3)
Three vessel disease	280 (24)	99 (7)
Diagnosis of subjects with normal coronary arteries		
Chest pain of unknown cause	72 (1)	80 (7)
Cardiomyopathy	5 (0)	4 (0)
Cardiac valvular disease	56 (3)	67 (9)
Miscellaneous	2 (0)	6 (0)

[†]The number in brackets are the number of subjects positive for *C. pneumoniae* DNA.
*67 men and 72 women were found to have normal coronary arteries or mild irregularities only.
[#]6 men and 9 women were found to have normal coronary arteries or mild irregularities only.

MI = myocardial infarction. UAP = unstable angina. PTCA = percutaneous transluminal angioplasty.

Table 9-2. The association of coronary artery disease with cardiac risk factors and circulating *C. pneumoniae* DNA.

	Male			Female		
	CAD (n=669)	No CAD (n=135)	OR (95% CI)*	CAD (n=244)	No CAD (n=157)	OR (95% CI)*
Age (yrs)	62.4 ± 10.1[†]	57.6 ± 12.1[†]		66.6 ± 9.4[‡]	62.6 ± 10.9[‡]	
Hypercholesterolaemia	514 (76.8%)	81 (60.0%)	2.2 (1.5 - 3.3)	208 (85.2%)	105 (66.9%)	2.9 (1.8 - 4.6)
Hypertension	221 (33.0%)	26 (19.3%)	2.1 (1.3 - 3.3)	113 (46.3%)	48 (30.6%)	2.0 (1.3 - 3.0)
Diabetes	79 (11.8%)	9 (6.7%)	1.9 (0.9 - 3.8)	34 (13.9%)	9 (5.7%)	2.7 (1.2 - 5.7)
Family History of CAD	359 (53.7%)	49 (36.3%)	2.0 (1.4 – 3.0)	150 (61.5%)	67 (42.7%)	2.1 (1.4 – 3.2)
Carstairs Score[§]						
Upper third	210 (32.8%)	42 (33.3%)	1.0 (0.7 - 1.5)	78 (33.5%)	47 (32.6%)	1.1 (0.7 - 1.7)
Lower third	203 (31.7%)	41 (32.5%)	1.0 (0.7 - 1.5)	86 (36.9%)	57 (39.6%)	1.0 (0.6 - 1.5)
Smoking (Current/Ex)	523 (78.2%)	90 (66.7%)	1.8 (1.2 - 2.7)	130 (53.3%)	81 (51.2%)	1.1 (0.7 -1.6)
Circulating *C. pneumoniae* DNA +ve	59 (8.8%)	4 (2.9%)	3.2 (1.1 - 8.9)[¶]	20 (8.2%)	17 (10.8%)	0.7 (0.4 - 1.5)
Seropositive[#]	492 (74.8%)	97 (73.5%)	1.1 (0.7 – 1.6)	148 (62.2%)	97 (63.8%)	1.0 (0.6 – 1.4)

* Unadjusted Odd's ratio (95% confidence interval)

[†‡] P < 0.001 (2 sample t test)

[§] Carstairs score available for 766 men and 377 women.

[¶] 3.4 (1.2 - 9.6) after adjusting for age (continuous variable), month of recruitment, family history of CAD, smoking (current, ex, never), hypercholesterolaemia, hypertension and diabetes (categorical variables) with conditional multiple logistic regression.

3.3 (1.1 - 9.5) after adjusting for Carstairs score (thirds) in addition (n=766).

[#]Serology results available for 790 men and 390 women.

Table 9-3. Haematological variables in men with coronary artery disease according to the presence of circulating *C. pneumoniae* DNA.

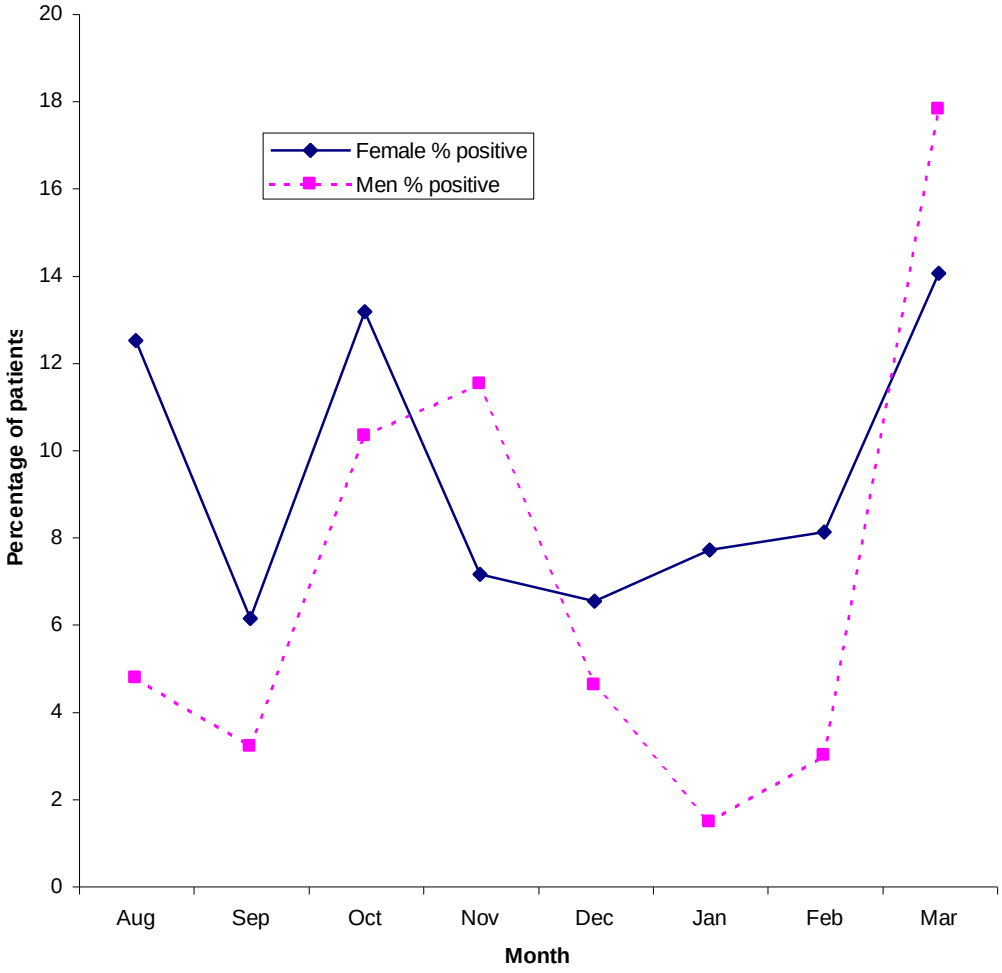
	CPn +ve (n = 59)	CPn -ve (n = 595)	P*	P†
Full blood count (g/l)	141.6 ± 15.8	140.3 ± 15.5	0.5	0.6
White cell count (10^9/l)	8.1 ± 3.1	7.8 ± 2.2	0.2	0.2
Platelets (10^9/l)	238.4 ± 69	221.9 ± 57	0.04	0.03§
Neutrophils (10^9/l)	5.1 ± 2.5	4.8 ± 1.9	0.2	0.2
Lymphocytes (10^9/l)	2.1 ± 0.8	2.1 ± 0.8	0.8	0.9
Monocytes (10^9/l)	0.7 ± 0.4	0.6 ± 0.3	0.08	0.06
Eosinophils (10^9/l)	0.2 ± 0.2	0.2 ± 0.1	0.8	0.8
Basophils (10^9/l)	0.07 ± 0.06	0.07 ± 0.05	0.8	0.8
Indirect fibrinogen (g/l)	4.5 ± 1.5 (n = 51)	4.1 ± 1.2 (n = 541)	0.08	0.06

*2 sample t test (2 tailed)

†Multiple linear regression adjusting for age (continuous variable), month of recruitment and smoking (current, ex or never).

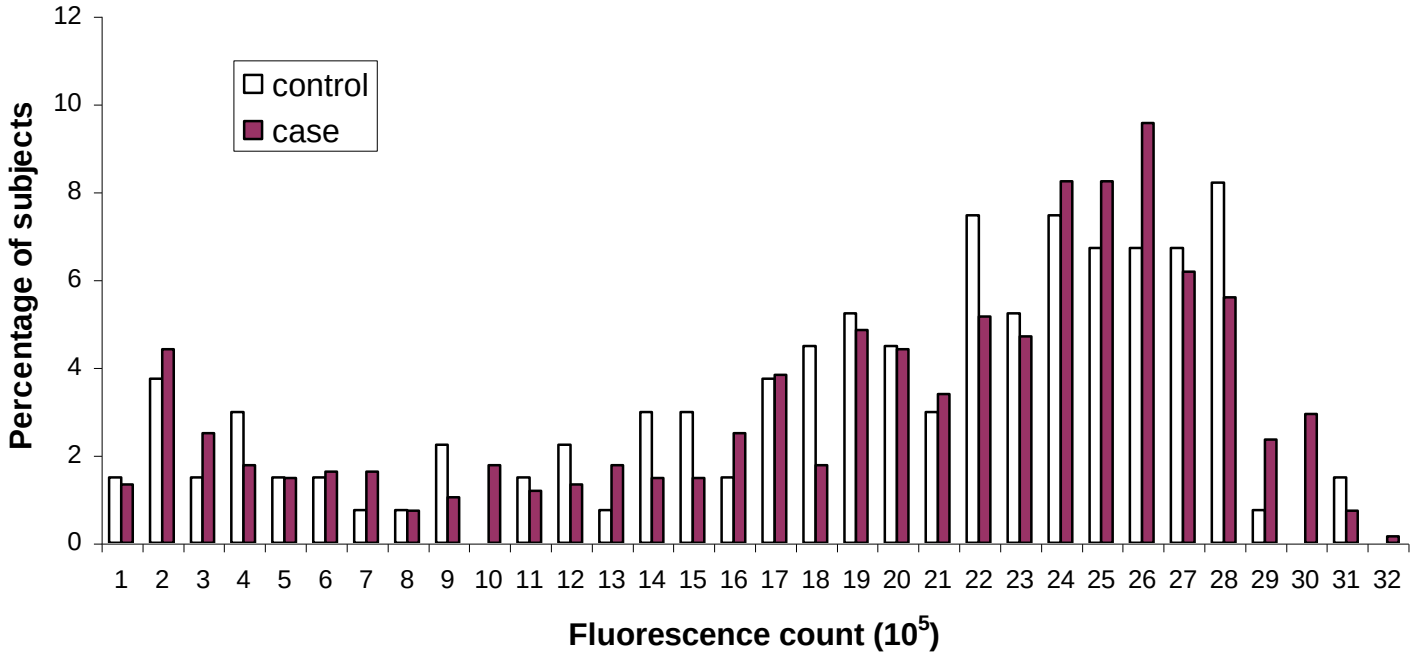
CPn = *C. pneumoniae*.

Figure 9-1. Monthly variation in the prevalence of subjects with *C. pneumoniae* DNA in their circulating buffy coat cells.



	Number of Subjects per month							
	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
Men	22	94	165	113	87	138	67	118
Women	8	49	76	56	46	65	37	65

Figure 9-2. Distribution of *C. pneumoniae* IgG levels in male subjects



Chapter 10 The distribution of *Chlamydia pneumoniae* DNA in human coronary arteries at post mortem and the histological severity (Stary grading) of associated atherosclerotic plaque.

Most pathological studies of *C. pneumoniae* and atherosclerosis have attempted to show that the organism is more prevalent in diseased compared with normal arteries since this may indicate that the organism is a cause of atherosclerosis. Unfortunately, atherosclerosis is ubiquitous although in three studies where atherosclerotic and control blood vessels were obtained from age and sex matched individuals, evidence for the organism was found in between 15 to 19% of diseased vessels and in only 0 to 5% of control vessels[98;99;122]. Pathological studies suffer from the fact that they are cross sectional and cannot determine whether infection preceded or followed the development of atherosclerosis. Another approach that can be taken is to see whether the distribution of *C. pneumoniae* in any individual matches the distribution and severity of atherosclerosis. If *C. pneumoniae* is found at all atherosclerotic sites rather than in just a few, then this would be stronger evidence that it is a cause of atherosclerosis. However, focal infection may still be important since it could potentially result in focal disease such as plaque rupture. We have systematically obtained both mildly and severely diseased segments of all three coronary arteries from subjects at autopsy. PCR was then used to detect for *C. pneumoniae* and the distribution of the organism was correlated with the extent and severity of atherosclerosis.

Methods

Specimen collection. All 3 coronary arteries plus segments of the lung and myocardium were collected from 33 consecutive adult subjects at autopsy. Twenty one subjects (63.6%) had died from causes related to coronary artery disease and 12 had died from non-cardiac causes. Post mortems were carried out within 48 hours of death and the heart was aseptically removed prior to removal of other organs. Operators were gowned and masked at all times. In a laminar flow cabinet, each coronary artery was inspected and one centimetre segments of the most and least diseased regions were sampled. Half of each segment was frozen in liquid nitrogen prior to detection of *C.*

pneumoniae DNA by the polymerase chain reaction (PCR) and the remainder was processed for histology.

Polymerase Chain Reaction. Extraction of DNA from blood vessels, checking for the presence of PCR inhibitors and *C. pneumoniae* PCR was carried out as described in appendixes 1, 2 and 3.

Histology. Sections of coronary arteries adjacent to those studied by PCR were stained with haematoxylin and eosin. Most sections were also stained with Schmorl's stain for lipofuschin and with Alizarin for calcium. The severity of atherosclerosis was graded using the Stary classification[185]. This classification ranges from grade 0 (normal) to grade 6 (plaque rupture or fissure or haemorrhage) with grade 4 representing lesions that are the earliest which are macroscopically visible (Table 10-1).

Results

Characteristics of the 33 recruited subjects are shown in Table 10-2. Three coronary arteries were obtained from 30 subjects and 2 coronary arteries from 3 subjects giving a total of 96 arteries. Two segments were available from all but five of these arteries resulting in 187 segments. Segments with atherosclerosis of Stary grade 3 or less were considered to have mild disease while those of grade 4 or greater were considered to have moderate or severe disease. To assess the reproducibility of Stary grading, 48 segments were re-coded and then re-graded by the same specialist cardiac pathologist. Thirty-four segments were graded identically on both occasions, with the remaining segments differing by only 1 grade (Kappa test, p=0.78). On classifying into mild or severe groups, only 3 of 48 segments changed groups on repeat grading.

Only one of 187 segments was Stary grade 0. Thirty one subjects had at least one atherosclerotic lesion in each artery of Stary grade 4 or more and 26 of these subjects were positive for *C. pneumoniae* DNA in their coronary arteries. The remaining 2 subjects had mild atherosclerosis of Stary grade 3 or less and neither had evidence of *C. pneumoniae* DNA. There was no statistical difference in age, sex or cause of death in subjects with or without *C. pneumoniae* DNA in their coronary arteries.

Distribution of *C. pneumoniae* in coronary arteries. As already stated, all but two subjects had lesions in every artery of at least grade 4. However, subjects were just as likely to have *C. pneumoniae* in one (8/26) as in two (9/26) or three (9/26) coronary arteries. Considering the individual arteries, 41 of 96 had both mild and moderately or severely diseased segments. In these arteries, the mild segments were just as likely to be positive for *C. pneumoniae* as the more severe segments (Table 10-3, McNemar test, p=1.0). Similarly, arteries which had mild disease only were just as likely to have *C. pneumoniae* as arteries which had moderate or severe disease only (9/14 vs. 20/36, χ^2 test, p=0.77). When all arterial segments were considered as a group, no correlation was observed between the Stary classification and the presence of *C. pneumoniae* DNA (Table 10-4, χ^2 test, p=0.57). Thus, in one subject with severe atherosclerosis throughout, *C. pneumoniae* was found at only one site whereas in another subject who had generally mild disease, every arterial segment was positive for *C. pneumoniae*.

The effect of PCR inhibitors.

DNA samples extracted from coronary artery segments with moderate or severe disease were more likely to contain PCR inhibitors than samples extracted from mildly diseased segments (40/114 vs. 15/73, odds ratio (OR) 2.1, 95% confidence interval (CI) 1.1 to 4.1). Inhibition was associated with the presence of lipid (44/128 versus 10/54, OR 2.3, CI 1.1 to 5.0) and especially calcium (42/103 vs. 12/79, OR 3.8, CI 1.9 to 8.0) and was eliminated in all but 1 case by ten-fold dilution. 16 of 55 (29.1%) inhibited samples were positive for *C. pneumoniae* compared with 52 of 132 (39.4%) uninhibited samples (χ^2 test, p=0.17). We tested each coronary artery segment on three separate occasions but repeat PCR did not always give a consistent result. Nine inhibited samples were positive once, 7 twice and none three times. For uninhibited samples, 34 were positive once, 8 twice and 10 three times.

Discussion

PCR inhibition.

In this study, we controlled carefully for the presence of PCR inhibition. The prevalence of PCR inhibition was high, especially with DNA samples obtained from coronary arteries with severe atherosclerosis and those that were calcified. This

illustrates the importance of checking for inhibition as has also been demonstrated by others[154]. Inhibition was eliminated in all but one case by ten-fold dilution, but this was associated with a non-significant reduction in chlamydial detection from 39% to 29%. As reported by others[86;107], repeated testing of samples by PCR did not always produce consistent results. We attribute this to the low amounts of *C. pneumoniae* DNA present in coronary arteries and the effects of sampling errors arising from the small sample volumes or of PCR inhibitors. Our strategy of testing all samples in triplicate should have reduced underestimation of the prevalence of *C. pneumoniae* arising from this.

Distribution of *C. pneumoniae* in atherosclerotic coronary arteries.

The main finding of this study was that *C. pneumoniae* DNA was common in coronary arteries but its distribution did not correlate with either the extent or severity of atherosclerosis. Only one previous study has looked for *C. pneumoniae* at multiple sites from the coronary tree[107]. Although severity of atherosclerosis was not graded, the findings of this study were also consistent with a patchy distribution of *C. pneumoniae*. Some studies have also reported a lack of association between severity of atherosclerosis and *C. pneumoniae*. In a study of 60 Alaskan natives dying mainly from non-cardiac causes (mean age 34.1), a high *C. pneumoniae* IgG titre of ≥ 256 an average of 8.7 years before death was associated with the presence of *C. pneumoniae* in coronary arteries. However, there was no difference in the severity of atherosclerosis in subjects with *C. pneumoniae* compared to those without[106]. *C. pneumoniae* has also been detected in vessels not usually associated with atherosclerosis such as the internal mammary artery and saphenous vein[108;182]. Other studies though, have reported that *C. pneumoniae* was more prevalent in severely diseased compared with mildly diseased arterial segments. In one study, 15% of carotid endarterectomy samples were found to be positive for *C. pneumoniae* whereas the organism could not be detected in macroscopically normal segments adjacent to the diseased areas[98]. Similarly, in an autopsy study of young persons where samples were age and sex matched[99], *C. pneumoniae* could not be found in normal segments but was found in 2 of 11 segments with intimal thickening and in 6 of 7 samples with atheroma.

Significance of the presence of *C. pneumoniae* in coronary arteries.

In this study, the finding that *C. pneumoniae* vascular infection is focal and not associated with the extent or severity of atherosclerosis does not disprove a role for the organism in CAD. After all, if *C. pneumoniae* causes atherosclerosis, its presence would be expected to precede that of disease. However, a focal distribution favours a model where the organism colonises diseased areas of the vascular tree. Evidence which supports this hypothesis is that *C. pneumoniae* has been associated with other diseases such as bronchial carcinoma, rheumatoid arthritis, chronic obstructive pulmonary disease, Alzheimer's disease and sarcoidosis and one common factor amongst them is the presence of damaged tissue. Nevertheless, even if *C. pneumoniae* colonises atherosclerotic lesions rather than being a cause of them, it is still possible that it is pathogenic. Infection by an intracellular organism which eventually kills the cell it inhabits may be expected to enhance disease progression. Results from rabbit models indicate that intranasal inoculation with *C. pneumoniae* results in aortic changes consistent with early atherosclerosis[186;187] or accelerates its development[8]. It is likely that the question of whether *C. pneumoniae* causes or exacerbates CAD can only be answered by well-controlled animal studies or by large scale antibiotic intervention trials.

Table 10-1. The Stary classification of human atherosclerotic lesions

Stary Nomenclature	Description
Grade I Initial Lesion	Only microscopic changes. Lipid in macrophages.
Grade II Fatty Streak	Lipid in macrophages and smooth muscle cells.
Grade III Preatheroma	Multiple deposits of pooled extracellular lipid. Microscopic evidence of tissue damage.
Grade IV Atheroma	Confluent mass of extracellular lipid (lipid core).
Grade V Fibroatheroma	Collagen and smooth muscle cap over lipid core.
Grade VI Complicated Fibroatheroma	Thrombosis and/or haemorrhage and/or erosion or fissure.

Table 10-2. Characteristics of patients with and without *C. pneumoniae* in their coronary arteries

	Subjects with *C. pneumoniae* in their coronary arteries (n=26).	Subjects without *C. pneumoniae* in their coronary arteries (n=7).
Male sex	14 (53.8%)	4 (57%)
Age (years)	72.4 ± 9.7	78.3 ± 7
Coronary deaths	17 (65.4%)	4 (57%)
Non coronary deaths	9 (34.6%)	3 (43%)
Presence of plaque rupture or haemorrhage or acute thrombosis.	12 (46.1%)	2 (28.6%)
C. pneumoniae in lungs	5 (19.2%)	1 (14.3%)
C. pneumoniae in myocardium	6 (23.1%)	0

Table 10-3. Prevalence of *C. pneumoniae* DNA in paired mild and severe segments from 41 coronary arteries.

		Severe lesions	
		C. pneumoniae +ve	*C. pneumoniae* -ve
Mild lesions	*C. pneumoniae* +ve	7	11
	C. pneumoniae -ve	10	13

Table 10-4. The prevalence of *C. pneumoniae* in atherosclerotic coronary artery segments according to the severity of disease (Stary classification).

Stary Classification	Mild lesions		Severe Lesions		
	≤2	3	4	5	6
Number of coronary artery segments positive for *C. pneumoniae*	18/41 (43.9%)	9/32 (28.1%)	17/45 (37.8%)	18/56 (32.1%)	6/13 (46.2%)

Chapter 11 The prevalence of *Chlamydia pneumoniae* in atherosclerotic and non-atherosclerotic blood vessels of patients attending for redo and first time coronary artery bypass graft surgery.

Tests such as the polymerase chain reaction (PCR), immunocytochemistry (ICC) and tissue culture have provided direct evidence that *C. pneumoniae* localises to blood vessels. It has been claimed that the organism is found more often in atherosclerotic than normal blood vessels. However, atherosclerosis is ubiquitous and few studies have investigated age matched control vessels. In this study, we have recruited patients undergoing redo and first time coronary artery bypass graft surgery (CABG). From both sets of patients, we obtained diseased vessels in the form of failed grafts or endarterectomy specimens as well as normal vessels in the form of new saphenous vein and internal mammary artery grafts. We hypothesised that if *C. pneumoniae* causes atherosclerosis, then in patients with graft failure, it should be more prevalent in failed grafts compared with new saphenous vein (SV) and new internal mammary artery (IMA) grafts. Also, all other things being equal, graft failure should occur earlier in those patients with vascular *C. pneumoniae* infection. Similarly, for patients requiring first time CABG, *C. pneumoniae* should be more common in diseased native coronary arteries compared with new grafts.

Method

49 consecutive patients presenting for elective redo (second) CABG were recruited between March 1996 and January 1998 following approval from the local research ethics committee. Another 9 consecutive patients presenting for first time CABG but who in addition, had coronary endarterectomy during their operation were also recruited. Demographic characteristics, smoking habit and medical history were recorded from each patient. From redo patients, a segment of one or more failed grafts was obtained. The decision as to whether or not to remove a failed graft and which failed graft to remove was left to the clinical discretion of the surgeon. From patients requiring first time CABG, endarterectomy specimens from native coronary arteries that were not grafted were obtained. In some cases, endarterectomy specimens were also

available from redo patients. From all patients, we attempted to obtain surplus segments of newly harvested saphenous vein and internal mammary artery grafts. Vessel specimens (1 to 5cms in length) were collected in the sterile environment of the operating theatre and processed immediately by dividing into 3 portions, one for histology, one for PCR and the final portion stored in liquid nitrogen for future reference. Samples for PCR were stored in liquid nitrogen if not processed immaediately.

PCR. DNA was extracted from each vessel (Appendix 1) and subjected to *C. pneumoniae* PCR after checking for the presence of PCR inhibitors (Appendix 2). The nucleotide sequence of positive PCR products was confirmed by Southern Blotting (Appendix 3). All DNA samples were tested by PCR in triplicate and negative and positive controls were included in every experiment.

Statistical analysis. The χ^2 test was used to compare proportions of categorical variables. The 2 sample t test (2 tailed, assuming equal variance) was used to compare continuous variables from 2 populations. A significance level of $p = 0.05$ was used.

Results

Redo CABG patients. The characteristics of the 49 patients who underwent redo CABG are shown in Table 11-1. The time interval between CABG operations was at least 6 years for all patients except for one (3 years). Therefore, this represents a group of patients with late graft failure. We obtained at least one failed vein graft from each of 47 patients. Of the other 2 patients, an endarterectomy sample was available from one but no diseased vessel was available from the other. Endarterectomy samples were available from 3 patients in total. We obtained at least one new saphenous vein graft from each of 41 patients. Of the other 8 patients, new IMA grafts were available for 2 but no undiseased vessel was available from 6 patients. New IMA grafts were available from 15 patients in total. 11 patients (22.4%) had *C. pneumoniae* DNA in a failed graft or endarterectomy specimen only, 2 patients (4%) in a new IMA or SV graft only and 5 patients (10.2%) had *C. pneumoniae* DNA in both failed and new grafts. The presence of *C. pneumoniae* DNA was not associated with age, gender, smoking, hypertension or

hyperlipidaemia (Table 11-1) and the interval between CABG operations was the same in patients with and without *C. pneumoniae* DNA in their vessels.

First time CABG patients. 9 patients were recruited with an average age of 70.4 ± 9.2 years (4 males, 5 females). At least one new SV graft and one endarterectomy specimen was obtained from every patient but a new IMA graft was obtained from only 5 patients. Four patients had an endarterectomy specimen that was positive for *C. pneumoniae* and of these, one also had a new IMA graft that was positive and another had a new IMA and a new SV graft that was positive.

It can be seen from Table 11-2 that in all patients, the prevalence of *C. pneumoniae* in diseased vessels (failed grafts and endarterectomy specimens) was similar to that in new IMAs but greater than that in new SV grafts ($p = 0.001$).

PCR inhibition. As in the post mortem study (Chapter 10), DNA samples extracted from atherosclerotic vessels were more likely to show inhibition to PCR compared with non-atherosclerotic vessels (29/68 vs. 19/71, $p = 0.05$). However, on repeat PCR for *C. pneumoniae*, positive atherosclerotic vessels were more likely to be positive on 2 or more occasions than positive non-atherosclerotic vessels (13/26 vs. 3/12) although this was not statistically significant. This may be because the quantity of *C. pneumoniae* DNA is greater in diseased vessels. Inhibition was eliminated in all cases by ten-fold dilution of the DNA sample. Although dilution inevitably dilutes the amount of DNA, 14 of 48 inhibited samples (29.2%) were positive at least once compared with 24 of 91 uninhibited samples (26.3%). Also, inhibited samples were just as likely to be positive on 2 or more occasions as uninhibited samples (7/14 vs. 9/24).

Discussion

Detection of *C. pneumoniae* in atherosclerotic vessels. All our specimens were tested in triplicate and although most were not positive on all occasions, we counted them as positive because our negative controls were consistently negative and it is likely that the inconsistent results were due to a combination of small amounts of DNA and low sample volumes. Inconsistent results have also been reported by other groups[107].

***C. pneumoniae* in arteries and veins.** The main finding of this study was that *C. pneumoniae* was more common in the arterial circulation (including failed venous grafts) than in saphenous veins but it was not more common in atherosclerotic vessels than in non-atherosclerotic IMAs. Another study has now also reported that *C. pneumoniae* can be found in IMAs[108]. At first sight, this appears to contradict previous studies. Thus, Muhlestein et al[129] detected *C. pneumoniae* in 79% of atherectomy specimens compared with 4% of non-atherosclerotic coronary arteries. A study of young adult deaths[99] reported similar findings. However, whereas these studies have compared patients with and without atherosclerosis, we have compared atherosclerotic and non-atherosclerotic blood vessels from patients with atherosclerosis. Taken together, these data may imply that *C. pneumoniae* is more common in blood vessels of patients with atherosclerosis but that in such patients, it may be present in any artery, including those which are not normally subject to atherosclerosis. Thus, of the seven redo patients in this study who had *C. pneumoniae* in a new graft, 5 also had *C. pneumoniae* in a failed graft. Likewise, both of the first time CABG patients who had *C. pneumoniae* in a new graft also had an endarterectomy specimen that was positive. In a post mortem study of *C. pneumoniae* in atherosclerotic coronary arteries[124], the organism was also detected in non-cardiovascular tissue (lung, liver and spleen), although less frequently. However, the prevalence of the organism in normal arteries and veins was not specifically addressed. It is uncertain as to why the prevalence of *C. pneumoniae* is greater in the arterial rather than the venous circulation. It has been hypothesised that *C. pneumoniae* is primarily a cause of respiratory infection and enters the circulation after being ingested by alveolar macrophages. In this case, organisms will enter the arterial circulation first after reaching the heart from the lungs. We speculate that chlamydiae may effectively be filtered out in the capillaries or alternatively, that haemodynamic factors such as a higher blood pressure are important in making arteries more susceptible to colonisation with *C. pneumoniae*.

Graft failure. Generally, IMA grafts last substantially longer than SV grafts as they are less prone to develop atherosclerosis[188]. However, we found that new IMA grafts were more likely than new SV grafts to be infected with *C. pneumoniae* (Table 11-2). Furthermore, redo patients whose failed grafts were found to be infected with *C. pneumoniae* did not present for their second operation any earlier than those patients

whose failed grafts were not infected (Table 11-1). Taken together, these data suggest that *C. pneumoniae* is not an important risk factor in graft failure. In this study, patients undergoing redo or first time CABG were of similar age and the prevalence of *C. pneumoniae* was also similar in both groups. In contrast, in a study of patients undergoing directional atherectomy[131], it was reported that *C. pneumoniae* was more likely to be found in restenotic compared with primary lesions although this was not statistically significant. However, whereas late graft failure is mainly due to atherosclerosis, the cause of restenosis following atherectomy is incompletely understood[189]. It was suggested that *C. pneumoniae* might be important in restenosis but another atherectomy study found that that the only clinical predictor of the presence of *C. pneumoniae* was the presence of a primary non-restenotic lesion[129].

Native coronary atherosclerosis. Several processes contribute to vein graft failure including thrombosis in the early stages and fibrointimal hyperplasia and graft atherosclerosis in the later stages. Our patients were mainly those with late graft failure requiring redo surgery. It could be argued that although graft atherosclerosis has many similarities to native coronary atherosclerosis, there are differences[188] and therefore, *C. pneumoniae* may still be important in native coronary atherosclerosis. However, in the 9 first time CABG patients, the presence of *C. pneumoniae* DNA in endarterectomy specimens was no greater than that in new IMA grafts. Also, there was a high prevalence of *C. pneumoniae* in the IMA from all patients and this is a vessel which is not prone to any form of atherosclerosis.

In summary, in patients undergoing first time or redo CABG, *C. pneumoniae* can be found in both atherosclerotic and non atherosclerotic arteries and to a lesser extent, in new saphenous vein grafts. This suggests that *C. pneumoniae* is not important in causing late graft failure or disease of native coronary arteries. However, cross sectional studies such as this give no information on the temporal sequence of events. It is not known whether *C. pneumoniae* infection preceded or followed the development of atherosclerosis. It is possible that if *C. pneumoniae* infects a pre-existing lesion, it may cause disease progression rather than initiate atherosclerosis itself. Thus, in New Zealand white rabbits fed a diet supplemented with small amounts of cholesterol, intranasal inoculation with *C. pneumoniae* was found to increase the size of

atheromatous aortic lesions and this could be prevented by azithromycin[8]. In two other studies using New Zealand White rabbits fed normal diets, intranasal inoculation resulted in aortic changes consistent with atherosclerosis but such changes were early and compatible with arteritis[186;187]. Aortic changes were not seen in a fourth study[190]. Two secondary prevention trials have also suggested that *C. pneumoniae* may have a role in atherosclerosis[9;10]. Patients given macrolide antibiotics were found to be at lower risk for further adverse cardiovascular events and this benefit was seen as early as one month[10]. However, both studies were small with just over 200 patients each and preliminary results after 6 months of follow up from a third, similar sized study have not shown any such benefit[183]. A fourth study recruited 34 men who had previously had CABG and randomised them to placebo or doxycycline for 4 months. No evidence of any effect on forearm basal nitric oxide production or *C. pneumoniae* antibody titres was seen[191]. It is evident that further research, including larger antibiotic intervention trials, is required to determine if *C. pneumoniae* plays a significant pathological role in atherosclerosis.

Table 11-1. Vascular risk factors & other characteristics of patients undergoing redo CABG

Characteristic	*C. pneumoniae* positive (n=19)	*C. pneumoniae* negative (n=30)	P value∗
Age (years) ± SD	67.1 ± 5.9	66.2 ± 6.5	0.66†
Male	18 (94.7%)	27 (90.0%)	0.56
Ex smoker‡	11 (57.9%)	22 (73.3%)	0.26
Hypertension	6 (31.6%)	10 (33.3%)	0.90
Hyperlipidaemia	9 (47.4%)	18 (60.0%)	0.39
Time between first and redo CABG (yrs)	12.8 ± 5.9	12.6 ± 3.4	0.89†

∗χ^2 test unless otherwise indicated

†Two sample t test

‡No patient claimed to be a current smoker

Table 11-2. The number of vessels (percentage) positive for *C. pneumoniae* DNA by PCR

	Failed Vein grafts	Coronary Endarterectomy Specimens	New SVGs	New IMA grafts
Redo CABG	21/55 (38.2%)	1/3 (33.3%)	5/42 (11.9%)	4/15 (26.7%)
First time CABG		4/10 (40.0%)	1/9 (11.1%)	2/5 (40%)
All patients	21/55 (38.2%)	5/13 (38.5%)	6/51 (11.8%)	6/20 (30.0%)

Summary

The recognition of smoking and hypercholesterolaemia as major risk factors have been landmarks in the management of atherosclerosis as prevention and treatment have lead to increased life expectancies for many patients. Nevertheless, it appears that atherosclerosis is a multifactorial disease and that one or more other risk factors remain to be discovered since the incidence and prevalence of atherosclerosis throughout the world cannot yet be fully explained. The report of a serological association between *C. pneumoniae* and atherosclerosis and the subsequent discovery that *C. pneumoniae* can be found in atherosclerotic tissue has lead to immense speculation that this organism may be important. The treatment of duodenal ulcers was revolutionised by the discovery of *H. pylori* as a major cause and there has been excitement that atherosclerosis could be another disease potentially treatable with antibiotics. In 1998 alone, there were at least twenty two published review articles which were generally in favour of the idea that *C. pneumoniae* is likely to be important in atherosclerosis[105;192-212]. In contrast, there was only one article which was critical[213].

There is no doubt that the initial serological and pathological reports warranted further investigation. Subsequently, authors have attempted to show that *C. pneumoniae* fulfils Koch's original postulates as a cause of atherosclerosis. These state, that to prove a microbial cause of disease:

1. The organism must always be associated with the clinical disease
2. The organism can be isolated from a case and grown in a series of pure cultures.
3. A late pure culture can reproduce the disease in a susceptible animal.
4. The organism can subsequently be re-isolated from the latter.

It becomes immaediately obvious that there are problems in attempting to fulfil these criteria for *C. pneumoniae*. It is technically difficult to culture *C. pneumoniae* from clinical samples and this has rarely been done in atherosclerosis (Chapter 6). It has been proposed that Koch's postulates should be updated so that modern methods of detecting infection such as serology should be taken into account[214]. However, although it is accepted that techniques such as PCR and immunocytochemistry can provide indirect

evidence of infection by living organisms, there is the problem that generally, clinical tissue is rarely available from patients with atherosclerosis. Serology has therefore been the main method of determining current infection but as has been discussed in Chapter 3, distinction between past and current infection is difficult and the evidence that atherosclerosis is associated with current infection is not compelling. Although the world-wide prevalence of atherosclerosis cannot be explained by the currently known cardiac risk factors, the prevalence of chlamydial infection as measured by serology would appear to have even less ability to explain this prevalence. Seropositivity appears to be uniformly high throughout the world (Table 1-2) and in fact is just as high in Japan, a country with a low prevalence of coronary artery disease as in England or Finland, countries with much higher levels of disease. This is despite the fact that stricter criteria were used to define seropositivity in Japan. Similarly, one group has found that the prevalence of *C. pneumoniae* in atheroma samples from young American subjects aged 15 to 34 who did not die from coronary artery disease was 85.7%[99]. However, the same group reported that the prevalence in coronary atherectomy samples obtained from American patients aged 35 to 81 was only 52.6%[131]. In a third study of patients aged 33 to 73, a third of whom died from coronary artery disease, they found that *C. pneumoniae* was in 34% of coronary arteries[124]. Yet, in a fourth study of younger Alaskan natives aged 15 to 57 years who did not die from coronary artery disease and who are a racial group with a low risk of atherosclerosis, the prevalence was also 36.7%[106].

There have been four studies of experimental *C. pneumoniae* infection in the New Zealand White rabbit[8;186;187;190] Intranasal inoculation with *C. pneumoniae* in rabbits fed a normal diet was found to induce aortic inflammatory changes consistent with early atherosclerosis[187] including foam cells[186] in two studies but not in a third[190]. In the largest study, in rabbits fed a diet supplemented with small amounts of cholesterol, lesion size was significantly increased in infected compared with uninfected rabbits and these changes could be prevented by azithromycin[8]. These results have been interpreted as showing that *C. pneumoniae* can at least exacerbate atherosclerosis. However, most of the histological changes seen were early and compatible with inflammation and there is a question of whether these changes are specific since inflammation was also found in lung, liver and spleen[186;190].

There have been 2 small antibiotic intervention trials where patients with unstable angina or myocardial infarction were given macrolide antibiotics[9;10]. Both reported beneficial effects in terms of reduction of further coronary events. However, one of these studies which reported after only thirty days of follow up and whose result was of borderline statistical significance[10] has subsequently found no difference in outcome between patients receiving antibiotics and placebo after ninty days of follow up[215]. A third study gave doxycycline to men who had had previous coronary artery bypass graft surgery[191]. No change in antibody titres or basal nitric oxide production was seen after 4 months of treatment. Preliminary results from a fourth antibiotic intervention trial in post infarction patients reported no benefit from antibiotic treatment after six months of follow up[183].

Therefore, there is little evidence that Koch's postulates have been fulfilled for *C. pneumoniae* in atherosclerosis. Overall, the results of our studies in this thesis do not provide evidence that *C. pneumoniae* is a cause of atherosclerosis. Two well controlled serological studies that were respectively the largest cross sectional and prospective study to date, showed no association between *C. pneumoniae* and atherosclerosis. Similarly, two pathological studies have shown that the presence of *C. pneumoniae* in atherosclerotic vessels is unrelated to the extent or severity of disease and in fact, can be found in the internal mammary artery, a vessel not prone to develop atherosclerosis. Buffy coat PCR is a more specific test for detecting current *C. pneumoniae* infection than serology and in a large study, we did find an association between coronary artery disease as diagnosed by coronary arteriography and current infection although this was not seen in women. The important question though, is whether this association is causative and it is likely that large intervention trials are required to answer this. It has been suggested that patients should be recruited into such trials regardless of their serological status since serology may not be an accurate discriminator of current infection[184]. In the buffy coat study, only 8.8% of men with coronary artery disease were positive for *C. pneumoniae* DNA in their buffy coat cells. This indicates that the prevalence of chronic active *C. pneumoniae* infection may be too low for unselective trials to demonstrate an effect. Furthermore, for reasons of appropriate prescribing alone, antibiotics should only be given to subjects in whom there is good reason to believe there is current *C. pneumoniae* infection, especially if, as has been suggested,

treatment may need to be for prolonged periods of up to a year[184]. Buffy coat PCR would be a test capable of identifying patients suitable for such antibiotic trials.

Although we did not find evidence to support the hypothesis that *C. pneumoniae* causes atherosclerosis, it is still possible that other organisms or infections in general may be important. In our study of mortality due to myocardial infarction in England and Wales, we found that the standardised mortality rate was associated with the percentage of households with four or more residents and that this association was independent of deprivation. Such overcrowding may be a surrogate marker for chronic infection. Chronic infections with organisms such as *C. pneumoniae*[11] and *H. pylori*[146] have been found to be associated with overcrowding in childhood. Infectious agents apart from *H. pylori* and *C. pneumoniae*, such as periodontal bacteria and cytomegalovirus have also been implicated in atherosclerosis[7] and a key question is whether individual agents or infections are general are important. All bacteria including *Chlamydia* share highly homologous heat shock proteins (HSP) capable of inducing autoimmune antibodies in humans due to the presence of cross reactive sites on human HSP. Macrophages in atherosclerotic lesions express high levels of HSP[216] and levels of specific antibodies to HSP are increased in the sera of patients with atherosclerosis[83]. One possibility is that the immune response to bacterial infection generally may interact with chlamydial or human HSP in vessel walls so exacerbating atherosclerosis. A recent study found that chlamydial and human HSP-60 co-localises in atherosclerotic lesions and that both types of HSP could induce $TNF_{\propto}$ (tumour necrosis factor) and matrix degrading metalloproteinase activity when incubated with mouse macrophages[169].

Atherosclerosis is a chronic disease which is ubiquitous and develops at an early age. Efforts to relate its development to infection by sub-clinical chronic infections, which are difficult to diagnose, are likely to be difficult especially if more than one organism is involved. However, intervention trials can determine the therapeutic benefits of antibiotics with respect to atherosclerosis and even if infection accounts for only a small percentage of cases of atherosclerosis, the impact on public health may be important as the clinical sequelae of atherosclerosis are extremely common.

Publications

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Appendix 1 DNA Extraction from Blood Vessels.

Digestion of blood vessel samples.

All preparatory work was carried out in a laminar flow cabinet. Blood vessel samples were taken from liquid nitrogen storage and placed into a 1.5 ml eppendorf tube containing a digestion solution consisting of 0.5 ml TRIS-EDTA pH 8.0, 0.5 ml 10% SDS and 400 μg Proteinase K (Table A 1). Samples were then incubated at 37°C for 24 to 48 hours in a warm room. For every 5 samples, a "mock extraction" control consisting only of digestion solution was used. A maximum of 10 samples was processed on each occasion.

Extraction of DNA.

500 μl of the digested sample was transferred to a new eppendorf tube containing 500 μl of phenol-chloroform while the remainder was stored at -20°C. All work with phenol-chloroform was carried out in a fume cabinet. The phenol-chloroform mixture was mixed by hand and centrifuged at 13000 revs/min for two minutes. Following centrifugation, the upper aqueous phase containing the DNA was carefully taken off using a fine pastette. Care was taken not to disturb the bottom organic phase or the protein layer at the interface. Extraction was then repeated with another 500 μl aliquot of phenol-chloroform. The volume of the final extract was estimated and to this was added 1/10 of the volume of 3M sodium acetate (Table A 1) and 3 volumes of cold absolute ethanol. This mixture was left at -20°C overnight so precipitating the DNA. The mixture was then centrifuged at 13000 revs/min at 4°C for fifteen minutes resulting in a pellet of DNA at the bottom of the eppendorf. The supernatant was discarded and 1 ml of 80% ethanol added to the DNA pellet. Centrifugation was repeated for 5 minutes and the supernatant carefully removed. The DNA pellet was then vacuum dried for 15 to 30 minutes and re-suspended in 50 μl of UHQ. The DNA solution was stored at -20°C prior to PCR.

Table A 1. Solutions used for DNA extraction from blood vessels.

Solution	Constituents	Quantity	Final concentration
TRIS-EDTA pH 8	Tris base EDTA 5M HCl UHQ	1.21 g. 0.37 g. Adjust pH to 8.0. Make up to 100ml.	0.1 M 0.01 M
10% SDS	SDS UHQ	10 g. 100 ml.	10%
3M sodium acetate	Sodium acetate 5M HCl UHQ	40.8 g. Adjust pH to 5.0. Make up to 100 ml.	3 M

Appendix 2 Polymerase Chain Reaction.

Detection of PCR inhibitors in extracted DNA

To check for the presence of PCR inhibitors in extracted DNA, 3 μl of extracted DNA was spiked with 5 pg of bacterial phage λ DNA and subjected to PCR with λ specific primers (Table A 2). If there was no amplification of λ DNA, inhibitors were considered to be present. The extracted DNA was then diluted ten-fold to reduce the concentration of inhibitors and another 3 μl was used to repeat the experiment. The sensitivity of the λ PCR was checked by using a positive control consisting of 5 pg of λ DNA in 3 μl of water.

***C. pneumoniae* PCR**

A nested PCR was used to detect *C. pneumoniae* DNA[155]. To prevent DNA contamination, different laboratories were used for pre and post PCR handling and PCR preparation was done in dedicated laminar flow hoods equipped with UV lamps for decontamination of equipment. Reagents were aliquoted with filter pipette tips. The primers (APNOU, APNOL, APN1 and APN2) and reaction conditions are shown in Table A 2. Negative controls consisting of water were used for every 5 samples and a positive control consisting of *C. pneumoniae* DNA equivalent to that from one to ten elementary bodies was used for each experiment. All PCR was carried out on a Perkin Elmer 9600 machine. PCR products were visualised with ethidium bromide after electrophoresis on a 2% agarose gel (Figure A 1).

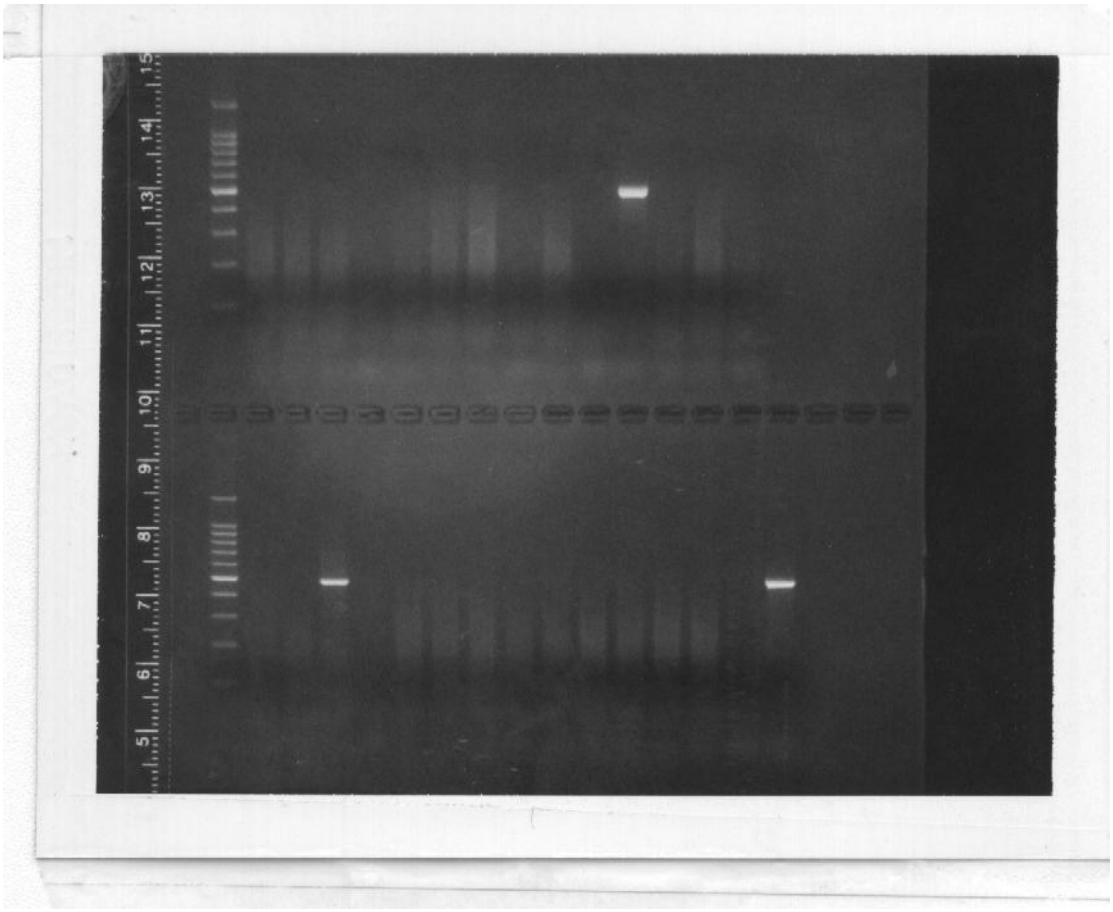
Table A 2. PCR primers and conditions.

PCR Target	Primer	Primer Sequence	Product size	Reagents in each 50µl reaction	PCR conditions
λ phage DNA	λ1	5'-ATG CAA TAT GCC ATT GCA GG-3'	647 bp	UHQ* 28.3 µl 10X NH_4 Buffer 5 µl 50 mM $MgCl_2$ 1.5 µl 10 mM dNTP 1 µl 10 µM λ1 5 µl 10 µM λ2 5 µl 5 pg/µl λDNA 1 µl 5 U/µl Taq# 0.2 µl Extracted DNA 3 µl	30 cycles Denaturation 94°C for 40s Annealing 48°C for 40s Extension 72°C for 45s
	λ2	5'-TTA TAC CTC TGA ATC AAT ATC AAC-3'			
C. pneumoniae OMP1 gene (round 1)	APNOU	5'-AAT TCT CTG TAA ACA AAC CC-3'	561 bp	UHQ* 33.3 µl 10X NH_4 Buffer 5 µl 50 mM $MgCl_2$ 1.5 µl 10 mM dNTP 1 µl 10 µM APNOU 3 µl 10 µM APNOL 3 µl 5 U/µl Taq# 0.2 µl Extracted DNA 3 µl	30 cycles Denaturation 94°C for 30s Annealing 52°C for 30s Extension 72°C for 30s Final extension cycle of 72°C for 7 mins
	APNOL	5'-ATT AAG AAG CTC TGA GCA TA-3'			
C. pneumoniae OMP1 gene (round 2)	APN1	5'-TGC CAA CAG ACG CTG GCG-3'	488 bp	UHQ* 33.3 µl 10X NH_4 Buffer 5 µl 50 mM $MgCl_2$ 1.5 µl 10 mM dNTP 1 µl 10 µM APN1 3 µl 10 µM APN2 3 µl 5 U/µl Taq# 0.2 µl First round products 3 µl	35 cycles Denaturation 94°C for 30s Annealing 63°C for 30s Extension 72°C for 30s Final extension cycle of 72°C for 7 mins
	APN2	5'-AGC CTA ACA TGT AGA CTC TGA T-3'			

*Ultra high quality water.

#Bioline, London.

Figure A 1. Ethidium bromide stained agarose gel of PCR products following *C. pneumoniae* PCR.



This gel shows *C. pneumoniae* PCR products from coronary artery specimens obtained for the post mortem study (Chapter 10). There are 15 lanes in the upper row and 16 lanes in the lower row. The first lane of each row contains a 100 base pair DNA ladder. Counting from the first lane of the upper row (excluding the ladders), every sixth lane contains a "mock extracted" control and every seventh lane contains a negative PCR control. The last lane of the bottom row is a positive PCR control. All other lanes are PCR products from coronary artery specimens. It can be seen that two of the specimens are positive.

Appendix 3 Southern Hybridisation.

Production of probe for Southern hybridisation

Southern hybridisation was used to confirm the nucleotide sequence of positive *C. pneumoniae* PCR products. A digoxigenin-deoxy-uridine triphosphate labelled DNA probe consisting of a 242 base pair VS4 fragment that spanned the omp1 target sequence was used. The probe was produced by a nested PCR using *C. pneumoniae* DNA as the template. The first round of the PCR used the APNOU/APNOL primer set as described in Appendix 2 and the reaction conditions of the second round are shown in Table A 3. A known volume (10 μl) of the final product was visualised by ethidium bromide staining after electrophoresis on a 2% agarose gel. A "ladder" consisting of known amounts of DNA was ran on the same gel and a comparison of the staining intensities allowed an estimation of the amount of labelled probe (**Figure A 2**).

Southern Hybridisation procedure

10 μl of *C. pneumoniae* PCR product was added to 10 μl of loading buffer consisting of 3:2 ratio of x20 SSC and formaldehyde (Table A 4). The mixture was heated at 96°C on a heating block for 10 minutes to denature the DNA before placing immaediately on ice. It was then dispensed onto a nylon membrane which had been soaked with x20 SSC. The DNA was cross-linked to the membrane using UV light (1200 J) following which the membrane was air-dried. Subsequently, the membrane was incubated with 50 mls of pre-hybridisation buffer at 68°C for one hour before incubation with 10 mls of hybridisation buffer at the same temperature overnight. Unbound probes were removed by washing the membrane twice with x2 wash at room temperature for 15 mins before a final two washes with x0.1 wash at hybridisation temperature for 15 minutes.

Detection of hybridisation products

Hybridisation products were detected colorimetrically using nitroblue tetrazolium (NBT) and X-Phosphate according to the manufacturer's instructions (Boehringer

Mannheim, Lewes, UK). Briefly, the membrane was washed sequentially in buffer 1 for 2 minutes, buffer 2 for 30 minutes (to block the membrane), conjugate solution for 30 minutes, twice in buffer 1 for 15 minutes (to remove unbound antibody) and twice in buffer 3 for 2 minutes. The membrane was then put into a sealed plastic bag with 10 mls of colour development solution and placed in the dark after making sure that all air bubbles were removed. A colour precipitate formed after a few minutes and was complete within 12 hours. The results were documented by drying and then photocopying the membrane.

Table A 3. Second round of nested PCR for making digoxigenin-deoxy-uridine triphosphate labelled probes against VS4 region of omp1 gene.

PCR Target	**Primer**	**Primer Sequence**	**Product size**	**Reagents in each 50µl reaction**		**PCR conditions**	
VS4 region of OMP1 gene	T1APN4L	5'-ATT TTA CGG AGA CTA TGT TT-3'	242 bp	UHQ*	33.3 µl	40 cycles	
				10X NH_4 Buffer	5 µl		
				50 mM $MgCl_2$	1.5 µl	Denaturation	94°C for 30s
				10 mM dNTP¶	1 µl	Annealing	50°C for 30s
	4APN10U	5'-TAG TGC CAT ACA TTG GAG TA-3'		10 µM T1APN4L	3 µl	Extension	72°C for 45s
				10 µM 4APN10U	3 µl		
				5 U/µl Taq#	0.2 µl		
				1:100 first round products	3 µl		

*Ultra high quality water.

#BIO-X-ACT (a proof reading Taq polymerase), Bioline, London.

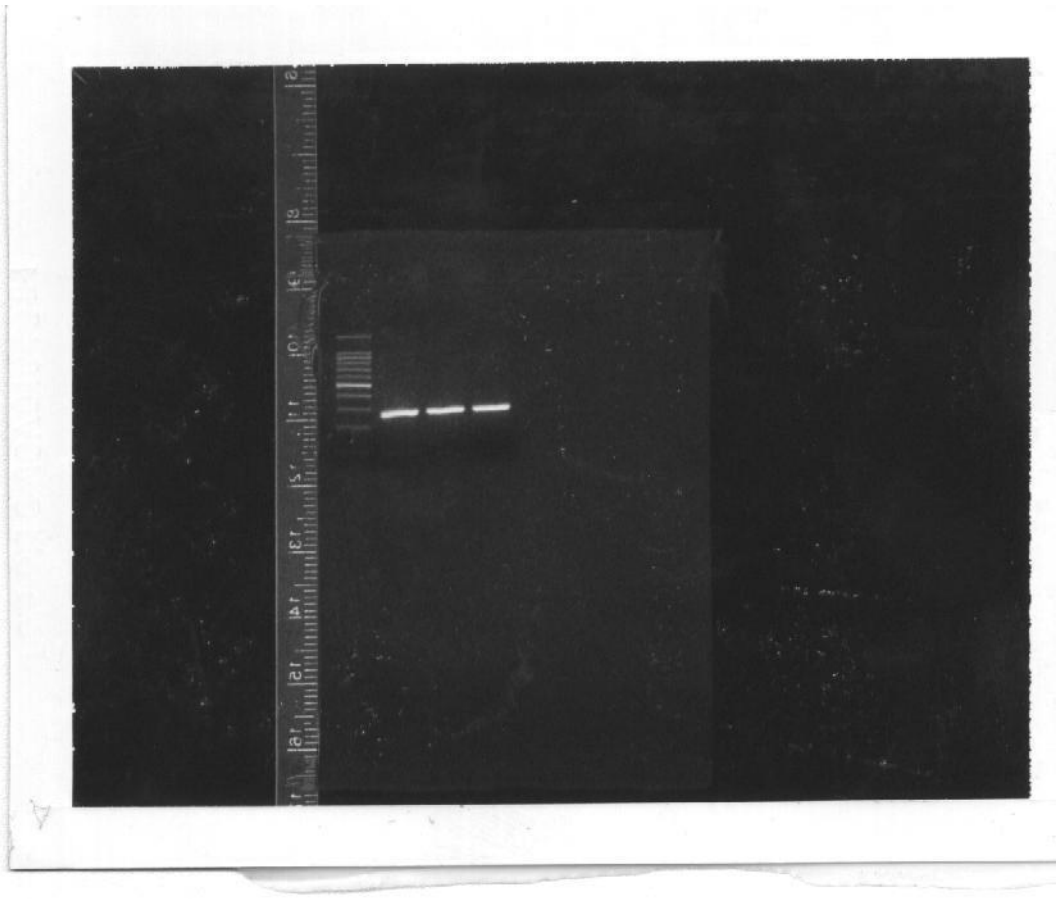
¶10 mM each of dATP, dCTP, dGTP, 6.5 mM of dTTP and 3.5 mM of Digoxigenin labelled dUTP (Boehringer Mannheim, Lewes).

(The recommended dTTP:dUTP ratio is approximately 2:1)

Table A 4. Solutions used in Southern Hybridisation

Solution	**Constituents**
20x SSC	3 mol/l NaCl 300 mmol/l sodium citrate, pH 7.0
Buffer 1	100 mM TRIS 150 mM NaCl, pH 7.5
Buffer 2	5 g blocking agent (Boehringer Mannheim, Lewes) in 50 mls Buffer 1 (1.0% w/v)
Buffer 3	100 mmol/l Tris-HCl, pH 9.5 100 mmol/l NaCl 50 mmol/l $MgCl_2$
Pre-Hybridisation Buffer	125 mls x20 SSC (x5 SSC) 50 mls Buffer 2 0.5 g N-lauroylsarcosine (0.1%) 0.1 g sodium dodecyl sulphate (0.02%) Make up to 500 ml with UHQ
Hybridisation Buffer	As for Pre-hybridisation buffer plus the addition of 10 ng/ml of digoxigenin-deoxy-uridine triphosphate labelled probe. The probe was denatured by heating at 100°C for ten minutes and chilled immaediately on ice before addition to the pre-hybridisation buffer.
x2 wash	10 mls x20 SSC (x2 SSC) 1 ml 10% SDS (0.1%) Make up to 100 mls with UHQ
x0.1 wash	0.5 mls x20 SSC (x0.1 SSC) 1 ml 10% SDS (0.1%) Make up to 100 mls with UHQ
Conjugate solution	12 μl conjugate (Anti-digoxigenin [Fab] conjugated to alkaline phosphatase) in 60 mls Buffer 2. Mixed by gentle inversion.
Colour development solution	90 μl of (7 mgs NBT + 70 μl DMF + 30 μl UHQ) and 70 μl of (5 mgs X phosphate + 100 μl DMF) and 20 mls of Buffer 3. Mixed by gentle inversion.

Figure A 2. Estimating the concentration of digoxigenin-labelled probe.



This ethidium bromide stained agarose gel has four lanes. In the first lane is a 100 base pair DNA ladder and the 600 base pair marker (brightest band) contains 100ng of DNA. The other three lanes each contain 10µl of digoxigenin-labelled probe. The staining intensity of these lanes is estimated at three times that of the 600 base pair marker. Therefore, each 10µl of digoxigenin-labelled probe contains approximately 300ng of DNA.