INVESTIGATING

THE ROLE OF INFECTIONS

IN HUMAN ATHEROSCLEROTIC

HEART DISEASE

By

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To Cathy,

Daniel, Nicole and Benjamin,

Zosia and Florian,

with all my love.

ABSTRACT

Human atherosclerosis is a disease of the blood vessel wall caused by an interplay between inflammatory, thrombotic, and lipid factors. A contributing or causal role for infection in that inflammatory response was first proposed in the 19th century, and with the advent of more sophisticated diagnostic techniques, a new search for a microbiologic etiology of human atherosclerosis has been rekindled.

In this thesis, I examine methods for investigating whether infections contribute to human atherosclerotic cardiovascular disease. I explore three different technologies: serology (measuring antibody), inflammatory markers (as risk markers and as surrogates for infections), and the direct measurement of bacterial or viral DNA in the bloodstream. I examine three different study designs: cross-sectional, case-control, and cohort.

Chlamydia pneumoniae, an obligate intracellular bacterium, is the primary focus of these studies. In addition, cytomegalovirus and other infections are included as controls, with the *a priori* expectation that these other infections would not be related to cardiovascular disease.

We found a relationship between cardiovascular disease and *C. pneumoniae* antibody status in a small case-control study, but found no independent association in a large, prospective study. Inflammatory markers were measured in the prospective study, and were associated with cardiovascular events, yet no clear association between inflammation and infection was found. However, in developing methods for directly detecting bacterial and viral DNA in the bloodstream, we found that serology itself was not associated with current detection of bacterial DNA. Furthermore, we found a strong relationship between *C. pneumoniae* and smoking, and conclude that future studies need to examine the interaction between infection, inflammation, and smoking status.

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PREFACE

This Ph.D. thesis is organized into four major sections and ten individual chapters, and consists of a "sandwich thesis" of eight chapters written as manuscripts, together with introductory and concluding chapters. I am first author on all submitted manuscripts, and played a lead or major supporting role in the study design, grant writing, laboratory measurement, analysis, and manuscript writing. Details for each study are outlined below. Publication or submission details are listed at the beginning of each chapter, where relevant.

Section 1 consists of one introductory chapter, in which I attempt to validate a scale of causality that I proposed in my MSc thesis. The current chapter is entirely my own work, although I have invited two infectious diseases colleagues (Drs. Mark Loeb and David Fisman) to collaborate with me to improve the scale, and to help rewrite the chapter as a manuscript for submission to a peer-reviewed general medicine journal.

Section 2 consists of three chapters exploring serologic and inflammatory markers as risk markers for cardiovascular disease. Chapter 2 consists of a case-control study, and has been published in the Canadian Journal of Cardiology. I proposed and pilot tested this study as part of my MSc in Health Research Methodology, obtained funding form the Regional Medical Associates, Hamilton ON Canada, helped enroll patients, measured the five antibody serologic tests, analyzed all data, was lead author on the manuscript, and presented the preliminary results at a Canadian Cardiovascular Society annual meeting. Dr. Lisa Cronin played a major role in helping me to develop and execute the study. My co-authors helped me to improve the design, analyses, and manuscript.

Chapters 3 and 4 summarize a study of infectious and inflammatory markers, respectively, in over 3,000 Canadian Heart Outcomes Prevention and Evaluation (HOPE) study patients. Dr. Salim Yusuf and I entitled this the "Infectious and Inflammatory Markers in HOPE study" (IIM-HOPE), and obtained funding from the Heart and Stroke Foundation of Ontario to undertake the measurements and analysis of these data. I had originally proposed a more limited case-control study, on the basis of my M.Sc. thesis work, and I obtained a Research Fellowship from the Heart and Stroke Foundation of Canada (1998-2001) under the supervision of Dr. Yusuf. Dr. Yusuf proposed expanding the study to include the entire cohort of over 3,000 patients for whom blood was available. The full protocol was planned and discussed by Drs. Yusuf, Eva Lonn, Matthew McQueen, Judy Gnarpe, Hakan Gnarpe, Gunnar Olsson, and myself. Dr. Hakan Gnarpe proposed that I investigate which cytokines and adhesion moleculares may be associated with infection and cardiovascular outcome.

Dr. Yusuf was the Principal Investigator of the HOPE and IIM-HOPE studies, and responsible for over-seeing the study design, execution, and analysis of the parent study and for guidance on the sub-study. As his research fellow, I was responsible for the design and execution of the IIM-HOPE study, under his guidance. Blood was stored as part of the HOPE study, and all study outcomes were part of the HOPE study proper.

For the IIM-HOPE study, I performed the literature searches, wrote the first and final drafts of the grant, organized (together with Dr. McQueen and Mrs. Kim Hall, laboratory research co-ordinator) all facets of blood testing including performing pilot studies to determine which assays were used, wrote and guided the statistical analysis (performed by Dr. Qilong Yi), and was lead author on the two manuscripts. I have

presented IIM-HOPE study results, in part, at two HOPE study investigators meetings, the

Canadian Cardiovascular Society meeting (2001), in Halifax NS Canada, and at the American

Heart Association meeting (2001) in Anaheim CA.

Section 3 consists of five chapters developing and validating the role of blood-based

DNA tests of *Chlamydia pneumoniae* and CMV. Chapter 5 consists of the angiography study, a

cross-sectional study (with a small prospective component) that I designed together with Dr.

Madhu Natarajan (cardiology) and colleagues in Microbiology (Drs. Mahony and Petrich). I

obtained two grants from the Father Sean O'Sullivan Research Centre to carry out this

research. Due to the developmental nature of this work, a team involving numerous

individuals was required to develop the blood-based DNA tests. Drs Mahony and Petrich,

Ms. Sylvia Chong, and Dr. Lynne Rainen (from BD Vacutainer systems, Franklin Lakes NJ,

makers of the cell preparation tubes) were extensively involved in this process. Although

this study was the catalyst, Dr. Mahony played the primary role in overseeing the

development of the circulating *C. pneumoniae* DNA test, without which the studies I

summarize in Chapters 5 to 8 would not have been possible. Patients were recruited by

study nurses under the supervision of Dr. Madhu Natarajan, with particular enthusiasm and

extra work by Mrs. Corinne Tartaglia. The major result of this study was the development

of our circulating *C. pneumonaie* DNA assay for other epidemiologic studies. The primary

goal was simply to estimate the prevalence of circulating *C. pneumonaie* DNA detection. Two

incidental findings were that *C. pneumoniae* DNA detection was strongly associated with

current smoking and season, which I observed in analyzing this study. In subsequent

chapters, we were able to validate these major findings, the implications of which are

discussed in the individual chapters and in Chapter 9. Results have been presented in

abstract form by me (Clinical Virology Symposium, Clearwater Beach FL) and by Dr.

Natarajan (Canadian Cardiology Society meeting, 2000), and we published a full manuscript

in the Journal of Clinical Microbiology.

Chapter 6 consists of an analysis of *C. pneumoniae* detection by probit regression

modeling of test replicates. The inspiration for this analysis was a manuscript written by Dr.

Mahony, on which I was co-author, in which we compared five different DNA amplification

tests for the detection of *C. pneumoniae*. I was asked to analyze this study, and sought

statistical advice from Dr. Charles Goldsmith. For the paper, we used an analysis based on

repeated measures of categorical data. However, to better model the effect of different

concentrations, Dr. Goldsmith recommended a cumulative distribution function approach,

and helped me to develop the probit regression model. Probit regression had been used,

according to my literature search, in only four other papers in the microbiology literature.

The co-authors helped develop a study to develop and validate the model. I presented the

data at the International Conference on Antimicrobial Agents and Chemotherapy (ICAAC),

Toronto ON, 2000, and published the manuscript in the Journal of Clinical Microbiology.

The paper has been cited by other investigators dealing with *C. pneumoniae* DNA detection in

samples such as cerebrospinal fluid or blood, and provides an important basis for explaining

the sampling problems of *C. pneumoniae* DNA detection. I believe that this will become

increasingly important as molecular diagnostics moves towards quantitation, in which

replicate testing has become an informal standard.

The respirology study described in Chapter 7 was designed by Drs. Astrid Petrich,

Richard Leigh, James Mahony, Frederick Hargreave and myself. A major motivation was my

observation in the angiography study, of a relationship between current smoking status and

circulating *C. pneumoniae* DNA detection. In addition, we wished to have a source of tests to further develop important technical details such as optimal extraction methods and volumes. The study was funded as part of Dr. Astrid Petrich's grant of chlamydia-like organisms in respiratory samples. Dr. Richard Leigh and I were primarily responsible for designing, executing, and analyzing the study, whereas Dr. Petrich supervised the laboratory component. Dr. Richard Leigh and Dr. Astrid Petrich each presented aspects of the study at the American Thoracic Society meeting (2000) and the American Society of Microbiology meeting (2001), and I performed all statistical analyses and was lead author for the manuscript. Dr. Astrid Petrich will be lead author on a companion manuscript, examining the detection of the novel chlamydia-like organism *Simkania negevensis* in respiratory specimens. This chapter validates two findings from the angiography study: an association with smoking (rather than with heart disease itself), and with season.

The angioplasty study in Chapter 8 was designed by Dr. Madhu Natarajan and colleagues to compare various angiocoagulation strategies for patients undergoing percutaneous coronary interventions. Dr. Natarajan and I designed a sub-study to measure infections and inflammatory markers. Peripheral blood mononuclear cells and plasma were obtained in study patients to measure circulating *C. pneumoniae* DNA and CMV DNA, and, in future, to measure C-reactive protein and soluble Intercellular Adhesion Molecule-1. The primary research question was whether these infections and inflammatory markers predicted clinical outcome (restenosis, hospitalization, cardiovascular events and death). We also wanted to compare the prevalence in cardiology patients with control patients.

Together with Drs. Janusz Kaczorowski and David Price, I developed the Family Practice prevalence study. These patients served as controls for the angioplasty study, and,

separately, will enable determining the influence of smoking, season, and other risk factors such as alcohol consumption on the detection of circulating *C. pneumoniae* DNA. The comparison between cardiology patients and family practice controls provides an estimate of whether *C. pneumoniae* DNA prevalence is higher in patients with cardiovascular disease. We found a lower prevalence, which was unexpected and difficult to explain. In the manuscript, and in future studies, I explore the possibility that cardiac medications directly affect the prevalence of *C. pneumoniae* DNA.

The final chapter of Section 3, chapter 9, summarizes current knowledge regarding *C. pneumoniae* DNA detection. Written specifically for this thesis, I invited microbiologists from our group and from Sweden (Dr. Jens Boman) to participate as co-authors. We plan to submit the manuscript for publication within three months.

In the fourth section, consisting of a single chapter, I conclude by summarizing the major findings and suggest future directions for the research. There were no co-authors on this chapter, and this will not be a separate manuscript.

ACKNOWLEDGEMENTS

List of Abbreviations used in this Thesis

ADV	Adenovirus
AIDS	Acquired immunodeficiency syndrome
AU	Arbitrary units
CAD	Coronary artery disease
CCU	Coronary Care Unit
CI	Confidence interval
CK	Creatine kinase
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
CP	*Chlamydia pneumoniae*
CPT	Cell preparation tube
CRP	C-reactive protein
CV	Cardiovascular
df	Degrees of freedom
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ECG	Electrocardiogram
EIA	Enzyme immunoassay
FEV_1	Forced expiratory volume in one second
FMD	Flow mediated vasodilation
HAV	Hepatitis A virus

HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HOPE	Heart Outcomes Prevention and Evaluation study
HP	*Helicobacter pylori*
HPV	Human papillomavirus
HR	Hazard ratio
HRM	Health Research Methodology
hsCRP	High sensitivity C-reactive protein
HSP	Heat shock protein
HSV	Herpes simplex virus
ID	Infectious Diseases
IFU	Inclusion forming units
IgG	Immune globulin G
IgM	Immune globulin M
IL-6	Interleukin-6
IMT	Intimal medial thickness
IU	International Units
log	Logarithm
MSc	Master of Science
MI	Myocardial infarction
MIF	Micro-immunofluoresence
MOMP	Major outer membrane protein
NPS	Nasopharyngeal swabs

OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RR	Relative risk
RT-PCR	Reverse transcriptase-polymerase chain reaction
SD	Standard deviation
SECURE	Study to Evaluate Carotid Ultrasound with Ramipril and Vitamin E
SEM	Standard error of the mean
sICAM-1	Soluble Intercellular Adhesion Molecule-1
TB	Tuberculosis
UA	Unstable angina
VCAM-1	Vascular Cell Adhesion Molecule-1

SECTION 1:

INTRODUCTION

Chapter 1

Validating the McMaster Causality Scale

Structure of this Thesis

In this introductory chapter, I consider some historical aspects of microbiological

enquiry, the paradigms under which causal investigations are undertaken and comprehended,

and the rules for proving a causal association. I then briefly examine 10 infection/ disease

pairs using Koch's postulates, Bradford Hill's guidelines, the McMaster causal scale, and

"expert opinion", in an attempt to validate the McMaster Causality Scale. Last, I apply these

guidelines to determine whether there is a causal association between *Chlamydia pneumoniae*

infection and human atherosclerotic heart disease.

The eight chapters that follow were written as manuscripts for publication. Three

have been published as of January 2002, three are currently undergoing peer-review, and the

remaining two manuscripts will be sent to journals for review by July 2002. In the tenth and

concluding chapter, I overview the findings of the individual studies to put them in a more

global context. I discuss the role of serologic and molecular studies within the thesis, and

the implications for further research. I complete the thesis with a consideration of the

process of studying epidemiologic markers, and conclude with a discussion of planned future

studies.

Introduction

With the development of sophisticated technologies for the detection of infectious agents, we are witnessing a massive resurgence of interest in a very old idea: that occult infections cause many chronic diseases. Books and articles in the scientific and lay press continually appear, claiming infections cause everything from cancer to behavioral problems. Is there any "evidence" that infections cause cardiovascular disease, cancer, or diabetes? More fundamentally, what do we mean by a "cause" and by "evidence"? What are the rules for declaring an association as causal? How good must this evidence be, before we act?

In this thesis, I do not propose resolving these difficult questions. Rather, I would like to contribute to the debate regarding appropriate methods for answering such questions, in the hope of improving the efficiency with which we are able to answer such questions in the future. My focus in this thesis is on the relationship between *Chlamydia pneumoniae* and other infections and human atherosclerotic cardiovascular disease. The methods, I believe, will be generalizable to the investigation of occult infections and chronic diseases across many medical disciplines.

In 1997, I completed a MSc in Health Research Methodology at McMaster University entitled: "A case-control study of the association of previous infection with *Chlamydia pneumoniae, Helicobacter pylori,* or cytomegalovirus, and the acute coronary syndromes"(1). In chapters 1 and 2 of that thesis, I reviewed the literature, as of August 1997, regarding the association between infection and human heart disease. To avoid redundancy and to focus on concrete study results, I provide only a limited review of *C. pneumoniae* and atherosclerosis in this thesis. This is found at the end of the current chapter. For a more detailed review of the association between serology, inflammation, and

cardiovascular disease, the interested reader may borrow the MSc thesis from the McMaster University Health Sciences Library. The only comprehensive review I undertake in this thesis is a systematic review of molecular epidemiologic studies of the direct detection of *Chlamydia pneumoniae* nucleic acids in peripheral blood (Chapter 9).

In chapter 3 of my MSc thesis, I reviewed the literature regarding rules and guidelines for demonstrating a causal association between an infectious agent and a disease, and proposed a guideline based on the strength of the evidence of association, and the strength of evidence of experimental modification (1). In this chapter, I describe the preliminary validation of this causation scale, which I will refer to as the "McMaster Causality Scale".

Historical Background

The nineteen century has been described as the "golden age of microbiology", during which the germ theory of disease was popularized and proven, microbiologic techniques for isolating and identifying bacteria were developed, and effective vaccines were developed. Two prominent figures from this era were Pasteur and Koch.

Louis Pasteur (1822-1895), recognized as the father of microbiology, carried out seminal work on fermentation, "pasteurization", disproving spontaneous generation, culture techniques for bacteria, immunology, and vaccine development (2). At a time of rampant infectious diseases, the development of techniques to diagnose illness, and of vaccines to treat or prevent them, were testament to the dynamic synergy between scientific and technological advances. Pasteur recognized the power of the media and the need to sway public opinion, and his famous experiments on disproving spontaneous generation and on anthrax vaccination were done before the European press. At a time that anthrax killed

thousands of cattle and other farm animals, Pasteur developed a vaccine for anthrax, and vaccinated a number of animals prior to exposure to *Bacillus anthracis*. Members of the press stayed for days at the farm and witnessed the death of unvaccinated animals, and the survival of vaccinated animals. The proof of causality, even in Pasteur's day, was not merely the development of diagnostic methods and prognostic studies, but also of developing and disseminating effective therapies. Rabies, a uniformly fatal disease, and Pasteur's fame spread as his live attenuated vaccine given to dog bite victims prevented otherwise certain death.

Robert Koch (1843-1910), microbiologist and Nobel prize laureate, developed the first effective culture media and stains for the diagnosis of *Mycobacterium tuberculosis* (3). He studied cholera, anthrax, and tuberculosis, and helped firmly establish microbiologic techniques and beliefs as a basis for medical and public health investigation. During his lifetime, the germ theory became widely accepted, although he, like Pasteur, recognized the need for knowledge as a road to effective therapy. He dedicated much of his life to developing a cure for tuberculosis, but was not successful. He is best remembered, however, for formulating the "rules" for proving a causal association, known as the Koch's postulates.

Koch's Postulates

In his work with tuberculosis, Koch developed his postulates to help convince a skeptical scientific community that *M. tuberculosis* was truly the cause of pulmonary tuberculosis. Tuberculosis was epidemic in European cities at the time, and a leading cause of death. His four postulates are (4):

1. *The parasite occurs in every case of the disease in question and under circumstances that can account for the pathological changes and clinical course of the disease.*
2. *It occurs in no other disease as a fortuitous and nonpathogenic parasite.*

3. *After being fully isolated from the body and repeatedly grown in pure culture, it can induce the disease anew.*
4. *The original organism must be isolated from the experimental animal.*

Reacting to disparate claims of isolating protean organisms from diseased patients, Koch emphasized the methods for ensuring high specificity for a causal association. The specificity has led to Koch's postulates being regarded as a lofty and often unattainable "gold standard". I argued in my MSc thesis that these postulates are neither sensitive, practical, nor sufficiently rigorous. I will return to these issues below, but first let us consider scientific paradigms of causation.

Paradigms

Koch's postulates equate one infection with one disease. They demand 100 % sensitivity and 100 % specificity. Thus, infection is both necessary and sufficient. Such a paradigm may have served well for the investigation of acute and common infectious diseases in the 19th century, but it may not serve well the investigation of chronic diseases.

By the early twentieth century, chronic diseases with no apparent infectious etiology were increasingly recognized. To the present day, many diseases are classified as infectious or non-infectious. Many chronic illnesses including atherosclerosis and cancer were investigated by curious microbiologists, and relegated to the "non-infectious" by the limitations of then-current microbiologic culture and of Koch's "necessary and sufficient" causality paradigm. Ironically, Koch himself recognized that his postulates could not be applied to cholera, as asymptomatic carriage was clearly demonstrated in violation of the specificity requirement of his second postulate.

The investigation of chronic diseases has proceeded, after the failure of 19th century microbiologic techniques, along a different paradigm. Its methods were statistical inference,

and multiple risk factors were identified and statistical associations "inferred". Careful observation and statistical modeling, and later the randomized controlled trial, became the criterion standard for proving a causal association between a treatment and outcome. Implicit in the methods used is a paradigm in which the causal agent, whether a therapy or a putative risk factor, is neither necessary nor sufficient. Rather, cause refers to a "contributing" cause, with an attributable risk that may be large or small. In some circumstances the attributable risk is 100 %, and in that setting we have a "necessary" causal agent. Recognizing a variety of genetic and environmental co-factors in many diseases, there are few circumstances in which we would recognize a putative cause as "sufficient".

This distinction between a 19th century paradigm of infections as sufficient and necessary, and contrasting this with chronic disease epidemiology with its emphasis on multiple causes, permeates the current debate on infections in heart disease.

Bradford Hill's Guidelines

In a speech before the Royal Society in Britain in 1965, Sir Austin Bradford Hill, the renowned statistician and epidemiologist who designed the first medical randomized controlled trial (streptomycin for tuberculosis), suggested guidelines for determining whether an association was causal (5). He advised that a number of the following needed to be met: strength of the association, consistency, specificity, temporality, biological gradient, plausibility, coherence, experiment, and analogy. Although he emphasized that experiment may be the most important of these, the nine are often treated as being equally important.

Other guidelines were developed in the infectious diseases by Rivers (6), Evans (7), Huebner (8), and Fredricks and Relman(4). Many of these were attempts to broaden Koch's

postulates, which were increasingly recognized as unworkable. However, none of these have been validated, and some lack substantially for content validity.

The McMaster Causal Scale (1)

For content validity, I distilled these numerous guidelines into two domains: association and experimental modification. Koch's first, second and fourth postulates are proofs of association, whereas postulate 3 is of experimental modification. Similarly, Bradford Hill's guidelines describe association (association, consistency, specificity, temporality, and biologic gradient) and experimental modification.

Next, I considered the need for developing a scale of the probability that a given association was causal, and to base the guidelines on recognized evidence-based principles. The McMaster Causality Scale (Table 1) has only two components:

1. Evidence of association
2. Evidence of experimental modification.

Each component is then scored between 0 and 3. For association, the highest score (3) is given for associations proven with well-designed cohort studies, using blinding and validated laboratory techniques including culture or two disparate technologies, for which a clinically-important and statistically-significant attributable risk is demonstrated. Scores of 1 or 2 are assigned to studies without prospective validation, or with less robust laboratory techniques. For experimental modification, the highest score (3) is assigned for adequately-powered, blinded, appropriately-controlled trials of a specific therapy which demonstrate clinically-important and statistically-significant prevention or treatment. Animal, volunteer experiments, and before/after studies would receive only 1 or 2 points, depending on the methodologic rigor.

To interpret the McMaster Causality Scale, the two components are summed and a total of 6 points interpreted as a definite cause, 5 as a probable cause, and 2 to 4 as various grades of possible cause.

There are two important difference between the McMaster Causality Scale and Koch's postulates. First, the McMaster Causality Scale does not require an infection to be either necessary or sufficient, in recognition of the multifactorial etiology of many diseases. Second, to be defined as a "definite cause" on the McMaster Causality Scale requires convincing randomized trial data in humans—in contradistinction to Koch's postulates, in which definitive experimental evidence consists of causing the disease in an animal model. A disease which perfectly fulfills Koch's postulates may score only 5 points, or "probable cause", on the McMaster Causality Scale.

A validation study for the McMaster Causality Scale

Where a reference standard exists, a new scale is best validated by comparison with that reference. This is known as criterion validity. One could use Koch's postulates or Hill's guidelines as reference standards, but there are many infectious diseases for which these are not considered useful. Koch's postulates cannot address the association of viruses with disease, for example, since viruses cannot be grown in cell-free cultures, or illnesses in which there are asymptomatic carrier states. Hill's guidelines include eight guidelines on "association", but only one guidelines on "experiment", despite Hill's own contention that the latter is most important (5).

If the current "gold standards" are recognized as inadequate, validation other than criterion validation will be required. Methods of validating include assessment of face validity, content validity, and construct validity.

I developed the McMaster Causality Scale for content validity and scalability, as discussed previously in this chapter. The only causal concept not embodied in this simple scale is that of mechanism, and I have chosen not to include this concept on the basis that for many well-accepted infection/disease associations, the mechanism is not well understood. The scale has considerable "face validity": if there is clear evidence that an infection is associated with a disease, and clear evidence that preventing or treating that infection changes the prevalence of that disease, most would consider the association causal.

Construct validation has been used extensively in the psychological and social sciences, as well as quality of life research and other constructs for which appropriate measurement instruments are being developed. To examine construct validity, I created 10 infection/diseases pairs for which at least some association had been reported. These varied from pairs such as *M. tuberculosis* and pulmonary tuberculosis, for which a causal association was assumed to be undisputed, to associations of viruses with various cancers. I scored these 10 pairs by Koch's postulates, Hill's guidelines, and the McMaster Causality Scale. To allow comparison with expert opinion (see below), the scores for Koch's postulates, Hill's guidelines, and the McMaster Causality Scale were converted to a scale of 1 (define causal association) to 4 (no association), as indicated by the formulas in the table legend.

Obtaining student and expert opinion by questionnaire

Next, a questionnaire was constructed to assess people's perceptions of a causal association, to triangulate responses obtained by questionnaire, with the results of Koch's postulates, Hill's guidelines, and the McMaster Causality Scale. A six-item questionnaire was pilot-tested at Health Research Methodology (HRM) student research rounds, which I presented in 1999. I refer to these as "Student Opinion". Nine responses were received of

10 in attendance (90 %), with assigning of numbers between 1 (definite cause), 2 (probable cause), 3 (possible cause) and 4 (not a cause). A 10-item questionnaire was e-mailed in June, 2001, to 50 members of the Hamilton Infectious Diseases, Microbiology, Public Health, and Infection Control community. I refer to these as "Expert Opinion". Twenty (40 %) of participants replied.

The results of these two questionnaires are summarized in Table 2. Student Opinion scores showed mean scores with large standard deviations, and assignment of only slightly lower scores (mean score 1.6) to infection/disease pairs for which causality is well established, such as *M. tuberculosis* and pulmonary tuberculosis, and pairs for which there is evidence of a lack of association, such as herpes simplex type 2 and cervical cancer (mean score 2.5). In Table 3, there was poor discrimination between infection/disease pairs for which causality is well accepted, and ones for which evidence exists of no causal association. The only statistically-significant difference was for *H. pylori*, in which the association with ulcers (2.0) was clearly stronger than with cancer (2.8). Despite some methodological expertise, it is likely that HRM students lack content expertise, resulting in wide variation of perception and a tendency to assign "probable" or "possible" to most associations.

Questionnaires answered by the Infectious Diseases community constituted Expert Opinion, with established content expertise. Many also have expertise in epidemiological methods. Diseases such as tuberculosis, leprosy, AIDS, and malaria, for which virtual unanimity within the Infectious Diseases community was assumed *a priori*, had a mean score of 1.0 (indicating "definite cause") to 1.1, with a very small standard deviation. The one disease association for which an association was initially demonstrated (9), HSV and cervical cancer, has subsequently shown to be confounded by association with human papillomavirus

and cervical cancer (10). As expected, Expert Opinion assigned the HSV/cervical cancer association a 3.4, indicating little evidence of causality.

Among infection/disease pairs for which causality is less clear, intermediate scores were assigned. *H. pylori* was felt to be a clear cause of duodenal ulcers (score of 1.4), whereas its relationship to gastric cancer was considered probable with a score of 2.2.

In table 4, I examine whether the differences between individual item responses were statistically significant. Because of the limited increments on the scale, a difference of 0.5 units was judged to be clinically important. For the first 3 pairs of comparisons in Table 4, tuberculosis (as a "definite cause") was compared with HSV / cancer (not a cause), HIV/AIDS (another "definite cause"), and *C. pneumoniae* / coronary artery disease (CAD). Expert Opinion assigned lower scores for the comparisons between TB and HSV/cancer, and TB and *C. pneumoniae*/CAD (*t*-tests). The consistency of the answers was sufficiently high that the 2.9 assigned to *C. pneumoniae* / CAD was statistically significantly more than the 2.0 assigned to HP / cancer, and less than the 3.5 assigned to HSV / cervical cancer. Respondents assigned the HPV / cancer association a 1.7, versus 3.5 for HSV / cervical cancer, a difference of 1.8 points on a scale where the maximum difference is 3.0. The comparison was highly statistically significant (P < 0.001). The association with *H. pylori* and duodenal ulcer was felt to be much stronger than that with cancer, although both were judged as at least "probable" causes. The difference of 0.8 was clinically important (delta > 0.5) and statistically significant (P < 0.001). Thus, Expert Opinion using a moderate number of respondents and a very limited scale has good precision for differentiating perceptions of causality.

Triangulation

The mean questionnaire Student and Expert Opinion scores for each of the 10 infection / disease pairs were compared with the Koch, Hill, and McMaster Causality Score values. The latter three were converted to a 1 to 4 point scale for easier comparison. In Table 5, the non-parametric correlations between these 3 scales, HRM Student Opinion, and Expert Opinion are examined. Koch scores correlated with McMaster Causality Scale scores (estimated rho = 0.79, P < 0.007) and Expert Opinion (estimated rho = 0.67, P = 0.03). Neither Hill's guidelines nor Student Opinion scores correlated with any of the other scales. The McMaster Causality Scale scores correlated with Koch's postulates (estimated rho = 0.79, P < 0.007) and with Expert Opinion scores (estimated rho = 0.83, P = 0.003).

Regression

To further examine the test properties of the McMaster Causality Scale, two linear regression models were constructed using either Koch's scores or Expert Opinion mean scores as the dependent variable. The McMaster Causality Scale scores, Hill's scores, and either Koch's scores or Expert Opinion mean scores were then assessed as independent variables. Variables were entered in a forward selection model.

For Koch's scores as the dependent variable, only McMaster Causality Scale scores were an independent predictor (R^2 = 0.67, F = 16.1, P = 0.004). Neither Hill's scores (P = 0.77) nor Expert Opinion scores (P = 0.47) were independent predictors in a multiple linear regression model.

Setting the Expert Opinion scores as the dependent variable, the McMaster Causality Scale scores were the only independent predictor (R^2 = 0.73, F = 21.2, P = 0.002). Neither Koch's scores (P = 0.77) nor Hill's scores (P = 0.31) were independent predictors.

Summary of McMaster Causality Scale Validation

This study provides the first formal validation for the proposed McMaster Causality Scale. The scale has face and content validity. The McMaster Causality Scale correlated with Koch's postulates and Expert Opinion scores, indicating construct validity. Setting either Koch's postulates or Expert Opinion scores as the criterion standard, the McMaster Causality Scale scores were the only independent predictor, thus demonstrating criterion validity.

Before this scale can be recommended for general use, however, work remains to be done in assessing its reproducibility, scalability, and inter-observer reliability for a wider variety of infection/ disease pairs. Nevertheless, this preliminary validation indicates that the scale may usefully contribute to the assessment of infections in chronic disease etiology.

The Association of *C. pneumoniae* with Atherosclerosis

Prior to determining whether the association between *C. pneumoniae* and atherosclerosis is causal, let us summarize pertinent data examining the association. I will examine, in order, pertinent pathological, animal model, sero-epidemiologic, and interventional data.

First, there are a number of pathological studies examining the association between *C. pneumoniae* and human atherosclerosis. Prior to establishment of a clear culprit organism, electron microscopy of atheroma demonstrated abnormalities resembling chlamydial elementary bodies (11). Subsequent studies of antigen detection with *C. pneumoniae* genus and species-specific monoclonal antibodies demonstrated antigen within atheroma (11,12), predominantly within macrophages. A number of laboratories have demonstrated chlamydial nucleic acid, predominantly DNA but occasionally RNA, within atheroma

(13,14). Finally, in a handful of cases, *C. pneumoniae* has been successfully cultures from carotid, coronary, and aortic atheroma (15-18). This is supported by *in vitro* data indicating that *C. pneumoniae* can infect cells which participate in atherosclerosis, including endothelial cells, macrophages, and smooth muscle cells. Furthermore, infection can induce heat shock protein expression and foam cell formation compatible with natural atherosclerosis (19). However, considerable inter-laboratory variation in nucleic acid detection of *C. pneumoniae* within atheroma was found, as well as occasional false-positives, in multi-centre comparisons (20). Furthermore, while *C. pneumoniae* has been studied the most intensively and found the most consistently, other infectious agents have been observed within atheroma including cytomegalovirus, *Porphyromonas gingivalis*, and *Streptococcus sanguis* (21). Hence, the finding of *C. pneumoniae* within atheroma may not be specific to the one organism.

Second, a number of animal models have been developed to demonstrate the interaction between *C. pneumoniae* infection and atherosclerosis. In mouse models, *C. pneumoniae* infection accelerates aortic atherosclerosis (22). In rabbit models, *C. pneumoniae* intranasal infection causes pneumonitis followed by the development *de novo* of aortic atherosclerosis (23). Repeated infections enhance atherosclerotic lesions (24), and early— but not late—treatment with azithromycin prevents lesion formation (25). However, none of these animal models replicates the complications of atherosclerosis, such as the acute coronary syndromes.

Third, over 30 sero-epidemiological studies have examined the relationship between the presence of chlamydial antibodies and cardiovascular disease. These have been summarized in two meta-analyses. In 1997, Danesh et al found an association between *C. pneumonia* antibody and clinical events, based primarily on case-control studies (26). In a

follow-up meta-analysis restricted to prospective studies, Danesh et al found a modest association between *C. pneumoniae* IgG antibody and cardiovascular events, which was not statistically significant after adjustment for other cardiovascular risk factors and socio-economic status (27). While this demonstrates that *C. pneumoniae* antibody status does not appear to be an independent predictor of cardiovascular disease, it addresses neither whether antibodies are a good measure of chlamydial exposure or current burden, nor whether *C. pneumoniae* interacts with other established risk factors. An accompanying editorial asked whether adjustment for socio-economic status represented over-adjustment in this analysis (28).

Fourth, there are a limited number of studies using other markers of infection. Saikku et al examined immune complexes, and found they were more strongly associated with heart disease than chlamydial IgG antibody (29). A number of investigators have examined the detection of *C. pneumoniae* in peripheral blood mononuclear cells and its relationship to cardiovascular disease (30-32). I review the molecular epidemiologic studies in Chapter 9 of this thesis.

Last, and perhaps most importantly, a number of studies have examined the association between antibiotic therapy and cardiovascular clinical events. In observational studies based on large administrative databases, an inconsistent protective effect of previous antibiotics in cases with incident heart disease versus controls has been found (33-35). In two of three small randomized trials, treatment with azithromycin or roxithromycin was associated with a reduction in clinical events (36-38). A number of large clinical studies are ongoing (39,40), and preliminary conference reports from two of these found no therapeutic benefit of antibiotics in patients presenting with myocardial infarction or unstable angina (M.

Dunne and P. K. Shah, American College of Cardiology meeting, 2002). These results are currently available only in abstract form, and will be discussed further in Chapter 10.

Assessing Causality for the Association between *C. pneumoniae* and Atherosclerosis

In Table 2, I assess the above data for a causal association using Koch's postulates, Bradford Hill's guidelines, and the McMaster Causality Scale. Koch's first postulate, of a consistent association between the infection and the disease, is met in part. Most laboratories have found an association between the presence of *C. pneumoniae* in atheroma. However, *C. pneumoniae* isolation is not specific to atherosclerosis, thus postulate 2 is not met. *Chlamydia pneumoniae* causes atherosclerosis in animal models, and the infection can, in some cases, be found within animal atheroma. Thus, 2.5 of the 4 postulates are satisfied, and the association is probably causal by Koch's criteria.

Of Bradford Hill's guidelines, guidelines 1 (strength of association), 4 (temporality), and 6 to 9 (plausibility, coherence, experiment and analogy) are satisfied. Guidelines 2 (consistency), 3 (specificity) and 5 (biological gradient) are not. Although this is no formal method of scoring these guidelines, satisfying 6 of the 9 guidelines suggests that the association is probably causal.

The McMaster Causality Scale scores 2 points for association (multiple studies with different technologies, but an absence of confirmation in prospective serological studies) and 2 points for experiment (animal studies and small clinical studies). Note that the scoring in Table 2 was established prior to the availability of the preliminary results of large studies, and is retained for valid comparison with Expert Opinion. 4 points is interpreted as "possible association" on the McMaster Causality Scale. Inclusion of results from large randomized clinical trials would assign only 1 point for experimental modification, in recognition of the

greater role of human clinical trials as evidence of human causality. I would now reassign 3 points to the Chlamydia pneumoniae/atherosclerosis relationship, or "possible association".

Expert Opinion was more conservative than any of these three scales, and assigned a mean score of 2.9 (SD 0.5) to the association of *C. pneumoniae* with coronary artery disease. A score of 3.0 corresponds to "possible association".

Thus, the association between C. pneumoniae and human atherosclerotic heart disease remains unproven. Time-honoured scales such as Koch's postulates and the causal guidelines of Bradford Hill would conclude this relationship is "probably causal", whereas the McMaster Causality Scale and Expert Opinion were more conservative and conclude this relationship is "possibly causal".

Summary

There is a large body of literature examining the association of *C. pneumoniae*, and other infections, with atherosclerotic heart disease. Interpretation of these data requires a set of causal guidelines, yet existing guidelines do not reflect perceived expert opinion.

In this chapter, I presented the McMaster Causality Scale, consisting of two domains: assessment of association, and assessment of experimental modification. I demonstrated face, content, construct, and criterion validation for this scale. By this measure, or by Expert Opinion, the association between *C. pneumoniae* and heart disease remains at best a "possible cause", despite the partial fulfillment of Koch's postulates.

In the remainder of this thesis, I explore one of these two causal domains: the association of *C. pneumoniae*, and other infections, with human cardiovascular disease. In the concluding chapter, I return to the suggested further steps which will be required to assess causality, including appropriate design issues for proving experimental modification.

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Table 1-1. The McMaster Causality Scale (Smieja, 1997).

Domain/points	3 Points	2 Points	1 Point	0 Points
Evidence of Association	Multi-center consistent prospective blinded association using culture or ≥ 2 non-related detection technologies	Prospective studies not meeting the 3 point criteria; multiple consistent cross-sectional studies;	Cross-sectional studies without adequate replication, using non-validated techniques	Poor design, measurement, or analysis
Evidence of Experimental Modification	Human RCT(s) of prevention or treatment with a pathogen-specific intervention and clinically important outcomes; or blinded RCT volunteer study	RCTs not meeting the 3 point criteria multiple before-after studies of putative effective therapies; animal models if clear parallel to human disease	Other animal models, volunteer experiments without appropriate controls; limited before/after studies; or non-specific therapies even if RCT	Poor design, outcome, end-points or post-hoc sub-group analyses

Total Points

Interpretation

6 points	Definite cause
5 points	Probable cause
2-4 points	Possible cause
0-1 points	No existing evidence for causality
< 0 points	Can assign negative points and score −6 to −1 with inferences such as "definitely not a cause", "probably not a cause", and so on.

Table 1-2: Assessment of Putative Causal Associations

Putative Infection/ Disease Association		Causation guidelines			Expert Opinion	
Infection	Disease	Koch (inverted 4)	Bradford Hill (inverted 4)	McMaster Scale (inverted 4)	Methodology Students (n = 9) mean (SD)	Infectious Diseases Staff (n = 20) mean (SD)
M. tuberculosis	Pulmonary tuberculosis	1, 2, 3, 4 (1.0)	1-4, 6-9 (1.3)	3, 3 (1.0)	1.6 (0.5)	1.1 (0.2)
M. leprae	Leprosy	1, 2 (2.5)	1-9 (1.0)	3, 2 (2.0)	-	1.0 (0.0)
HIV [a]	AIDS [b]	1, 2 (2.5)	1-6, 8 (1.7)	3, 3 (1.0)	1.8 (0.7)	1.1 (0.0)
H. pylori	Duodenal ulcer	1, 3 (2.5)	1, 2, 4, 6-9 (1.7)	3, 3 (1.0)	2.0 (0.0)	1.4 (0.6)
H. pylori	Gastric cancer	3 (3.3)	1, 4, 6-9 (2.0)	2, 2 (2.5)	2.8 (0.5)	2.2 (1.2)
Herpes simplex type 2	Cervical cancer	None (4.0)	6, 9 (3.3)	0, 0 (4.0)	2.5 (0.6)	3.4 (0.8)
Human papillomavirus	Cervical cancer	1 (3.3)	1, 2, 4, 6-8 (2.0)	3, 1 (2.5)	-	1.8 (0.8)
C. pneumoniae	CAD [c]	(1), 3, 4 (2.1)	1, 4, 6, 8, 9 (2.3)	2, 2 (2.5)	2.7 (1.0)	2.9 (0.5)
Plasmodium species	Malaria	1, 2, 3, 4 (1.0)	1-9 (1.0)	3, 3 (1.0)	-	1.0 (0.0)
Hepatitis B virus	Hepatocellular cancer	3, 4 (2.5)	1, 2, 4-9 (1.3)	2, 2 (2.5)	-	1.6 (0.8)

[a] HIV: human immunodeficiency virus; [b] AIDS: acquired immunodeficiency syndrome; [c] CAD: coronary artery disease.
A comparison of Koch's postulates, Bradford Hill's guidelines, the McMaster Causal Scale, and Expert Opinion for assessing the strength of evidence for causality in a putative infection/ disease pair.
Inverted 4 scale and expert opinion scale: *1 = definite cause, 2 = probable cause, 3 = possible cause, 4 = not a cause.
For Koch's postulates, inverted 4 score = 4-3*(number of postulates/4)
For Bradford Hill's guidelines, inverted 4 score = 4-3*(number of guidelines satisfied/9)
For McMaster Causal Scale: 6 = definite cause (inverted 4 scale = 1), 5 = probable cause (inverted 4 scale = 2), 2-4 = possible cause (inverted 4 scale = 2.5, 3.0, or 3.5 for scores of 4, 3 or 2 respectively), and 0-1 = not a cause (4).

Table1-3. Pairwise Comparisons of Responses among Health Research Methodology Students

Comparator 1	Comparator 2	n	Mean 1 (SD)	Mean 2 (SD)	Difference (SD)	t-test	P-value
MTB[a]-tuberculosis	HSV[b]-cancer	6	1.67 (0.52)	2.50 (0.55)	0.83 (0.98)	2.1	0.09
MTB-tuberculosis	HIV[c]-AIDS[d]	8	1.63 (0.52)	1.75 (0.71)	0.13 (0.83)	0.4	0.69
MTB-tuberculosis	CP[e]-CAD[f]	5	1.80 (0.45)	2.60 (1.14)	0.80 (1.48)	1.2	0.29
HSV-cancer	CP-CAD[g]	5	2.40 (0.55)	2.60 (1.14)	0.20 (1.30)	0.3	0.75
HP[h]-ulcer	HP-cancer	5	2.00 (0.00)	2.80 (0.45)	0.80 (0.45)	4.0	0.02

[a] *Mycobacterium tuberculosis*

[b] HSV, herpes simplex virus-type 2

[c] HIV: human immunodeficiency virus-type 1

[d] AIDS: acquired immunodeficiency syndrome

[e] CP: *Chlamydia pneumonia*

[f] CAD: coronary artery disease

[g] HP: *Helicobacter pylori*

Table 1-4. Pairwise Comparisons of Responses in the Infectious Diseases Communty

Comparator 1	Comparator 2	n	Mean 1 (SD)	Mean 2 (SD)	Difference (SD)	t-test	P-value
MT[a]-tuberculosis	HSV[b]-cancer	18	1.06 (0.24)	3.44 (0.78)	2.39 (0.78)	13.0	< 0.001
MT-tuberculosis	HIV[c]-AIDS[d]	20	1.05 (0.22)	1.10 (0.31)	0.05 (0.22)	1.0	0.33
MT-tuberculosis	CP[e]-CAD[f]	15	1.00 (0.00)	2.87 (0.52)	1.87 (0.52)	14.0	< 0.001
HP[g]-cancer	CP-CAD	14	2.00 (1.18)	2.86 (0.53)	0.86 (1.17)	2.7	0.02
HSV-cancer	CP-CAD	15	3.53 (0.52)	2.87 (0.52)	0.67 (0.82)	3.2	0.007
HSV-cancer	HPV-cancer	18	3.44 (0.78)	1.67 (0.77)	1.78 (1.26)	6.0	< 0.001
HP-ulcer	HP-cancer	18	1.39 (0.61)	2.22 (1.17)	0.83 (0.79)	4.5	< 0.001

[a] *Mycobacterium tuberculosis*

[b] HSV, herpes simplex virus-type 2

[c] HIV: human immunodeficiency virus-type 1

[d] AIDS: acquired immunodeficiency syndrome

[e] CP: *Chlamydia pneumonia*

[f] CAD: coronary artery disease

[g] HP: *Helicobacter pylori*

Table 1-5. Correlation between Causal Guidelines and Expert Opinion

	Hill Causality Guidelines	McMaster Causality Scale	Student Opinion[a]	Expert Opinion[b]
Koch (P)	0.13 (0.73)	0.79 (0.007)	0.71 (0.11)	0.67 (0.03)
Hill (P)	-	0.29 (0.41)	0.75 (0.08)	0.44 (0.21)
McMaster (P)		-	0.74 (0.09)	0.83 (0.003)
Students (P)			-	0.75 (0.08)

Non-parametric correlations (estimated Spearman's rho, SPSS 10.0) between causal guidelines and expert opinion regarding the strength of

a causal association for 10 infection-disease pairs (see text and table 1).

[a] Health Research Methodology students (n=9), McMaster University.

[b] Questionnaire to Infectious Diseases, Microbiology, and Infection Control community in Hamilton (n=20).

SECTION 2:

SEROLOGICAL AND INFLAMMATORY MARKERS

Chapter 2

A Case-Control Study of Serologic Markers

This chapter consists of a published manuscript, describing a case-control study of heart disease patients (acute coronary syndromes or chronic angina undergoing coronary antiography) versus outpatient clinic controls. I have made no changes from the published manuscript. The full citation is:

> **Smieja M**, Cronin L, Levine M, Goldsmith CH, Yusuf S, Mahony JB. Previous exposure to *Chlamydia pneumoniae, Helicobacter pylori*, and other Infections in Canadian Patients with Ischaemic Heart Disease. Canadian Journal of Cardiology 2001;17(3):270-276.

I presented the study results as a poster presentation at the Canadian Cardiovascular Society Meeting, October 19-23, 1999, Quebec City QC.

Our main study finding was of an association between *C. pneumoniae* IgG serology and heart disease, with a stronger odds ratio at higher antibody levels, in an age and gender-adjusted logistic regression model.

Previous Exposure to *Chlamydia pneumoniae*, *Helicobacter pylori*, and other Infections in

Canadian Patients with Ischaemic Heart Disease.

Running title: *Chlamydia* and heart disease

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Word Count: Summary: 53; Abstract: 227; Text: 2604

Abstract:

Objective: Previous exposures to *Chlamydia pneumoniae* (CP), *Helicobacter pylori* (HP), or

cytomegalovirus (CMV) have been associated with atherosclerotic heart disease. We studied

these associations in Canadian patients, with concurrent measurement of exposure to five

infections.

Design: Case-control study.

Setting: Coronary care units and angiography suite, Hamilton ON.

Patients: 107 consecutive patients presenting with myocardial infarction (MI) or unstable

angina (CCU patients), or with previous angina or MI (angiography suite), compared with

107 family practice or outpatient clinic controls.

Interventions: Recorded cardiovascular risk factors, and measured serology for CP, HP,

CMV, adenovirus (ADV), and hepatitis A virus (HAV). Statistical analysis by logistic

regression, adjusted for age and gender.

Main results: Exposure to CP was more frequent in cases than controls (85.4 % vs. 70.3 %,

adjusted odds ratio {OR} 2.3, 95 % confidence interval: 1.1 to 5.1, P = 0.03). Dividing CP

IgG absorbance into quarters, with the lowest quarter as the referent group, the adjusted

ORs were 2.8, 3.0, and 4.3 respectively for the 2nd, 3rd and 4th quarters (P = 0.001 for

trend). Seroprevalences of HP (61.7 %), CMV (64.0 %), ADV (75.6 %) or HAV (59.2 %)

were high, with no association with disease.

Conclusions: We found an association between heart disease and previous exposure to

Chlamydia pneumoniae, with a step-wise increase in ORs at higher antibody levels, whereas no

association was found with HP, CMV or other infections. Prospective validation of this

association is needed.

Introduction

The pathogenesis of coronary atherosclerosis has been described as an inflammatory microangiopathy, with a complex interaction of hyperlipidemia, inflammation and thrombosis(1,2). The critical roles of hyperlipidemia and thrombosis have been empirically demonstrated, and current treatment strategies for acute and chronic coronary artery disease are based on therapeutic interventions targeted at these underlying factors. However, the role of inflammation remains poorly understood and largely unexploited therapeutically.

Inflammatory proteins such as C-reactive protein and fibrinogen are higher in patients with acute coronary syndromes, and predict vascular events in patients with and without vascular disease at study baseline (3,4). Both are acute phase reactants that increase with injury or infection. It has been speculated that chronic infection is a potential cause of the inflammatory component of human atherosclerosis (5).

A number of infections, including *Chlamydia pneumoniae* (CP), *Helicobacter pylori* (HP), and cytomegalovirus (CMV) have been associated with human atherosclerosis (6,7). CP has been studied most extensively. In previous sero-epidemiologic studies, higher antibody levels to CP were associated with acute myocardial infarction, chronic angina, and coronary atherosclerotic narrowing at angiography (8-10). CP antigen and DNA were detected in coronary atheroma, carotid artery wall, and aortic aneurysm, and culture of CP from atheroma has been reported (8, 11, 12). In animal models, CP causes or accelerates aortic atherosclerosis in rabbits and mice (13-15). In two small controlled trials, treatment of post-MI patients with anti-chlamydial antibiotics reduced vascular end-points including unstable angina, MI, and vascular death (16,17). A third, larger study did not confirm this finding, and large randomized controlled trials of antibiotics in heart disease are ongoing (18,19).

However, antibiotics are often not specific for the organisms that they treat, and may in the case of macrolides or tetracyclines have anti-inflammatory effects which are independent of their antibiotic effects (20).

Helicobacter pylori has also been studied extensively, particularly in the United Kingdom, although the data are not convincing. In a systematic review, an inconsistent association of HP antibody with heart disease was found (6,7). Few studies have measured both CP and HP together, and those that did found that CP, but not HP, was associated with CAD (12,21). There was no association between HP and cardiac events or carotid thickening in two prospective studies (22,23). HP has been associated with socio-economic status and childhood infections, and thus the association with HP may be a confounded association (6).

CMV is strongly associated with accelerated atherosclerosis of patients with cardiac transplants (24), and may be associated with coronary artery restenosis and thrombosis after angioplasty or atherectomy (25). In a rat model, rat CMV increases neo-intimal cell proliferation after balloon injury to the carotid artery (26). A role in non-immunocompromised human vascular disease remains unproven.

A role for adenovirus in human atherosclerosis has not previously been sought. However, adenovirus (ADV) is a common cause of upper and lower respiratory tract infections, and has been associated with cardiomyopathy (27).

In this study, we sought to determine whether previous exposure to CP, HP, CMV or ADV was common in Canadian patients with coronary artery syndromes or chronic angina, and whether exposure was more common in cases than controls. As a serologic control and marker of socio-economic status, we also measured antibodies to hepatitis A

virus (HAV). Additionally, among the cases only, we examined the association among

infectious serologic markers, and between serologic status and coronary risk facts.

Methods

Patients and controls

Patients were recruited from all four Hamilton Coronary Care Units (Hamilton

General Site, Henderson General Site, and McMaster University Medical Centre site of the

Hamilton Health Sciences Corporation, and St. Joseph's Hospital) and from the Regional

Angiography Suite (Hamilton General Site), between March 1997 and May 1998. Patients

admitted with unstable angina (UA) or acute myocardial infarction (MI), or patients

presenting for coronary angiography with a history of UA or MI, were eligible. Unstable

angina was defined by symptoms and ECG changes, and the attending cardiologist's

assessment. Myocardial infarction was defined by symptoms, ECG changes, and total CK

and CK-MB patterns. Troponin was not in routine use at the time of the study. During

weekdays and subject to research staff availability, consecutive eligible patients were

approached by cardiology clinical trials study nurses or by the study investigators.

Information regarding age, gender, and a history of previous cardiac disease, smoking,

diabetes mellitus (fasting > 7.8 mol/L, random > 11.1 mol/L, or on treatment),

hypercholesterolemia (total cholesterol > 5.2 mmol/L or on treatment), and hypertension (>

140/90 mm Hg. on 3 separate occasions or on treatment) were obtained from the patient

and chart. Age and gender frequency matched anonymous control sera were obtained from

outpatients seen through family practice and outpatient clinics at McMaster University

Medical Centre between May 1997 and May, 1998. No information other than age and

gender was obtained for controls. The protocol was approved by the Research Ethics

Boards at the Hamilton Health Sciences and St. Joseph's Hospitals, and informed written

consent was obtained from all case patients.

Laboratory methods

Serum obtained from cases and controls was stored at –20 °C until serologic testing,

and all assays were batch-processed to minimize freezing and thawing cycles. All antibody

measurements were performed by enzyme immunoassay for IgG antibody, with negative

and positive controls: *Chlamydia pneumoniae* (Sero-CP, Savyon Diagnostics, Kiryat Minrav,

Israel); *Helicobacter pylori* (Hycor Biomedical, Kassel, Germany), and cytomegalovirus,

adenovirus, and hepatitis A virus (DiaSorin Inc., Stillwater MN). Fewer than 214 patient

samples were processed per assay, either due to unavailability of patient samples at the time

of assay, or due to the testing in multiples of 96 with variable numbers of patient and quality

control sera. For all serologic assays, extensive validation with known positive and known

negative sera was performed prior to processing patient samples. In brief, a sample of serum

was diluted, incubated with antigen bound wells in 96-well microtitre plates, then reacted

with an enzyme-linked anti-human IgG antibody. Absorbances were measured in a

spectrophotometer. All kits were processed according to the manufacturer's package insert.

Statistical analysis

All laboratory data were dichotomized as positive or negative according to the

manufacturers' specifications. Indeterminate values were treated as negative results. We

used logistic regression modeling (SPSS for Windows v. 10.0, SPSS Inc. Chicago IL) with

case status as the dependent variable, CP, HP, CMV, ADV or HAV serostatus as the

explanatory variables, and age and gender as co-variates.

We performed a number of secondary analyses. CP absorbance results were divided into quarters and an association between sequential quarters and case/control status was sought. Student's *t*-test was used to compare the mean logarithm of CP absorbance by case and by gender. To explore the relationship between cardiovascular risk factors and serologic status, we modeled CP, HP, and CMV status by logistic regression with age, gender, hypertension, diabetes mellitus, hypercholesterolemia, and smoking status (ever/ never) entered in a forward step-wise fashion. *A priori*, a relationship between CP and heart disease was expected, but not with other infections. To account for multiple testing, an α value of 0.05 for the primary CP analysis, and 0.01 for all other analyses, was set as the level for finding a statistically significant difference.

Results

Description of patients

There were 107 case-patients recruited: 71 (66.4 %) with acute coronary syndromes, and 36 (33.6 %) with chronic angina undergoing angiography. Mean age was 60.7 years (min-max 38-81 years), and 75.7 % were male. Fifty-three (49.5 %) of patients were admitted for myocardial infarction (MI) and 18 (16.8 %) for unstable angina (UA). Medical history included the following: diabetes mellitus in 21.5 %; hypertension in 49.5 %; hypercholesterolemia in 54.2 %; current smokers 29.0 %; former smokers 49.5 %; and never smokers 21.5 %. A history of previous MI was present in 42.1 %; and of UA in 56.1 %, including 26.1 % with a history of both MI and UA. All but 2 patients (105/107, 98.1 %) had a recent or previous history of MI or UA.

Chlamydia pneumoniae serology

Serology results are reported in the Table. CP-IgG antibody was present in 85.4 % of cases and 70.3 % of controls, for a crude OR of 2.5 (P = 0.02). Adjusting for age and gender, the OR was 2.3 (95 % CI: 1.1 to 5.1, P = 0.03). Dividing CP-IgG absorbance by quartiles (see Figure 1), and using the lowest quarter as the referent group (OR = 1.0), the adjusted ORs for the 2nd, 3rd and 4th quarters were 2.8, 3.0, and 4.3 respectively (P = 0.02, 0.01, 0.001 respectively; P = 0.001 for linear trend).

The level of CP IgG antibody, as a continuous measure, was associated with gender and case status (see Figure 2). Cases had a higher CP antibody absorbance than controls (log CP-IgG: 0.34 vs. 0.18, t = 2.9, 174 degrees of freedom {df}, P = 0.004), and men had higher CP antibody absorbance than women (log CP-IgG: 0.32 vs. 0.14, t = 3.0, df = 174, P = 0.002). CP antibody absorbance was similar in patients with acute coronary syndromes and chronic symptoms (log CP-IgG: 0.33 vs. 0.36, t = 0.4, df = 94, P = 0.70).

Among the 107 cases, the association of serology and cardiovascular risk factors was examined. CP serologic status (positive/ negative) was strongly associated with male gender (OR = 9.0, 95 % CI: 2.4 to 33.6, P = 0.001) but not with age, smoking status, hypertension, diabetes mellitus or hypercholesterolemia, nor with laboratory evidence of previous exposure to HP, CMV, ADV or HAV.

Helicobacter pylori serology

Laboratory evidence of exposure to HP was demonstrated by HP-IgG antibodies in 61.7 % of the study group, including 57.0 % of cases and 66.3 % of controls, for an adjusted OR of 0.7 (95 % CI: 0.4 to 1.2, P = 0.17). HP serostatus was associated with age (OR = 1.6 per decade, 95 % CI: 1.2 to 2.1; P = 0.002) but not with gender or with a history of diabetes

mellitus, hypertension, hypercholesterolemia, or smoking. HP serology was positive more
commonly with previous exposure to HAV (adjusted OR = 2.8, 95 % CI: 1.4 to 5.6,
P = 0.003).

Cytomegalovirus serology

CMV exposure as determined by CMV-IgG antibody status was present in 64.0 % of
the study group, including 60.2 % of cases and 69.3 % of controls, (adjusted OR = 0.7, 95 %
CI: 0.4 to 1.3, P = 0.25). CMV serostatus was associated with age (OR = 2.0 per decade,
95 % CI: 1.2 to 3.3, P = 0.01) and current smoking status (OR = 7.8, 95 % CI: 2.1 to 28.6,
P = 0.002), but not with gender or with a history of diabetes mellitus, hypertension, or
hypercholesterolemia. After adjustment for age, CMV was not associated with HP or HAV.

Adenovirus serology

ADV-IgG antibody was present in 75.6 % of the study group, including 70.0 % of
cases and 79.0 % of controls, (adjusted OR = 0.6, 95 % CI: 0.3 to 1.3, P = 0.20). ADV was
associated with a history of hypertension (OR = 3.7, 95 % CI: 1.1 to 12.4, P = 0.04), but not
with age, gender, diabetes, hypercholesterolemia, smoking status, or with serologic evidence
of CP, HP, CMV or HAV infection.

Hepatitis A serology

HAV-IgG serology, indicating previous infection with hepatitis A virus, was present
in 59.2 % of the study group, including 60.4 % of cases and 58.0 % of controls, (adjusted
OR =1.1, 95 % CI: 0.6 to 2.2, P = 0.69). HAV status was strongly associated with age
(OR = 2.1 per decade, 95 % CI: 1.5 to 2.9, P < 0.001), but was not associated with gender,
diabetes, hypercholesterolemia, or smoking status.

Discussion

In previous studies, exposure to the respiratory pathogen, *Chlamydia pneumoniae*, was common among adults, with an increased prevalence in men and smokers (28-30). In our study, serologic evidence of previous CP exposure was present in 85 % of Canadian patients with heart disease, and was more common in heart disease patients than in controls, independent of age, gender, and previous exposure to other infections.

Potential limitations of our study were the choice of diagnostic test cut-point, the limited information of potential confounding factors in controls, the relatively small sample size, and temporal association. These issues will be addressed in order.

Diagnostic tests are imperfect, and controversy surrounds the appropriate cut-off points for measuring CP exposure. The gold standard for measuring CP antibody is the technically-demanding method of micro-immunofluorescence (MIF) end-point titration (31), whereas we used a commercially-available enzyme immunoassay (EIA). This assay has 95 % sensitivity and 97 % specificity, compared with MIF (manufacturer's package insert, based on laboratory data from an independent laboratory). We have found similar CP seroprevalence (78-85 %) in 3 studies with Canadian heart disease patients using two different EIAs or MIF (unpublished data).

Dividing the antibody levels into quarters indicated that higher levels were even more strongly associated with heart disease—with an OR of 4 in the highest quarter compared with the lowest quarter. At any cut-point, there was an association between CP-IgG antibody and heart disease. Thus, our results appear robust to the choice of cut-off value for defining CP seropositivity.

Our choice of controls enabled investigation for other infections, but information on potentially confounding risk factors other than age and gender was not obtained. Whereas a larger and more expensive case-control study could address that issue, a superior method is to conduct a prospective study—as we are currently doing.

The sample size was sufficient to demonstrate that CP serostatus was associated with disease, and that the other 4 infections were not. The upper 95 % confidence intervals were 1.3 or less for the associations between heart disease and HP, CMV, or ADV. Since some control patients may have had heart disease, we may have slightly under-estimated any relationship, but an OR above 1.5 is unlikely.

In a case-control design, susceptibility to infection in those with heart disease cannot be separated from susceptibility to heart disease in those with infection (temporal sequence), nor can we fully exclude confounding by smoking status or unmeasured coronary risk factors. However, we found no association between smoking status (either as current or ever smokers) and CP IgG status, making confounding by smoking status less likely. The increased prevalence of CP IgG positivity in men has been found in other studies (32), although the explanation remains unclear.

While other case-control studies have obtained similar results of an association between CP serology and heart disease (6), recent prospective studies utilizing a nested case-control design (33-35) and a meta-analysis of prospective studies (35) could find no such association between CP serology and vascular events over up to 12 years of follow-up. The meta-analysis has been criticized for over-correcting for potential confounders such as socio-economic status (36). Furthermore, CP antibody levels wane over 3 to 5 years, and re-

infection is common (28). Thus, baseline antibody levels may not be predictive over many years but still predictive over a shorter time interval.

It remains possible that the association of CP serology with heart disease is confounded by some other factor such as smoking, genetic predisposition or socio-economic status, but the presence of CP antigen and DNA within atherosclerotic plaques suggests that the association of infection with vascular disease is real (although not necessarily causal).

We found that previous exposures to HP, CMV, or ADV were no more common in patients with heart disease than in controls. The association of HP exposure and heart disease in previous studies may have been confounded by an association of both with socio-economic status. Indeed, we were able to demonstrate an association between HP and HAV status—both markers of childhood socio-economic status.

HP, CMV and HAV were all associated with age, and to some extent, with one another. These reinforce the importance of controlling for age and gender in the analyses. The association between CMV and smoking was, to our knowledge, a novel finding, and requires confirmation. The association between ADV and hypertension, however, was of borderline statistical significance and likely represents a chance finding.

In conclusion, in a case-control study of Canadian heart disease patients, we found a relationship between previous exposure to *Chlamydia pneumoniae* and acute or chronic heart disease. We are currently examining these infectious markers prospectively within the Heart Outcomes and Prevention Evaluation (HOPE) study (37), to ascertain whether exposure to these infections predicts anatomical disease progression and vascular events.

Acknowledgements

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Table 2-1. Seroprevalence of infections in over-all study group, cases and controls. Age and gender adjusted odds ratio obtained from logistic regression modeling (SPSS).

	Prevalence	Cases	Controls	OR[a]	95 % CI[b]	P-value
CP[c]	78.8 %	82/96 (85.4 %)	52/74 (70.3 %)	2.3	1.1, 5.1	0.03
HP[d]	61.7 %	57/100 (57.0 %)	67/101 (66.3 %)	0.7	0.4, 1.2	0.17
CMV[e]	64.0 %	62/103 (60.2 %)	52/75 (69.3 %)	0.7	0.4, 1.3	0.25
ADV[f]	75.6 %	46/65 (70.0 %)	78/99 (78.8 %)	0.6	0.3, 1.3	0.20
HAV[g]	59.2 %	55/91 (60.4 %)	51/88 (58.0 %)	1.1	0.6, 2.2	0.69

[a] OR, odds ratio.

[b] CI, confidence interval.

[c] CP, *Chlamydia pneumoniae.*

[d] HP, *Helicobacter pylori.*

[e] CMV, cytomegalovirus.

[f] ADV, adenovirus.

[g] HAV, hepatitis A virus.
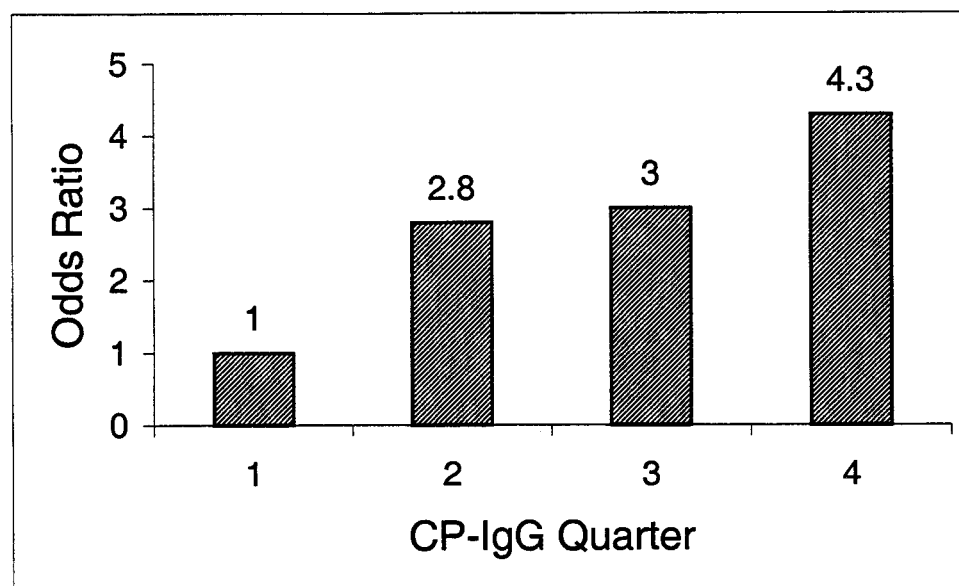
Figure 2-1. Odds ratio for heart disease by quarter for *C. pneumoniae* IgG absorbance.

CP-IgG absorbence measured by enzyme immunoassay. P = 0.001 (test for trend).
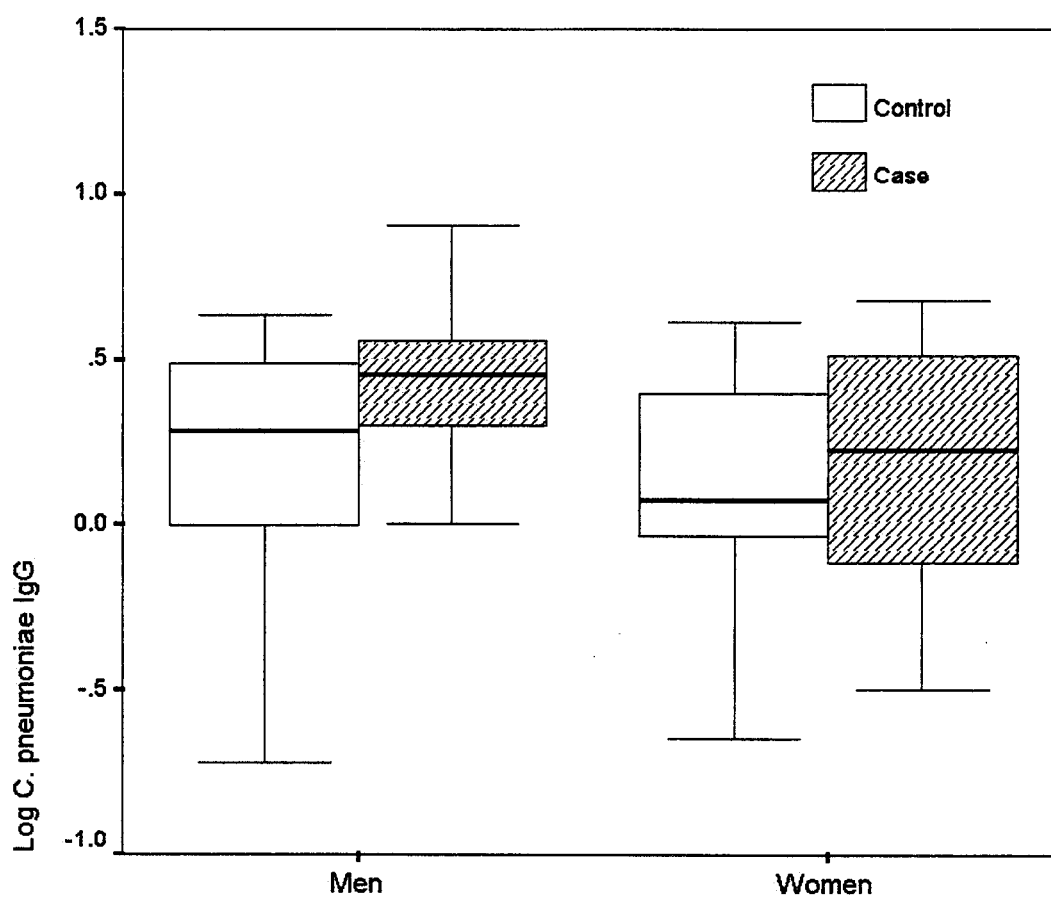
Figure 2-2. Boxplots of *C. pneumoniae* IgG serology (logarithm of absorbance) by case

status and gender. Heavy line in box indicates median; box indicates 25th to 75th

percentiles, and bars indicate 10th to 90th percentiles. Cases had higher *C. pneumoniae*

IgG levels than controls ($t = 2.90$, df = 174, P = 0.004), and men had higher levels than

women ($t = 3.01$, df = 174, P = 0.002) in a linear regression model (SPSS).

Chapter 3

A Prospective Study of Serologic Markers

In Chapters 3 and 4, I report the findings from the Inflammatory and Infectious

Markers in the HOPE study cohort (IIM-HOPE study), funded by the Heart and Stroke

Foundation of Ontario, on which I am co-investigator.

Chapter 3 presents the IIM-HOPE results of infections and cardiovascular events

among 3, 168 HOPE study patients. We found a relationship with cytomegalovirus, but not

with *C. pneumoniae*, other individual infections, or with the total burden of infectious

exposures. We also found no over-all relationship between infection and atherosclerotic

progression.

Chapter 3 is the third draft of a manuscript for submission, in February 2002, to

Circulation. The full citation is:

> **Smieja M**, Gnarpe J, Lonn E, Gnarpe H, Olsson G, Yi Q, Dzavik V, McQueen M,
> Yusuf S. Multiple infections, subsequent cardiovascular events, and atherosclerotic
> progression in the Heart Outcome and Prevention Evaluation (HOPE) study.
> Submitted to Circulation (February 2002).

I presented the results, in part, as a poster session at the Canadian Cardiovascular

Congress, October 21-24, 2001, in Halifax NS, and as an oral presentation at the American

Heart Association meeting, November 11-14, in Anaheim CA. The poster session was

awarded the Canadian Cardiovascular Society student award for best poster in the clinical

category.

Multiple Infections, Subsequent Cardiovascular Events, and Atherosclerotic

Progression in the Heart Outcome and Prevention Evaluation (HOPE) Study.

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Text: Intro 264; methods 888; results 1378; discussion 1220

Abstract:

Background—Limited prospective epidemiologic data are available on the relationship between exposure to *Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus (CMV), and hepatitis A virus (HAV), individually or as a total pathogen score, and human cardiovascular (CV) disease.

Methods and Results—We analyzed enrollment sera from 3,168 Canadian patients in the Heart Outcomes Prevention and Evaluation (HOPE) study for antibodies to *Chlamydia pneumoniae*, *Helicobacter pylori*, CMV and HAV, and measured the relationship between serostatus and 494 adjudicated trial outcomes of MI, stroke, or CV death over 4.5 years follow-up. We also examined the association between serostatus and carotid artery intimal medial thickness (IMT) at study baseline and after 4.5 years follow-up in a sub-study of 715 patients. CV events were associated with CMV serostatus (covariate-adjusted hazard ratio {HR} = 1.24, 95 % confidence interval {CI}: 1.01, 1.53). Neither *C. pneumoniae*-IgG (adjusted HR = 0.87, 95 % CI: 0.68, 1.10), *C. pneumonia*-IgA (adjusted HR = 1.10, 95 % CI: 0.90, 1.34), *H. pylori* IgG (HR = 0.99, 95 % CI: 0.82, 1.19), nor HAV IgG (HR = 1.01, 95 % CI: 0.83, 1.24) predicted CV events. Total pathogen score was not associated with CV events (adjusted HR for 4 versus 1 or 0: 1.16, 95 % CI: 0.83, 1.62). Infections were not clearly associated with the extent or progression of carotid IMT.

Conclusions—Exposure to CMV, but not to *C. pneumoniae*, *H. pylori*, or HAV, was associated with a small excess risk of subsequent CV events in HOPE study patients. Total pathogen score did not predict increased vulnerability to MI, stroke or CV death.

Condensed Abstract

There are limited prospective data on the relationship between exposure to multiple infections and subsequent cardiovascular events. Among 3,168 HOPE study patients, baseline antibody to *C. pneumoniae*, *H. pylori*, cytomegalovirus and hepatitis A virus was measured. Cytomegalovirus was associated with a small excess risk of CV events during 4.5 years of follow-up, whereas the remaining infections were not. A total pathogen score was not associated with subsequent CV risk, and no infection was clearly associated with greater extent or progression of carotid artery atherosclerosis.

Key Words: Infection, cardiovascular diseases, atherosclerosis, prognosis

Introduction

A possible role for infections in atherosclerosis of the coronary or carotid arteries has been intensely scrutinized since the demonstration of herpesvirus-induced atherosclerosis in chickens in 1978(1). Human atherosclerotic heart or cerebro-vascular disease has been associated with previous exposure to the bacteria *Chlamydia pneumoniae*(2;3), *Helicobacter pylori*(4), or *Porphyromonas gingivalis*(5;6), and with the viruses cytomegalovirus (CMV)(7), herpes simplex virus type 1 and 2 (HSV-1, HSV-2)(8;9), enteroviruses(10), or hepatitis A virus (HAV)(9;11), but prospective studies remain limited.

Over the last three decades, attention has focused on the individual contributions of these infections to atherosclerosis. The herpesviruses (HSV and CMV) and the obligate intra-cellular bacterium *C. pneumoniae* have been studied in animal models and in cross-sectional, prospective, and pathologic human studies(12-14). Large randomized trials of antibiotics for secondary prevention of cardiovascular (CV) disease are ongoing(15;16).

Recently, Zhu and colleagues proposed and demonstrated that the aggregate sum of infectious exposures, expressed as a total pathogen burden, was a stronger prognostic marker than individual infections in cross-sectional(17)and prospective studies(9). Rupprecht and colleagues also found a prospective relationship between pathogen burden and CV outcome(18).

Our primary study objective was to determine whether exposure to *C. pneumoniae* and to three other infections, individually or as a total pathogen score, were a prospective risk marker for CV events among patients with pre-existing CV disease or at high risk of disease. Secondary objectives were to explore the relationship between infections and CV risk

factors, and to examine the relationship between infections and the extent and progression of carotid artery atherosclerosis.

Methods

Description of the HOPE study and SECURE sub-study

The Heart Outcomes Prevention and Evaluation (HOPE) study was a multi-centre randomized clinical trial of ramipril, vitamin E, both or neither for the prevention of CV events among 9,541 patients with previous coronary artery disease, stroke, peripheral vascular disease, or high risk diabetes (19;20). Baseline enrollment blood samples from 3,168 Canadian HOPE study patients were stored at the HOPE central laboratory in Hamilton ON Canada.

In the Study to Evaluate Carotid Ultrasound with Ramipril and Vitamin E (SECURE), a HOPE sub-study, 732 patients were randomized to one of two doses of ramipril, or to placebo, and to vitamin E or placebo, and had serial 12-segment mean maximal carotid intimal medial thickness (IMT) measured at study baseline and at 2 and 4.5 years, as reported previously (21). For 715 patients, baseline sera and baseline ultrasound measurements were available, and for 679 of these, follow-up ultrasound studies were also available.

The HOPE, SECURE, and Inflammatory and Infectious Markers in HOPE study protocols were approved by the Research Ethics Board at McMaster University and the Hamilton Health Sciences, and written informed consent was obtained.

Serologic methods: Chlamydia pneumoniae

All serologic testing was performed by staff blinded to clinical outcomes. Serum was stored at −70 °C until ready for testing, and batch assayed for *C. pneumoniae* after initial

thawing. All remaining assays were performed after one additional freeze-thaw cycle. Serum

was assayed for *C. pneumoniae* IgG and IgA antibodies by microimmunofluoresence (MIF)

end-point titration as described previously (22;23). Doubling dilutions of sera in PBS pH 7.4

were incubated overnight at 4°C with *C. pneumoniae* antigen (LabSystems Oy, Helsinki,

Finland), conjugated for 30 minutes at 37°C with FITC-conjugated anti-human IgG or IgA

(Dakopatts, Denmark). All sera found to be positive for IgA in a screening test were treated

with GullSorb (Gull Laboratories, USA) to remove IgG antibodies, then retested. Slides

were read using a Zeiss microscope with a UV light source at 400X magnification by one

experienced microbiologist (JG). All tests were run using strict quality control and test runs

accepted only if the high and low titer IgG and IgA controls were within one titer step of the

predetermined values. Antibody levels were expressed as inverted titers. *A priori*, previous

exposure to *C. pneumoniae* was defined by IgG $\geq$ 32 or IgA $\geq$ 16, and recent exposure was

defined as IgG $\geq$ 512 or IgA $\geq$ 64.

Helicobacter pylori, cytomegalovirus, and hepatitis A virus

Serum IgG antibodies to *Helicobacter pylori*, CMV, and HAV were determined in 3135,

3153, and 3128 patients, respectively, using 96-well microtitre plate enzyme immunoassay

(EIA) and an automated washer and reader (Biotek Instruments Inc, Winooski VT). Fewer

than 3168 assays were performed due to inadequate blood volume, and were considered

missing completely at random. *H. pylori* IgG antibody were measured by Hycor HP (Hycor

Biomedical, Kassel, Germany). Results of > 40 arbitrary units (AU) were considered positive,

and values of 27-40 AU were considered indeterminate. CMV IgG antibody was measured

by using a quantitative CMV-IgG assay (DiaSorin, Saluggia, Italy). The assay incorporates

four control calibration sera set to proposed World Health Organization reference standards (1995), and results of > 0.4 International Units (IU)/mL were considered positive. HAV-IgG was determined by a qualitative assay (DiaSorin, Stillwater MN), and results of > 20 mIU/mL were considered positive. In all assays, we used cut-off values recommended by the manufacturers.

Statistical analysis

The primary study outcome measure was the HOPE study primary event cluster of incident MI, incident stroke or CV death. Secondary outcomes were MI alone, stroke alone, or the primary event cluster combined with revascularization procedures.

Laboratory results were dichotomized as positive/ negative for primary analysis for all assays except *H. pylori* IgG. Indeterminate values for CMV and HAV were 0.3 % and 2.3 %, respectively, and classified as negative. For *H. pylori*, results were analyzed as negative, indeterminate, or positive, as the indeterminate category included 5.1 % of patients. Kaplan-Meier time-to-event curves were plotted for the cohort of 3,168 patients, and serostatus tested by log-rank P. In addition, simple and multiple Cox proportional hazards modeling was performed in SAS 6.02 adjusting simultaneously for age, gender, smoking status (current, former or never), ramipril randomization, diabetes mellitus, hypertension, and history of hypercholesterolemia. For *C. pneumoniae*, a relationship between higher antibody titres and CV events was sought. In addition, interactions were sought between *C. pneumoniae* and smoking. For total pathogen score, 0 to 4 was assigned according to the individual patient's seropositivity to *C. pneumoniae* (IgA $\geq$ 16), *H. pylori* (> 40 AU), CMV (> 0.4 IU/mL), or HAV (> 20 mIU/mL).

Baseline carotid IMT was calculated as the mean of 12 arterial thickness measurements. Carotid IMT progression was measured as the slope of the mean maximum IMT for each patient from the five serial ultrasounds, by least squares regression, after verifying the absence of significant deviations from linearity. The baseline carotid IMT and carotid IMT progression were used as the dependent variable (log-transformed) in a linear regression model. Serostatus for *C. pneumoniae* IgG or IgA, *H. pylori*, CMV and HAV were entered as independent dichotomous variables. An adjusted model with age, gender, smoking, and ramipril was examined.

SAS 6.02 (SAS Institute, Cary NC) was used for all analyses. An alpha level of 0.05 was set as the level of significance for the primary hypothesis of *C. pneumoniae* serology and the primary CV outcome, and 0.01 for all other infections and for sub-group analyses, to account for multiple testing.

Results

Patient description

The patient characteristics among the 3,168 patients were similar to those in the over-all HOPE study, which has been described in detail previously (19). Patients had mean age 65.4 year; 77.6 % were male; 13.9 % were current smokers, 63.0 % former smokers, and 23.1 % never smokers; 34.2 % had diabetes, 41.4 % had hypertension; 49.0 % had a history of hypercholesterolemia; 57.9 % had a previous MI; 9.5 % had a previous stroke or transient ischemic attack, 16.9 % had peripheral vascular disease; and 9.6 % had no previous vascular event. In addition to randomization to ramipril, vitamin E, both or neither, patients were taking other CV medications including ASA (79.0 %), lipid-lowering drugs (40.1 %), beta-blockers (43.4 %) and calcium channel blockers (18.3 %). The patient characteristics among

the 732 SECURE patients were similar to those in the over-all HOPE study, and have been

described in detail elsewhere (19;21;24).

Cardiovascular Events

For patients with sera available, the adjudicated primary event cluster of incident MI,

stroke, or CV death occurred in 494 of 3,168 patients (15.6 %) during a mean follow-up of

4.5 years. MI alone occurred in 364 patients (11.5 %), stroke alone in 107 (3.4 %), and the

composite measure of MI, stroke, CV death or revascularization in 980 (30.9 %).

Chlamydia pneumoniae and CV events

C. pneumoniae IgG antibodies, at a pre-defined reciprocal titre of $\geq$ 32, were present in

2,627 of 3,168 (82.9 %) patients. By time-to-event analysis, *C. pneumoniae* IgG serostatus was

not associated with CV outcome (Log rank test = 0.86). Using Cox proportional hazards

models (Table 1), an unadjusted hazard ratio (HR) of 0.90 (95 % CI: 0.71, 1.13) was

obtained, and an adjusted HR of 0.87 (0.68, 1.10) after accounting for co-variates (age,

gender, smoking, ramipril assignment, diabetes mellitus, hypertension and

hypercholesterolemia). *C. pneumoniae* IgG $\geq$ 32 was not associated with the secondary end-

points of MI alone, stroke alone, or the primary end-point with revascularization (Table 1).

To examine the influence of different antibody levels, *C. pneumoniae* IgG titres were divided

approximately into quarters (Figure 1A, Log rank test = 0.16 for 4th versus 1st quarter). No

association was found at any level of IgG antibody titre and CV outcomes (data not shown).

C. pneumoniae IgA antibodies, at a pre-defined cut-off titre of $\geq$ 16, were present in

1,995 of 3,168 patients (63.0 %). There was no clear relationship between *C. pneumoniae* IgA

serostatus and CV outcomes (Log rank test = 0.13). Using Cox proportional hazards

modeling (Table 1), there was no association after co-variate adjustment for either the primary outcome, or for any of the secondary outcomes. Dividing *C. pneumoniae* IgA titres by titres, a relationship was sought between higher antibody titres and CV (Figure 1B and Table 1), with no clear relationship with higher titres (log rank test = 0.09). *C. pneumoniae* IgA titres of ≥ 512, which represented 9.5 % of the patients, were associated with an unadjusted HR of 1.39 (1.04, 1.88) for the primary outcome and 1.34 (1.06, 1.61) for incident MI alone, but neither of these associations was statistically significant after covariate adjustment.

To measure recent exposure to *C. pneumoniae*, a composite measure of *C. pneumoniae* IgG titre ≥ 512 or *C. pneumoniae* IgA titre of ≥ 64 was examined (Table 1). There was a weak relationship between this composite measure of *C. pneumoniae* exposure and the primary event cluster of MI, stroke, or CV death: unadjusted HR = 1.19, (95 % CI: 1.00, 1.42), adjusted HR = 1.11 (95 % CI: 0.92, 1.34).

The association of CV risk factors with serologic status was sought by multiple logistic regression, with serostatus as the dependent variable (see Table 2). Recent exposure to *C. pneumoniae*, as defined by the composite antibody measure above, was strongly associated with male gender (odds ratio {OR}= 1.69, 95 % CI: 1.42, 2.01, P < 0.0001) and with smoking status (OR {current versus never}= 1.93, 95 % CI: 1.51, 2.47, P < 0.001; OR {former versus never}= 1.52, 95 % CI: 1.28, 1.82, P < 0.001), but not with age, diabetes mellitus, hypercholesterolemia or hypertension.

Interaction between C. pneumoniae and Smoking

We sought an interaction between *C. pneumoniae* IgA titres and current smoking

status by including smoking-by-serology interaction terms in the Cox models. Interaction

terms were statistically significant (data not shown). Among 2,727 former or never smokers,

C. pneumoniae IgA ≥ 512 was associated with an adjusted HR of 1.59 (95 % CI: 1.14, 2.22) for

the primary study outcome, compared with 1.21 (0.98, 1.49) for IgA 32-256 and 1.00 for

IgA < 32. Among 441 current smokers, *C. pneumoniae* IgA was not associated with CV

outcomes: adjusted HR (IgA ≥ 512) = 0.69 (95 % CI: 0.35, 1.34); HR (IgA 32-256) = 0.70

(95 % CI: 0.46, 1.08).

Helicobacter pylori

H. pylori IgG antibody (> 40 AU) was present in 1,934 of 3,135 patients (61.7 %),

and indeterminate (27-40 AU) in 5.1 %. *H. pylori* serostatus was not associated with CV

outcome (Figure 2A and Table 1), with an HR of 0.99 (0.82, 1.19). *H. pylori* serostatus was

not associated with MI alone, stroke alone, or the primary outcome measure with

revascularization. By multiple logistic regression (Table 2), *H. pylori* IgG serostatus was

related to age (OR = 1.26 per decade) and with smoking status (OR = 1.93 and 1.42,

respectively, for current and former smokers, all P < 0.001), but not with gender or with

other CV risk factors.

Cytomegalovirus

CMV IgG antibody > 0.4 IU/mL was present in 2,220 of 3,153 patients (70.4 %).

CMV serostatus was associated with an excess of CV events (log-rank test = 0.03, Figure

2B). In Cox proportional hazards models (Table 1), CMV serostatus was associated with an

unadjusted HR of 1.26 (95 % CI: 1.03, 1.54, P = 0.02), and an adjusted HR of 1.24 (1.01, 1.53, P = 0.04). CMV was associated with the outcomes of MI alone, and with the primary events combined with revascularization. CMV IgG seropositivity (Table 2) was associated with male gender (OR = 2.01) and age (OR = 1.23 per decade, both P < 0.001), but not with smoking or with other CV risk factors (Table 2).

Hepatitis A virus

Hepatitis A virus IgG antibody was present in 2,377 of 3,128 patients (76.0 %). HAV serostatus was not associated with CV events (log-rank test = 0.33, Figure 2C). In Cox models (Table 1), HAV was not associated with the primary outcome (HR = 1.01, 95 % CI: 0.83, 1.24), or with any of the secondary outcomes. HAV IgG antibody was more frequent with older age (OR = 1.23 per decade, P < 0.001), and with male gender (OR = 1.28, P = 0.04), but not with gender, smoking, or other CV risk factors (Table 2).

Total pathogen score

A total pathogen score consisting of one point each for *C. pneumoniae* status (IgA $\geq$ 16), *H. pylori* status (> 40 AU), CMV (> 0.4 IU/mL), and HAV (> 20 mIU/mL), (total 0 to 4 points), was examined for an association with CV events (Figure 3 and Table 3). The unadjusted event rates were associated with increasing total pathogen score (X^2 for trend = 5.26 on 1 degree of freedom, P = 0.02). As only nine events occurred in the group with a pathogen score of 0, groups 0 and 1 were combined for regression models. In Cox models, pathogen scores of 2, 3 or 4 were associated with a small, non-statistically significant excess hazard rate, and this was further attenuated on covariate-adjustment. The highest pathogen score was associated with an adjusted HR of 1.16 (95 % CI: 0.83, 1.62) for CV events, compared with a burden score of 0 or 1. Similarly, no relationship between total

pathogen score and the secondary end-points of MI alone, stroke alone, or the primary end-point combined with revascularization procedures.

Infections and Carotid IMT

Neither *C. pneumoniae* IgG ≥ 32, IgA ≥ 16, nor the composite measure of IgG ≥ 512 or IgA ≥ 64, was associated with either baseline carotid IMT or with progression of carotid IMT over 4.5 years (Table 4). The highest antibody titres (≥ 512) of either IgG or IgA were associated with baseline extent of carotid IMT, but this association was not statistically significant after adjusting for age, gender, smoking, and ramipril allocation. There was no association between antibody titre and 4.5 year IMT progression. Similarly, *H. pylori* IgG, CMV IgG, or HAV IgG serostatus was not associated with either the extent, or 4.5-year progression, of carotid IMT (Table 4).

Discussion

In a large cohort of clinical trial patients with previous CV events or at high risk of events, we determined the prognosis associated with exposure to a number of infections that had been previously associated with human atherosclerotic disease. We found a modest association between trial-adjudicated CV events and CMV serostatus, but no consistent association with exposure to *C. pneumoniae*, *H. pylori*, or hepatitis A virus. Furthermore, we found no association with total pathogen score, nor between any of the infections and either the extent, or progression, of carotid atherosclerosis.

In a recent meta-analysis, Danesh and colleagues found a total of 15 prospective studies including 3,169 cases, consisting primarily of case-control studies nested within primary prevention cohorts (13). They estimated a pooled, covariate-adjusted odds ratio of

1.15 (95 % CI: 0.97, 1.36) for the association between *C. pneumoniae* IgG and cardiac events. Our study results are compatible with this meta-analysis, with an estimated CV risk of 0.87 for IgG and 1.10 for IgG or IgA, respectively, and complement existing data in three important ways. First, the HOPE patients primarily had pre-existing disease, and were at high risk for CV events compared with the community studies. Thus, they predominantly represented a secondary prevention cohort, and firmly establish that *C. pneumoniae* antibodies have little prognostic value in patients with established CV disease. Second, we carried out a cohort study, rather than a nested case-control, with time-to-event data. We were thus able to estimate the influence of serologic and cardiovascular variables with considerably more precision than previous studies. Third, we also measured *C. pneumoniae* IgA, which has been used in few previous prospective studies. *C. pneumoniae* IgA may be a better marker for recent exposure to infection, and *C. pneumoniae* IgA titres, or a combination of high titre IgG and IgA, may be better predictors of "chronic chlamydial infection". While we found a modest relationship with *C. pneumoniae* IgA at high titre and among former or never smokers, these associations were not robust to co-variate adjustment. Thus, neither chlamydial IgG, IgA, or a combination of the two were independently associated with subsequent CV events.

Our findings demonstrate that chlamydial antibodies had no prognostic value in high-risk patients, but do not prove that *C. pneumoniae* plays no role in atherogenesis, atherosclerotic progression, or the acute coronary syndromes. Most HOPE study patients had pre-existing CV disease, hence our study cannot examine the role of these infections in the genesis of atherosclerosis and first CV events. While we found no independent association between *C. pneumoniae* antibodies and CV events, infections may be co-factors together with established CV risk factors. We found that chlamydial antibodies were

significantly more common among men and smokers, validating associations noted by others (25;26). We specifically sought an interaction between *C. pneumoniae*, smoking, and CV events, hypothesizing synergy between smoking and infection. While the interaction term was statistically significant, the relationship between *C. pneumoniae* IgA and CV events persisted only in former smokers or never smokers. Further analysis showed this risk only among former smokers (data not shown). This may simply represent a spurious sub-group analysis, but two other explanations are possible. Higher chlamydial titres may relate to a more recent cessation of smoking, and hence a higher residual risk of smoking-associated CV disease. Thus, smoking may be a confounding factor in the *C. pneumoinae*-heart disease association, as originally suggested by Hahn (25). Alternatively, smoking and chlamydial infection may be part of the same causal pathway, such that no excess risk is associated with chlamydial infection after controlling for smoking.

In this study, we also measured CMV, *H. pylori*, and HAV antibodies, with the *a priori* expectation that no association would be demonstrated. Thus, the modest association between CMV and CV disease may be spurious, and indeed the P-value of 0.03 did not cross our threshold of P < 0.01 for a secondary analysis. However, the result was robust to adjustment for CV risk factors, and in keeping with other reports of an association between CMV and native vessel or post-transplant atherosclerosis (27). In a companion manuscript, we measured four inflammatory markers in this same cohort of patients (Smieja et al, unpublished), and found that fibrinogen and soluble Intercellular Adhesion Molecule-1 (sICAM-1) were associated with CV events. CMV status remained statistically significantly associated with CV events when added to the fully-adjusted model including inflammatory,

clinical, and metabolic co-variates (HR = 1.25, 95 % CI: 1.00, 1.55, P = 0.048), indicated that CMV status was independent of inflammation as measured by these markers.

H. pylori, a major cause of peptic ulcers and gastritis, was originally associated with CV disease in case-control studies (4), but no independent role could be verified in prospective nested case-control studies (28;29). Our study validates these negative studies. We believe that further epidemiologic studies of *H. pylori* and CV disease are unlikely to be fruitful.

Hepatitis A virus is a hepatotropic RNA virus that affects the majority of the world's population, and is a self-limited illness, usually in early childhood. HAV is a strong measure of childhood socio-economic status, since infection is common in childhood, and transmission is predominantly by the fecal-oral route. We (30) and others (31) used HAV antibody testing as a "serologic control" in CV studies. We previously found no association between HAV serostatus and coronary disease in a case-control study of Canadian patients (30), and found no association in this study. These findings are at odds with two studies by Zhu and colleauges (11). HAV may be a proxy for other CV risk factors or for childhood socio-economic status, and the ethnic heterogeneity of our study patients compared with the Utah cohort may explain the differences between studies. Further data are needed.

We examined the hypothesis that total pathogen burden, rather than exposure to any single infection, was associated with CV disease. Zhu et al found that a pathogen score of 0 to 6, including *C. pneumoniae*, *H. pylori*, HAV, CMV, and HSV types 1 and 2, predicted incident MI and death in a cohort of Utah angiography patients (9). Rupprecht et al found that a score of > 5 of 8 bacteria or viruses, also including Epstein-Barr virus (EBV), and *Mycoplasma pneumoniae* and *Hemophilus influenzae*, compared with a score of 3 or fewer,

predicted 12.6 % CV mortality versus 3.7 % in a cohort of 1018 patients undergoing

coronary angiography (18). In our study, a total pathogen score based on the first 4 of these

infections did not predict CV outcome after adjustment for clinical co-variates. HOPE

study patients were likely more heterogeneous than the Utah cohort, and the additional viral

serologies that we did not measure were important component of those scores. To test this

hypothesis, we examined HSV-1 antibodies in a nested case-control sub-group of 772

HOPE patients (Smieja et al 2001, unpublished). We found that the addition of HSV-1

serostatus to the other four infections may improve the strength of the association (adjusted

odds ratio = 1.77 for score of 5 vs. 0 and 1 combined, 95 % CI: 0.83, 3.79). Examination of

the total pathogen burden concept is required in other populations, alongside investigations

to determine whether antibody represents non-specific immune stimulation, or specific viral

or bacterial re-infection, re-activation, or persistence.

Last, we found no association between the four infections and either the extent, or

the progression, of carotid atherosclerosis as measured by a highly-reproducible measure of

carotid IMT. Our results are compatible with the cross-sectional study of Markus et al (32)

who found no over-all relationship between *C. pneumoniae* IgG or IgA and carotid IMT

among 983 apparently health people. The Atherosclerosis Risk in Communities study

investigators found a relationship only among younger patients (33). Our data did not

address a role for these infections in younger patients without pre-existing disease.

We conclude that, among patients with pre-existing CV disease at high risk for CV

events, *C. pneumoniae*, *H. pylori*, and HAV antibodies, alone or in aggregate, were not

associated with future CV events, whereas CMV IgG serostatus had a modest association.

No infections was associated with progression of atherosclerosis.

Acknowledgements

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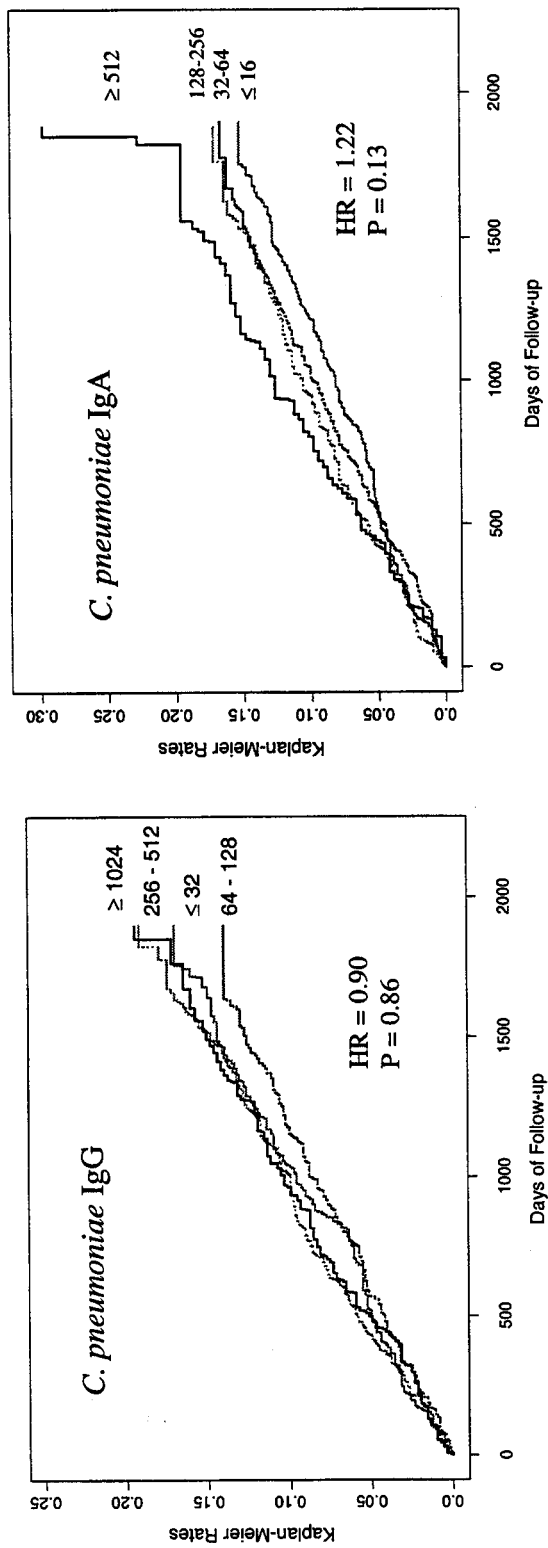
Figure 3-1: Kaplan-Meier plots for cumulative cardiovascular events by titre levels of *C. pneumoniae* IgG (left panel) and IgA (right panel) among 3,168 Canadian HOPE study patients. Hazard ratio (HR) for highest versus lowest titres.
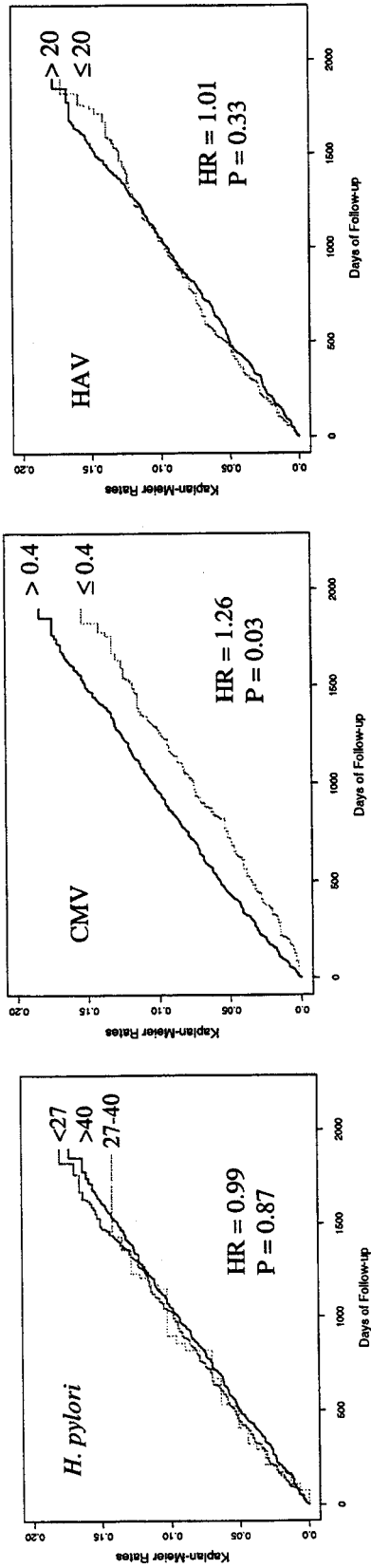
Figure 3-2: Kaplan-Meier plots for cumulative cardiovascular events by presence or absence of IgG antibody to *H. pylori* (left panel), cytomegalovirus (CMV, middle panel), and hepatitis A (HAV, right panel) among 3,128 to 3,153 Canadian HOPE study patients. Hazard ratio (HR) for antibody positive versus negative.
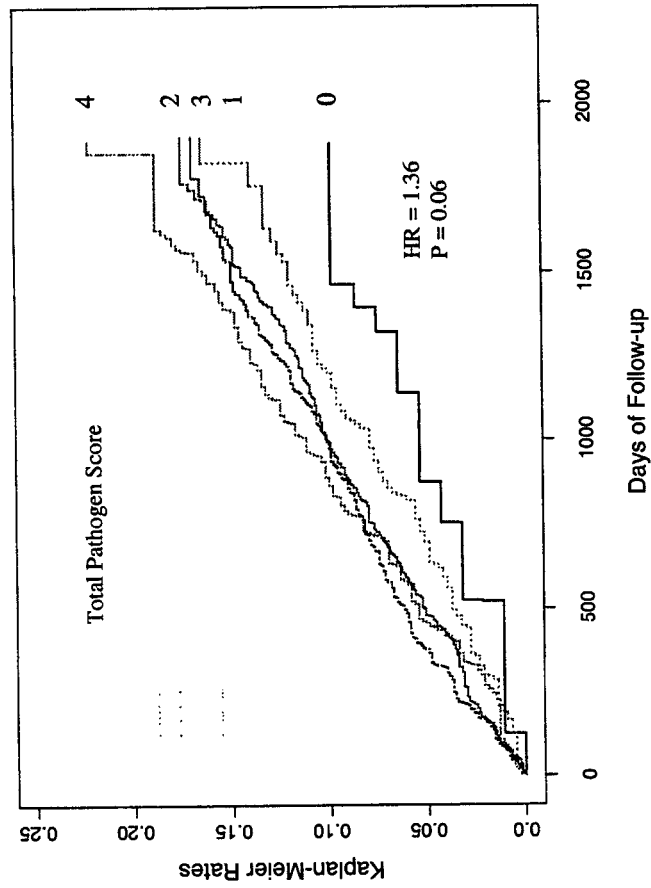
Figure 3-3. Kaplan-Meier plots for cumulative cardiovascular events by total pathogen score (*C. pneumoniae* IgA $\geq$ 64; *H. pylori* > 40 AU; CMV > 0.4 mIU/mL; and HAV > 20 IU/mL) among 3,168 Canadian HOPE study patients. Hazard ratio (HR) for pathogen score of 4 versus 0 or 1.

Table 3-1: Hazard ratios for 4.5-year cardiovascular outcomes by serostatus to *C. pneumoniae*, *H. pylori*, cytomegalovirus and hepatitis A virus among 3,168 HOPE study patients.

Serology	Prevalence (%)	Hazard Ratio (95 % Confidence Interval) for Outcome			
		Primary Outcome (MI, stroke, or CV death)	Myocardial infarction	Stroke	MI, Stroke, CV death or revascularization
Number of Events (%)		494 (15.6)	365 (11.4)	107 (3.4)	905 (28.6)
CP[a]-IgG ≥ 32	2627 / 3168 (82.9)	0.90 (0.71, 1.13)	1.02 (0.78, 1.34)	0.61 (0.40, 0.95)	0.96 (0.81, 1.13)
Adjusted [b]		0.87 (0.68, 1.10)	0.96 (0.72, 1.27)	0.66 (0.41, 1.05)	0.90 (0.76, 1.08)
CP-IgA ≥ 16	1995 / 3168 (63.0)	1.22 (1.01, 1.47)[f]	1.25 (1.01, 1.56)[f]	0.92 (0.62, 1.36)	1.06 (0.92, 1.21)
Adjusted		1.10 (0.90, 1.34)	1.11 (0.89, 1.41)	0.92 (0.61, 1.40)	0.96 (0.84, 1.11)
CP-IgG ≥ 512 or IgA ≥ 64	1616 / 3168 (51.0)	1.19 (1.00, 1.42)	1.31 (1.06, 1.61)[f]	0.95 (0.65, 1.39)	1.05 (0.92, 1.20)
Adjusted		1.11 (0.92, 1.34)	1.21 (0.98, 1.51)	0.95 (0.64, 1.42)	0.99 (0.86, 1.13)
CP-IgA < 32	1250 / 3168 (39.5)	1.00	1.00	1.00	1.00
CP-IgA 32-256	1618 / 3168 (51.1)	1.11 (0.92, 1.34)	1.16 (0.93, 1.45)	0.84 (0.56, 1.26)	0.99 (0.87, 1.15)
Adjusted		1.06 (0.87, 1.29)	1.08 (0.86, 1.35)	0.81 (0.54, 1.22)	0.94 (0.82, 1.09)
CP-IgA ≥512	300 / 3168 (9.5)	1.39 (1.04, 1.88)[f]	1.34 (0.94, 1.91)	1.15 (0.61, 2.17)	1.26 (1.01, 1.57)[f]
Adjusted		1.22 (0.90, 1.66)	1.18 (0.82, 1.68)	1.08 (0.57, 2.06)	1.14 (0.91, 1.43)
CMV[c] (> 0.4 IU/mL)	2220 / 3153 (70.4)	1.26 (1.03, 1.54)[f]	1.32 (1.04, 1.67)[f]	1.04 (0.68, 1.58)	1.15 (0.99, 1.33)
Adjusted		1.24 (1.01, 1.53)[f]	1.33 (1.04, 1.58)[f]	0.93 (0.61, 1.42)	1.17 (1.01, 1.36)[f]
HP[d] (> 40 AU)	1934 / 3135 (61.7)	0.99 (0.82, 1.19)	0.97 (0.78, 1.20)	1.02 (0.69, 1.51)	1.03 (0.90, 1.18)
HAV[e] (> 20 mIU/mL)	2377 / 3128 (76.0)	1.01 (0.83, 1.24)	1.00 (0.79, 1.27)	1.02 (0.65, 1.60)	0.99 (0.85, 1.15)

[a] CP, *C. pneumoniae*

[b] Adjusted for age, gender, smoking, ramipril, diabetes, hypertension, hypercholesterolemia

[c] HP, *H. pylori*; [d] CMV, cytomegalovirus; [e] HAV, hepatitis A virus. [f] P < 0.05. All other P > 0.05.

Table 3-2: Cardiovascular determinants of *C. pneumoniae*, *H. pylori*, cytomegalovirus and hepatitis A virus serostatus among 3,168 HOPE study patients.

Serology	Odds ratio (95 % Confidence Interval)[a]			
	C. pneumoniae	*H. pylori*	Cytomegalovirus	Hepatitis A virus
Threshold	IgG ≥ 512 or IgA ≥ 64	IgG > 40 AU	IgG > 0.4 IU/mL	IgG > 20 mIU/mL
Number positive / total (%)	1616 / 3168 (51.0)	1934 / 3134 (61.7)	2220 / 3152 (70.4)	2337 / 3127 (76.0)
Age (per decade)	1.11 (1.00, 1.23)	1.26 (1.13, 1.41)[c]	1.23 (1.10, 1.39)[c]	1.23 (1.08, 1.41)[d]
Gender (male)	1.69 (1.42, 2.01)[c]	1.01 (0.84, 1.21)	2.01 (1.62, 2.50)[c]	1.28 (1.04, 1.58)[e]
Current Smoking	1.93 (1.51, 2.47)[c]	1.93 (1.49, 2.49)[c]	0.96 (0.74, 1.26)	1.10 (0.83, 1.45)
Former Smoking	1.52 (1.28, 1.82)[c]	1.42 (1.19, 1.69)[c]	0.98 (0.80, 1.19)	1.14 (0.93, 1.40)
Diabetes mellitus	0.98 (0.84, 1.14)	0.97 (0.83, 1.14)	1.19 (1.00, 1.41)	1.07 (0.91, 1.27)
Hypercholesterolemia[b]	1.03 (0.89, 1.18)	1.12 (0.97, 1.30)	0.88 (0.75, 1.03)	1.07 (0.91, 1.27)
Hypertension	1.00 (0.86, 1.16)	0.97 (0.83, 1.14)	1.04 (0.89, 1.22)	1.07 (0.91, 1.25)

[a] Odds ratio for association between CV risk factors and seropositivity for infections, by multiple logistic regression (SAS).

[b] History of fasting cholesterol ≥ 5.2 μmol/L

[c] $P < 0.001$; [d] $P < 0.01$; [e] $P < 0.05$.

Table 3-3: Hazard ratios for 4.5-year cardiovascular outcomes by total pathogen score among 3,168 HOPE study patients.

Pathogen score[a]	Total (%)[b]	Hazard Ratio[c] (95 % Confidence Interval) for Outcome			
		Primary outcome (MI, stroke, or CV death)	Myocardial infarction	Stroke	Primary outcome or Revascularization
Total	494 / 3168 (15.6)				
Score = 0	9 / 94 (9.6)	1.00[d]	1.00	1.00	1.00
Score = 1	57 / 433 (13.2)	1.00[d]	1.00	1.00	1.00
Score = 2 unadjusted	153 / 958 (16.0)	1.25 (0.91, 1.73)	1.23 (0.85, 1.78)	0.82 (0.43, 1.57)	1.14 (0.91, 1.43)
Adjusted[e]		1.21 (0.88, 1.66)	1.19 (0.83, 1.72)	0.79 (0.41, 1.51)	1.13 (0.90, 1.41)
Score = 3 unadjusted	178 / 1148 (15.5)	1.28 (0.94, 1.74)	1.23 (0.87, 1.76)	1.01 (0.55, 1.83)	1.13 (0.92, 1.41)
Adjusted		1.21 (0.89, 1.65)	1.18 (0.83, 1.60)	0.92 (0.51, 1.68)	1.10 (0.89, 1.37)
Score = 4 unadjusted	97 / 535 (18.1)	1.36 (0.98, 1.90)	1.38 (0.95, 2.01)	1.03 (0.53, 1.98)	1.16 (0.92, 1.47)
Adjusted		1.16 (0.83, 1.62)	1.20 (0.82, 1.76)	0.83 (0.43, 1.61)	1.07 (0.85, 1.36)

[a] Pathogen burden score assigned 1 point each for: *C. pneumoniae* IgA $\geq$ 64, *H. pylori* > 40 AU, CMV > 0.4 IU/mL, or HAV > 20 IU/mL.

[b] Unadjusted X^2 for linear trend: 5.26 (df = 1), P = 0.02

[c] Numbers denote hazard ratios (HR) with 95 % confidence intervals for association between total pathogen score and cardiovascular events (Cox proportional hazards models, SAS). All P > 0.05.

[d] Categories 0 and 1 combined due to low number of events for score = 0.

[e] Adjusted for age, gender, smoking, diabetes mellitus, hypercholesterolemia, hypertension, and ramipril allocation.

Table 3-4. Serologic markers, baseline extent, and 4.5-year progression of carotid intimal medical thickness (IMT) among 679 to 715 SECURE study patients.

Antibody	Prevalence (%)	Baseline Carotid IMT[a] (mm), n = 715			Slope Change in Carotid IMT (mm/year), n = 679		
		Risk Factor Absent	Unadjusted (SE)	Adjusted[b] (SE)	Risk Factor Absent	Unadjusted (SE)	Adjusted[b] (SE)
CP[c]-IgG ≥ 32	570 of 715 (79.7)	1.13	1.16 (0.03)	1.14 (0.03)	0.018	0.018 (0.004)	0.017 (0.004)
CP-IgA ≥ 16	469 of 715 (65.6)	1.14	1.16 (0.03)	1.09 (0.03)	0.014	0.019 (0.003)	0.019 (0.003)
CP-IgG ≥ 512 or IgA ≥ 64	406 of 715 (56.8)	1.08	1.13 (0.03)	1.12 (0.02)	0.014	0.019 (0.003)	0.018 (0.003)
CP-IgG < 32	145 of 715 (20.3)	1.13	1.13	1.13	0.018	0.018	0.018
CP-IgG 32-64	180 of 715 (25.2)	1.13	1.14 (0.04)	1.10 (0.04)	0.018	0.016 (0.005)	0.016 (0.005)
CP-IgG 128-256	239 of 715 (33.4)	1.13	1.14 (0.04)	1.06 (0.03)	0.018	0.018 (0.004)	0.017 (0.004)
CP-IgG ≥ 512	151 of 715 (21.1)	1.13	1.22 (0.04)[g]	1.20 (0.04)	0.018	0.020 (0.005)	0.020 (0.005)
CP-IgA < 32	246 of 715 (34.4)	1.14	1.14	1.14	0.014	0.014	0.014
CP-IgA 32-64	230 of 715 (32.2)	1.14	1.12 (0.03)	1.11 (0.03)	0.014	0.021 (0.004)	0.020 (0.004)
CP-IgA 128-256	152 of 715 (21.3)	1.14	1.16 (0.03)	1.15 (0.03)	0.014	0.020 (0.004)	0.020 (0.004)
CP-IgA ≥ 512	87 of 715 (12.2)	1.14	1.24 (0.04)[g]	1.20 (0.04)	0.014	0.015 (0.005)	0.016 (0.005)
HP[d]-IgG > 40 AU	382 of 692 (55.2)	1.13	1.17 (0.03)	1.16 (0.03)	0.021	0.016 (0.003)	0.015 (0.003)
CMV[e]-IgG > 0.4 IU/mL	517 of 703 (73.5)	1.16	1.15 (0.03)	1.14 (0.03)	0.021	0.017 (0.004)	0.017 (0.003)
HAV[f]-IgG > 20 mIU/mL	419 of 681 (61.6)	1.21	1.18 (0.03)	1.16 (0.03)	0.026	0.016 (0.034)	0.013 (0.003)

[a] Carotid IMT obtained by 12-segment (see text).
[b] Adjusted for age, gender and smoking (current, former or never)
[c] CP, C. pneumoniae; [d] HP, H. pylori; [e] CMV, cytomegalovirus; [f] HAV, hepatitis A virus.
[g] P = 0.01. All other P > 0.05. Analysis by linear regression (SAS).

Chapter 4

A Prospective Study of Inflammatory Markers

In Chapter 4, my co-authors and I examine the relationship of four inflammatory markers and cardiovascular outcome. We found that soluble Intercellular Adhesion Molecule-1 (sICAM-1) and fibrinogen were independent predictors of cardiovascular outcome, but not of atherosclerotic progression. This manuscript was submitted alongside Chapter 3 to Circulation in February 2002. The full citation is:

> **Smieja M**, Yusuf S, Lonn E, Gnarpe H, Gnarpe J, Olsson G, Held C, Dzavik V, Yi Q, McQueen M. Multiple inflammatory markers, subsequent cardiovascular events, and atherosclerotic progression in the Heart Outcome and Prevention Evaluation (HOPE) study. Submitted to Circulation (February 2002).

I presented the results as a poster session at the American Heart Association meeting, November 11-14, 2001, in Anaheim CA. The poster was a finalist for best poster in the Population Sciences category.

Multiple Inflammatory Markers, Subsequent Cardiovascular Events, and Atherosclerotic

Progression in the Heart Outcomes Prevention and Evaluation (HOPE) Study.

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Abstract:

Background: Limited prospective data are available to compare the relationship of multiple inflammatory markers (high sensitivity C-reactive protein {hsCRP}, fibrinogen, soluble Intercellular Adhesion Molecule-1 {sICAM-1}, and Interleukin-6 {IL-6}, with cardiovascular (CV) events and with atherosclerotic progression among high-risk patients.

Methods and Results: Using blood samples obtained from 3,142 patients in the Heart Outcomes Prevention and Evaluation (HOPE) study at baseline, we examined the relationship between baseline hsCRP, fibrinogen, sICAM-1, and IL-6 concentrations and 491 adjudicated trial outcomes of incident myocardial infarction, stroke, or CV death which occurred during mean follow-up of 4.5 years. In a sub-study of 608 patients, we also examined whether these markers predicted atherosclerosis measured by carotid intimal medial thickness (IMT) progression utilizing serial B-mode ultrasonography. The highest third of hsCRP (hazard ratio {HR} versus lowest third = 1.24, 95 % confidence interval {CI}: 1.02, 1.52), fibrinogen (HR = 1.40, 95 % CI: 1.17, 1.69), or sICAM-1 concentration (HR = 1.46, 95 % CI: 1.21, 1.77) significantly predicted CV events, whereas IL-6 did not (HR = 1.21, 95 % CI: 0.95, 1.54), after adjusting for CV risk factors (age, gender, smoking, ramipril, hypertension, diabetes, body-mass index, total:HDL cholesterol ratio, and glucose). In a forward selection model, fibrinogen and sICAM-1 (both P < 0.001), but not hsCRP, were independently associated with outcome. None of the inflammatory markers predicted greater carotid IMT extent or progression.

Conclusions: Among high-risk patients, elevation of fibrinogen or sICAM-1 predicted CV events. These effects were unrelated to atherosclerotic progression.

Condensed Abstract:

There are limited prospective data on the relationship between multiple inflammatory markers and subsequent cardiovascular events. Among 3,142 HOPE study patients, baseline blood concentrations of four inflammatory markers was measured. Fibrinogen and soluble intercellular adhesion molecule-1 (sICAM-1) were found to be independent risk markers for subsequent CV events, whereas C-reactive protein (CRP) and interleukin-6 were not. None of the inflammatory markers predicted greater carotid IMT extent or progression.

Key Words: inflammation, cardiovascular diseases, prognosis, fibrinogen, cell adhesion molecules.

Introduction

Human atherosclerosis has been described as an inflammatory and fibroproliferative microangiopathy, with an over-zealous "response-to-injury" secondary to arterial wall damage (1). In histo-pathological studies of human or experimental atherogenesis, all stages of disease are characterized by an inflammatory cell infiltrate, predominantly of macrophages and T-lymphocytes, and by inflammatory proteins such as C-reactive protein (CRP), Interleukin-6 (IL-6), and cell adhesion molecules (2-5).

Studies of circulating inflammatory molecules may provide important prognostic information, identify patients who will benefit from particular therapies, and suggest directions for the development of new therapeutic strategies (6-8). In meta-analyses of prospective primary and secondary prevention studies, the highest versus lowest third of CRP or fibrinogen concentrations were each associated with an increased relative risk of CV events of 1.7 to 2.0 (9;10). However, those studies were undertaken prior to the widespread availability of high sensitivity (hs)CRP assays, and the comparative prognostic ability of various inflammatory markers remains unclear. Two other circulating inflammatory molecules—IL-6 and soluble Intracellular Adhesion Molecule-1 (sICAM-1)—may play important roles within atheroma (11;12), but prospective data on these novel risk markers are limited (13-15).

Our primary study objective was to determine which of four circulating inflammatory markers—hsCRP, fibrinogen, sICAM-1, or IL-6—best predicted cardiovascular (CV) events in a high-risk population. Secondary objectives were to examine the determinants of elevated inflammatory marker concentrations, and to measure the association between inflammatory marker concentrations and atherosclerotic progression.

Methods

Description of the HOPE study and SECURE sub-study

The Heart Outcomes Prevention and Evaluation (HOPE) study was a multi-centre randomized clinical trial of ramipril, vitamin E, both or neither for the prevention of CV events among 9,541 patients with previous coronary artery disease, stroke, peripheral vascular disease, or high risk diabetes (16;17). Baseline enrollment blood samples from 3,142 Canadian HOPE study patients were stored at the HOPE central laboratory in Hamilton ON Canada.

In the Study to Evaluate Carotid Ultrasound with Ramipril and Vitamin E (SECURE), a HOPE sub-study, 732 patients were randomized to one of two doses of ramipril, or to placebo, and to vitamin E or placebo, and had serial 12-segment mean maximal carotid intimal medial thickness (IMT) measured at study baseline and at 2 and 4.5 years, as reported previously (18;19). Missing sera were assumed missing completely at random. For 608 of these patients, baseline sera and complete ultrasound measurements throughout the study were available, and for 607 patients, matching blood drawn two years after randomization was available.

The HOPE, SECURE, and Inflammatory and Infectious Markers in HOPE study protocols were approved by the Research Ethics Board at McMaster University and the Hamilton Health Sciences, and written informed consent was obtained.

Inflammatory Markers

All inflammatory markers were assayed using serum or plasma that had been continuously frozen at −70 °C since blood collection and processing. For 117 patients, three

or fewer of the markers were measured due to insufficient sample volume, and data were assumed missing completely at random.

Serum hsCRP was measured in 3,025 patients using a high sensitivity automated rate nephelometric immunoassay (Dade Behring high-sensitivity CRP, BNII Nephelometer System, Marburc, DE). For 2,418 patients, a single baseline specimen was assayed. For 607 patients, matched baseline and 2 year sera were thawed and processed in the same run for determination of hsCRP concentration changes during treatment.

Plasma fibrinogen was measured in 3,142 patients by the Clauss method on the Sigma Amax (Sigma, St. Louis MS), using reagent from Diagnostica Stago (Asmieres-Sur-Seine, France).

Plasma sICAM-1 was assayed in duplicate and averaged in 3,057 patients using the Parameter human soluble ICAM-1 immunoassay (R&D Systems, Minneapolis MN) and an automated ELISA washer and reader (Biotek Instruments Inc, Winooski VT). Briefly, 10 μL of EDTA plasma was incubated for 1.5 hours at room temperature, conjugated for 30 minutes at room temperature, and read at 450 nm with 620 nm correction. Six internal calibrators (0-1000 μg/L) were run in duplicate to determine a 4-point regression calibration curve for each 96 micro-titre plate.

Plasma Interleukin-6 concentrations were assayed in duplicate and averaged in 3,060 patients using the Quantikine HS high sensitivity human IL-6 immunoassay (R&D Systems, Minneapolis MN), and an automated ELISA washer and reader (Biotek Instruments Inc, Winooski VT). Briefly, 200 μl of EDTA plasma was incubated overnight (16 hours) at room temperature, conjugated (60 minutes) and amplified (30 minutes) at room temperature, and

read in a spectrophotometer at 490 nm wavelength with 650 nm correction. Six internal

calibrators (0-10 ng/L) were run in duplicate to establish a 4-point regression calibration

curve for each 96-microtitre plate.

Other biochemical measurements

We measured fasting study baseline serum lipids (total cholesterol, HDL cholesterol,

and triglycerides), and glucose in 3,151 patients, and uric acid (which was measured last, and

omitted if the sample had insufficient volume), in 2,963 patients.

Statistical analysis

The primary study end-point was the HOPE study primary event cluster of incident

MI, stroke or CV death. We also examined the secondary outcomes of MI alone, stroke

alone, or the primary event cluster combined with revascularization procedures. Kaplan-

Meier curves were plotted for the cohorts of 3,025 to 3,142 patients, and inflammatory

marker concentrations (divided by tertiles of the study group) tested by the log-rank test

using SAS 6.02 (SAS Institute, Cary, NC). Inflammatory marker concentrations, divided into

thirds, were individually evaluated in unadjusted Cox proportional hazards models for the

primary and three secondary outcomes. For more complex Cox models, all markers were

dichotomized to enable use of all patients in the analyses, and to reduce complexity. hsCRP

was dichotomized with at-risk defined as > 2.0 mg/L (0.2 mg/dL), corresponding to "high"

and "highest" CV risk in apparently healthy adults (20). For fibrinogen, sICAM-1 and IL-6,

contiguous thirds were combined if the hazard ratios (HR) were within 5 % of one another.

Thus, fibrinogen and sICAM-1 were dichotomized at the second tertile, and IL-6 at the first

tertile. Two adjusted Cox models were constructed for each marker. In the first adjusted

model ("clinical co-variates"), nine clinical co-variates were simultaneously forced in: age,

gender, smoking status (current, former or never), ramipril randomization, diabetes mellitus,

history of hypertension, history of hypercholesterolemia, body mass index (> 25 kg/m^2 for

men, > 27 kg/m^2 for women) and use of lipid lowering agents. In the second adjusted

model ("clinical and metabolic co-variates"), two additional variables were included: study

baseline fasting glucose and total cholesterol: HDL cholesterol ratio. Fasting triglycerides

and uric acid concentrations were not independent predictors of outcome, and were not

included in the adjusted models. To examine the relative contributions of the 15 clinical,

metabolic, and inflammatory variables, a stepwise forward selection Cox model including all

four inflammatory markers, the nine clinical variables, and two metabolic variables, was

constructed for the primary study outcome, with P < 0.20 for entry into the model. Clinical

and metabolic determinants of elevated inflammatory marker levels were examined by

multiple logistic regression, setting the dichotomous inflammatory marker as the dependent

variable. For carotid IMT progression, the annualized slope of the mean maximal carotid

IMT measured at baseline, 2 years, and 4.5 years into the study was used as the dependent

variable in a linear regression model, as previously described (19). An alpha level of 0.05 was

set as the level of significance.

Results

Patient description

The patient characteristics among the 3,142 patients were similar to those in the

over-all HOPE study, which has been described in detail previously (16). Patients had mean

age 65.4 year; 77.6 % were male; 13.9 % were current smokers, 63.0 % former smokers, and

23.1 % never smokers; 34.2 % had diabetes, 41.4 % had hypertension; 49.0 % had a history

of hypercholesterolemia; 57.9 % had a previous MI; 9.5 % had a previous stroke or transient

ischemic attack, 16.9 % had peripheral vascular disease; and 9.6 % had no previous vascular

event. In addition to randomization to ramipril, vitamin E, both or neither, patients were

also on other CV medications including ASA (79.0 %), lipid-lowering drugs (40.1 %), beta-

blockers (43.4 %) and calcium channel blockers (18.3 %).

Cardiovascular Events

The primary event cluster of incident MI, stroke, or CV death occurred in 491 of

3,142 patients (15.6 %) during a mean follow-up of 4.5 years. For secondary analyses, MI

alone (363 events, 11.6 %), stroke alone (107 events, 3.4 %), and the composite measure of

MI, stroke, CV death or revascularization (904 events, 28.8 %), were also analyzed.

Lipid and metabolic risk markers

Fasting serum total:HDL cholesterol ratio (hazard ratio {HR} = 1.12 per unit

change, 95 % CI: 1.05, 1.20) and glucose (HR = 1.06 per mmol/L, 95 % CI: 1.02, 1.09)

predicted the primary outcome cluster of MI, stroke, or CV death, in a Cox proportional

hazards regression model. Neither fasting triglycerides (HR = 0.92 per mmol/L, 95 % CI:

0.84, 1.01) nor uric acid (HR = 1.001 per mmol/L, 95 % CI: 1.000, 1.002) were independent

predictors of the primary outcome cluster.

C-reactive Protein

Serum hsCRP concentration was divided into thirds (tertiles: 1.63, 4.29 mg/L).

Cumulative event Kaplan-Meier plots by thirds of hsCRP concentration are shown in Figure

1. Setting the lowest third as the referent group, the middle third (HR = 1.21, 95 % CI: 0.95,

1.54) and upper third (HR = 1.37, 95 % CI: 1.09, 1.75) of hsCRP concentrations were

associated with the primary event cluster in unadjusted analyses. Stroke alone was predicted

by hsCRP, but not MI alone or the cluster of primary events combined with revascularization (Table 1).

The highest third of hsCRP concentration remained a significant predictor of the primary outcome cluster (HR = 1.24, 95 % CI: 1.02, 1.52) after adjustment for clinical co-variates (age, gender, smoking, ramipril allocation, cholesterol-lowering drugs, diabetes mellitus and history of hypercholesterolemia) and metabolic co-variates (fasting total:HDL cholesterol and glucose). Similarly, hsCRP ≥ 2.0 mg/L was a predictor of the primary outcome cluster (HR = 1.24, 95 % CI: 1.02, 1.52), as detailed in Table 2, after adjustment for clinical co-variates. Additional adjustment for metabolic co-variates (fasting glucose and total:HDL cholesterol) attenuated this association (HR = 1.19, 95 % CI: 0.98, 1.46).

Serum hsCRP concentrations were correlated with plasma fibrinogen concentrations (Pearson correlation coefficient = 0.46, P < 0.0001) and IL-6 (0.36, P < 0.0001), and weakly correlated with sICAM-1 (0.09, P < 0.0001). In a multiple logistic model, hsCRP concentrations ≥ 2.0 mg/L were independently associated with gender, current or former smoking, fasting total:HDL cholesterol, fasting glucose, and with hypertension, but not with age, diabetes mellitus, and a history of hypercholesterolemia (Table 3). hsCRP concentrations were lower among patients taking lipid-lowering drugs.

Changes in hsCRP Concentrations

Among 607 patients with duplicate serum hsCRP concentrations collected 2 years apart, there was a moderate correlation between paired samples (Pearson correlation = 0.28, P < 0.001; estimated Spearman rho = 0.61, P < 0.001). No over-all change in hsCRP concentration was found between the two time periods: baseline mean (SEM): 3.85 (0.25)

mg/L and 2-year, 3.77 (0.19) mg/L, mean difference = -0.09 (0.27) mg/L, P = 0.76. The

2-year changes in hsCRP were: -0.44 (0.47) mg/L among patients randomized to ramipril 10

mg/day, +0.36 (0.47) mg/L among patients randomized to ramipril 2.5 mg/day, and -0.17

(0.48) mg/L among patients on placebo (P = 0.47 for difference among groups). Among

patients taking a lipid-lowering drug at study baseline, the mean {SEM} hsCRP was lower

(3.44 {0.23}mg/L) than among remaining patients (4.17 {0.35} mg/L, P = 0.17). The

difference between groups was statistically significant at 2-year follow-up (3.17 {0.33} mg/L

versus 4.08 {0.24}, respectively, P = 0.02), although the difference in change scores was not

(-0.24 {0.46} mg/L versus 0.00 {0.33} mg/L, P = 0.68).

Fibrinogen

Higher concentrations of plasma fibrinogen, divided into thirds (tertiles: 3.24, 3.76

g/L), were associated with the primary outcome in unadjusted (Figure 1 and Table 1) and

covariate-adjusted analyses (Table 2). Fibrinogen concentrations correlated with hsCRP

(Pearson correlation 0.45) and IL-6 (0.31), and very weakly correlated with sICAM-1 (0.08,

all P < 0.0001). In a multiple logistic regression model, fibrinogen concentrations

dichotomized at the second tertile (≥ 3.76 g/L) were independently associated with age,

gender, current or former smoking, hypertension, and fasting total:HDL cholesterol, but

not with other CV risk factors (Table 3).

Intercellular Adhesion Molecule-1

Higher concentrations of plasma sICAM-1, divided into thirds (tertiles: 278.4 and

363.9 µg/L), were associated with the primary event cluster in unadjusted and in the two

covariate-adjusted models (Figure 2 and Table 1). The highest third (≥ 363.9 µg/L) was

associated with a HR of 1.46 (95 % CI: 1.21, 1.77), after adjusting for clinical and metabolic

risk markers. Plasma sICAM-1 concentrations were very weakly correlated with hsCRP

(Pearson correlation coefficient = 0.09), fibrinogen (0.08), and IL-6 (0.12, all P < 0.0001). In

a multiple logistic regression model, sICAM-1 concentrations ≥ 363.9 µg/L were

independently associated with age, gender, current or former smoking, and fasting glucose,

but not with other CV risk factors (Table 3).

Interleukin-6

Higher concentrations of plasma IL-6, divided into thirds (tertiles: 2.45 and 4.13

ng/L), were associated with the primary outcome only in unadjusted analyses (Figure 2 and

Table 1). In covariate adjusted analyses, IL-6 was not an independent predictor of the

primary outcome cluster. IL-6 concentrations correlated with hsCRP (Pearson correlation

0.36) and fibrinogen concentrations (0.31), and very weakly correlated with sICAM-1

concentration (0.09, all P < 0.0001). In a multiple logistic regression model, IL-6

concentrations dichotomized at the first tertile (≥ 2.45 ng/L) were associated with age,

current and former smoking, total:HDL cholesterol, fasting glucose, and with a history of

hypertension (Table 3). A history of lipid-lowering drugs was associated with a lower IL-6

concentrations.

Forward Selection Model of Multiple Inflammatory Markers

In a forward selection model including all clinical, metabolic, and inflammatory risk

markers, fibrinogen ≥ 3.76 g/L and sICAM-1 ≥ 363.9 µg/L entered the model as

independent predictors (Table 2), whereas neither hsCRP ≥ 2.0 mg/L nor IL-6 ≥ 2.45 ng/L

were statistically significant (P > 0.2). Of all 15 clinical, metabolic, and inflammatory

variables, age, gender, sICAM-1, current smoking and fasting glucose entered the model, in that order (all P < 0.001), followed by fibrinogen (P = 0.001), ramipril (P = 0.006), and a history of hypertension (P = 0.03). No other variable was a statistically significant predictor of CV events. Alternatively, with all inflammatory markers entered as thirds, and all 15 variables forced into the model, the top versus bottom third of hsCRP was not associated with CV events (HR = 1.13, 95 % CI: 0.84, 1.53).

Interaction with Ramipril and Vitamin E

We examined whether there was an interaction between randomization to ramipril and baseline inflammatory marker status, for predicting the primary event cluster. The effectiveness of ramipril was greater in the highest thirds of hsCRP, fibrinogen, and sICAM-1 concentrations, compared with the lowest thirds (Table 4). However, the individual interaction terms between ramipril and hsCRP, fibrinogen, sICAM-1 or IL-6 were not statistically significant. Ramipril versus placebo efficacy, by sub-groups defined by an inflammatory risk score with one point assigned to each of fibrinogen $\geq$ 3.76 g/L or sICAM-1 $\geq$ 363.9 μg/L, was: HR (risk score 2) = 0.74 (95 % CI: 0.49, 1.12); HR (1) = 0.69 (95 % CI: 0.52, 0.91); and HR (0) = 0.92 (95 CI: 0.70, 1.21).

We also sought an interaction between inflammatory marker concentration and vitamin E efficacy. The vitamin E-by-hsCRP interaction terms were statistically significant: vitamin E-by-CRP(middle third): HR = 2.03, (95 % CI: 1.24, 3.30, P = 0.005); and vitamin E-by-CRP(upper third): HR = 2.07 (95 % CI: 1.28, 3.33, P = 0.003). Interaction terms between vitamin E and fibrinogen, sICAM-1, or IL-6 concentrations were not statistically

significant. Vitamin E versus placebo efficacy (see Table 4) among 1,008 patients with hsCRP in the lowest third was: HR for CV events = 0.62 (95 % CI: 0.44, 0.89, P = 0.01).

Carotid Intimal Medial Thickness

Carotid artery 12-segment mean maximal IMT (SEM) was 1.151 (0.023) mm at study baseline, and was not associated with hsCRP, fibrinogen, sICAM-1, or with IL-6 concentration (Table 5). The 4.5-year mean progression in carotid IMT was 0.017 (0.003) mm per year, and was not associated with any of the four inflammatory markers. The upper third of fibrinogen or sICAM-1 concentrations were associated with adjusted carotid IMT progression of 0.001 (95 % CI: -0.007, 0.008) and 0.003 (95 % CI: -0.004, 0.010) mm per year, respectively, accounting for 2.8 % (95 % CI: -38.9, 44.4) and 16.7 % (95 % CI: -21.6, 54.1) of total progression, respectively.

Discussion

In a large cohort of patients with previous CV events or at high risk for such events, we found that higher concentrations of inflammatory markers were associated with a higher incidence rate of the adjudicated primary event cluster of incident MI, stroke, or CV deaths during 4.5 years of follow-up. Fibrinogen and sICAM-1, but not hsCRP or IL-6, were associated independently of other clinical, metabolic, and inflammatory variables. However, no relationship with atherosclerotic progression was observed.

Our estimated hsCRP adjusted hazard ratio of 1.24 for the top third of concentration, compared with the lowest third, is lower than that reported in previous meta-analyses(9;10). Using high-sensitivity assays, hsCRP concentrations were a strong predictor of subsequent MI in the Physicians' Health Study (OR = 2.9) (21) and in the Women's Health Study (OR = 4.4)(22). Both of these studies involved primary prevention cohorts,

and data are more limited using hsCRP in patients with established CV disease. In a second, more recent meta-analysis of three secondary prevention studies, the top third of CRP was associated with a pooled relative risk of 1.9 (95 % CI: 1.5, 2.3) (10).

We used a commercially-available, high sensitivity CRP assay in our study which correlates closely with the in-house hsCRP assays used in earlier epidemiologic studies(23). Concurrent treatment may attenuate the predictive value of hsCRP, since most patients were taking ASA or other anti-platelet agents (79.0 %), and many were on lipid-lowering drugs (40.1 % at study baseline, 48.0 % by study end). Interactions between hsCRP and ASA (21) or pravastatin(24), with minimal efficacy of either drug in the absence of elevated hsCRP concentrations, have been previously demonstrated. Hence, studies of inflammatory markers among patients on effective concurrent medications may under-estimate the association with CV events, as previously demonstrated(25).

Statins have been shown to decrease hsCRP concentrations(25;26), and we found that a history of taking lipid-lowering agents was associated with lower hsCRP concentration (Table 3). Ramipril, however, was not associated with a statistically significant change in 2-year hsCRP concentrations, although its effectiveness may be greater at higher concentrations of hsCRP, fibrinogen, or sICAM-1 (Table 4). Conversely, patients in whom neither fibrinogen nor sICAM-1 concentrations were in the upper third may not benefit from ramipril (HR = 0.92, 95 % CI: 0.70, 1.21).

We also sought an interaction between vitamin E and baseline hsCRP levels. For the 1,008 patients with the lowest third of hsCRP concentration, vitamin E was associated with fewer CV events compared with placebo. A similar, but not statistically significant, pattern was seen with fibrinogen concentrations. Conversely, vitamin E was associated with

increased incidence of events at higher concentrations. While these results may be spurious, they are in keeping with a small study demonstrating that vitamin E had pro-oxidant effects among smokers taking a high polyunsaturated fat diet (27). Validation of these findings in other study cohorts is needed to determine whether vitamin E may be effective at lower hsCRP concentrations.

Our results for fibrinogen are compatible with the cited meta-analysis (9), and demonstrate that fibrinogen is a readily-obtained, low-cost valid predictor independent of other inflammatory markers, even among patients on current effective therapies.

We examined two novel markers, sICAM-1 and IL-6, for which limited prospective data were previously available. sICAM-1 predicted future MI in a nested case-control study of 474 cases and matched controls in the Physicians' Health Study (OR = 1.6)(13), and was associated with poorer prognosis in patients with stable angina pectoris (28). Our estimate for sICAM-1 is similar (adjusted HR = 1.40), and extends the observations to a high-risk group of patients. In the forward selection model of 15 clinical, metabolic and inflammatory variables, sICAM-1 was the third variable to enter the model—after age and gender. A prospective study of 1,246 patients undergoing coronary angiography compared Vascular Cell Adhesion Molecule (VCAM)-1 with sICAM-1 and E-selectin, and found VCAM-1, but not sICAM-1, to be associated with future CV events independently of the other inflammatory markers (HR = 2.1) (15). The authors suggest that VCAM-1 may be a superior risk marker among patients with overt CV disease, and further study is needed to determine whether VCAM-1 and ICAM-1 are more informative risk markers in patients with established CV disease.

Another study nested within the Physicians' Health Study cohort found that IL-6 was associated with future MI (RR = 2.3) (14). Unlike that study of apparently well individuals, we found that IL-6 was not prognostically important after adjusting for other clinical risk factors in HOPE study patients..

We also investigated the clinical and laboratory determinants of high inflammatory marker concentrations, and wish to make two specific points. First, hsCRP, fibrinogen and IL-6 correlated with one another, but only very weakly with sICAM-1. Thus sICAM-1, a measure of endothelial cell damage, may be measuring a different aspect of the inflammatory response. Second, all four markers showed a strong association with smoking status, with a gradient from non-smokers, to former smokers, to current smokers (see Table 3). An association between CRP and smoking has been previously demonstrated (29;30). It remains possible that assessment of inflammatory markers may, to some extent, be measuring CV risk associated with smoking status, and the interaction between smoking and inflammation requires further study.

In a companion manuscript, we report the association between previous infections, inflammation, and cardiovascular outcome. We found that three of these infections—*C. pneumoniae, H. pylori*, and hepatitis A virus—were not associated with CV events (Smieja et al, unpublished). Cytomegalovirus IgG sero-positivity predicted a small excess of CV events, but this was independent of hsCRP, fibrinogen, sICAM-1 or IL-6. We infer that the CV risk associated with elevated levels of inflammatory markers was not explained by exposure to these infections, to the extent that exposure is accurately measured by serologic tests.

We examined whether the four inflammatory markers predicted atherosclerotic progression as assessed by serial carotid artery ultrasonography in a sub-group of patients.

We found no relationship between baseline inflammatory marker levels and either the extent, or the progression, of carotid disease. A relationship between carotid IMT and sICAM-1 has been reported (31). The 40 percent higher CV event rate associated with the highest third of fibrinogen or sICAM-1 concentration contrasts with the estimated 2.8 to 16.7 % of atherosclerotic progression that these markers explain, but the confidence intervals are wide and compatible with 44 to 54 % progression for the two markers respectively. Nevertheless, the data suggest that the inflammatory state predisposes to CV events independently of anatomical disease progression, perhaps via thrombosis or propensity to plaque rupture.

We conclude that the inflammatory markers fibrinogen and sICAM-1 provide independent prognostic information in patients at high risk of CV events on multiple cardiac medications. Continued investigations of inflammatory markers may help to risk stratify, and to identify individuals likely to derive greater benefit from specific treatments.

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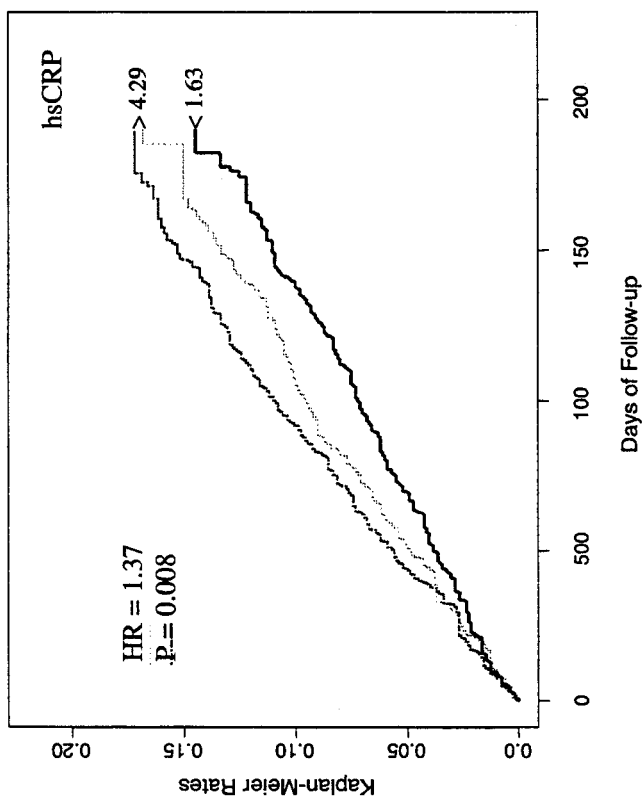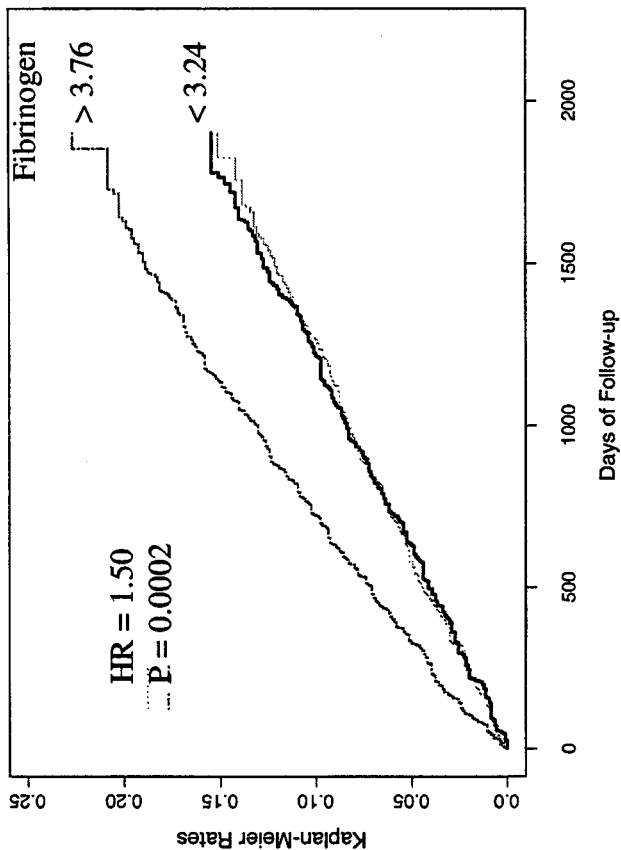
Figure 4-1. Kaplan-Meier curves for baseline high-sensitivity C-reactive protein (hsCRP, tertiles 1.63 and 4.29 mg/L), left, and fibrinogen concentrations (tertiles 3.24 and 3.76 g/L), right, divided into thirds, as risk markers for cardiovascular events (MI, stroke, or CV death) among 3,025 to 3,142 HOPE study patients. Unadjusted hazard ratio (HR) for CV events by highest third versus lowest third.
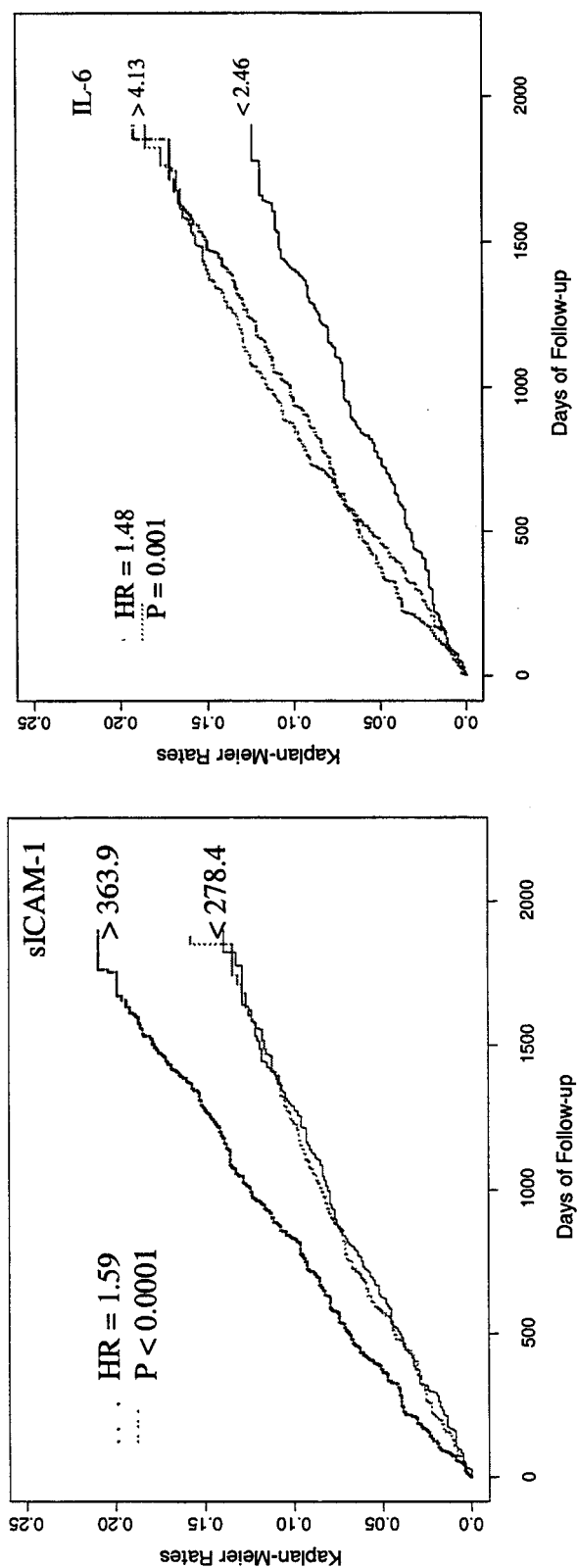
Figure 4-2. Kaplan-Meier curves for baseline soluble Intercellular Adhesion Molecule-1 (sICAM-1, tertiles 278.4 and 363.9 μg/L),

left, and Interleukin-6 concentrations (IL-6, tertiles 2.46 and 4.13 ng/L), right, divided into thirds, as risk markers for cardiovascular

events (MI, stroke, or CV death) among 3,057 to 3,060 HOPE study patients. Unadjusted hazard ratio (HR) for CV events by

highest third versus lowest third.

Table 4-1: Unadjusted hazard ratios for inflammatory markers and CV events among 3,001 to 3,116 HOPE study patients.

Inflammatory marker concentrations (by tertiles)	Hazard Ratios (95 % Confidence Intervals)			
	Primary Study Outcome (MI, stroke, or CV death)	MI alone	Stroke alone	Primary Study Outcome or Revascularization
hsCRP: <1.63 mg/L	1.00	1.00	1.00	1.00
1.63-4.29	1.21 (0.95, 1.54)	1.00 (0.76, 1.31)	2.51 (1.40, 4.49)	1.09 (0.92, 1.29)
>4.29	1.37 (1.09, 1.74)	1.12 (0.85, 1.46)	2.55 (1.43, 4.56)	1.17 (0.99, 1.38)
Fibrinogen: <3.24 g/L	1.00	1.00	1.00	1.00
3.24-3.76	0.96 (0.76, 1.20)	0.94 (0.72, 1.23)	1.07 (0.65, 1.75)	1.05 (0.89, 1.24)
>3.76	1.50 (1.22, 1.86)	1.48 (1.16, 1.89)	1.55 (0.98, 2.48)	1.32 (1.13, 1.55)
sICAM-1: <278.4 µg/L	1.00	1.00	1.00	1.00
278.4-363.9	1.01 (0.79, 1.29)	0.96 (0.73, 1.27)	1.21 (0.68, 2.15)	0.95 (0.80, 1.12)
>363.9	1.59 (1.28, 1.99)	1.43 (1.11, 1.85)	2.56 (1.54, 4.26)	1.23 (1.04, 1.44)
IL-6: <2.45 ng/L	1.00	1.00	1.00	1.00
2.45-4.13	1.50 (1.18, 1.90)	1.51 (1.15, 1.98)	1.69 (1.01, 2.84)	1.23 (1.04, 1.45)
>4.13	1.48 (1.16, 1.87)	1.45 (1.10, 1.91)	1.72 (1.03, 2.89)	1.20 (1.01, 1.42)

Table 4-2: Covariate-adjusted hazard ratios for primary CV events by baseline inflammatory marker concentrations among 3,001 to 3,116 HOPE study patients.

Inflammatory marker[a]	Hazard Ratios (95 % Confidence Intervals)			
	Unadjusted	Clinical covariates[b]	Clinical and metabolic[c]	Clinical, metabolic, inflammatory[d]
hsCRP (> 2.0 mg/L)	1.34 (1.10, 1.63)	1.24 (1.02, 1.52)	1.19 (0.98, 1.46)	-
Fibrinogen (> 3.76 g/L)	1.54 (1.28, 1.84)	1.41 (1.17, 1.69)	1.38 (1.15, 1.66)	1.38 (1.13, 1.67)
sICAM-1 (> 363.9 µg/L)	1.57 (1.30, 1.89)	1.46 (1.21, 1.77)	1.41 (1.17, 1.71)	1.40 (1.15, 1.69)
IL-6 (> 2.45 ng/L)	1.47 (1.19, 1.81)	1.24 (1.00, 1.53)	1.21 (0.997, 1.50)	-

[a] Inflammatory marker concentrations dichotomized (see text)

[b] clinical covariates; age, gender, ramipril, current smoking, diabetes, hypertension, hypercholesterolemia, body-mass index, lipid-lowering drugs forced into the model

[c] clinical and metabolic: clinical covariates and fasting glucose, fasting total:hdl cholesterol ratio forced into the model

[d] forward selection model of all clinical, metabolic and inflammatory variables significant at P < 0.20. Fibrinogen and sICAM-1 were significant at P < 0.001. hsCRP and IL-6 were not statistically significant (P > 0.20) and did not enter the model.

Table 4-3. Determinants of elevated inflammatory marker concentrations among HOPE study patients. Predictors of elevated blood high sensitivity C-reactive protein (hsCRP), fibrinogen, soluble Intercellular Adhesion Molecule-1 (sICAM-1) and Interleukin-6 (IL-6) concentrations among 3,001 to 3,116 Canadian HOPE study patients, determined by multiple logistic regression.

	Odds ratio (95 % confidence interval)			
	hsCRP	Fibrinogen	sICAM-1	IL-6
Threshold	> 2 mg/L	> 3.76 g/L	> 363.9 µg/L	> 2.45 ng/L
Number	3,001	3,116	3,031	3,034
Age (decade)	1.05 (0.93, 1.19)	1.47 (1.30,1.67)[a]	1.13 (1.00, 1.28)[c]	1.61 (1.42, 1.83)[a]
Gender (male)	2.10 (1.72, 2.58)[a]	1.78 (1.47, 2.15)[a]	1.59 (1.32, 1.93)[a]	1.25 (1.02, 1.54)[c]
Current smoking	2.49 (1.90, 3.26)[a]	2.99 (2.30, 3.90)[a]	3.34 (2.55, 4.38)[a]	2.56 (1.92, 3.40)[a]
Former smoking	1.53 (1.27, 1.84)[a]	1.32 (1.08, 1.60)[b]	1.68 (1.37, 2.06)[a]	1.44 (1.19, 1.73)[b]
Hypertension	1.36 (1.16, 1.59)[b]	1.19 (1.02, 1.40)[c]	1.15 (0.98, 1.35)	1.23 (1.05, 1.45)[c]
Diabetes	0.90 (0.72, 1.12)	1.11 (0.89, 1.38)	1.10 (0.88, 1.37)	1.14 (0.91, 1.44)
Lipid lowering drugs	0.84 (0.71, 0.99)[c]	1.08 (0.91, 1.28)	0.94 (0.79, 1.11)	0.81 (0.68, 0.95)[c]
Total:HDL Cholesterol (per unit)	1.10 (1.05, 1.16)[a]	1.13 (1.08, 1.19)[c]	1.03 (0.98, 1.07)	1.07 (1.01, 1.12)[b]
Glucose (mmol/L)	1.10 (1.05, 1.14)[a]	1.02 (0.98, 1.06)	1.08 (1.04, 1.12)[a]	1.08 (1.04, 1.13)[b]

[a] $P < 0.0001$; [b] $P < 0.01$; [c] $P < 0.05$

Table 4-4. Ramipril and Vitamin E efficacy for preventing cardiovascular outcomes (incident MI, stroke or CV death) by baseline inflammatory marker concentration among 3,001 to 3,116 Canadian HOPE study patients.

| | Hazard Ratios (95 % Confidence Intervals) | | | | | |
| | Ramipril versus Placebo | | | Vitamin E versus Placebo | | |
	Lowest Third	Middle Third	Upper Third	Lowest Third	Middle Third	Upper Third
hsCRP	0.81 (0.57, 1.15)	0.81 (0.58, 1.17)	0.65 (0.47, 0.88) [a]	0.62 (0.44, 0.89) [b]	1.26 (0.91, 1.75)	1.28 (0.94, 1.75)
Fibrinogen	0.94 (0.68, 1.29)	0.79 (0.57, 1.11)	0.70 (0.53, 0.92) [a]	0.82 (0.59, 1.13)	0.97 (0.69, 1.35)	1.05 (0.80, 1.38)
sICAM-1	0.85 (0.60, 1.20)	0.99 (0.70, 1.39)	0.67 (0.50, 0.89) [a]	1.09 (0.77, 1.54)	0.89 (0.63, 1.25)	0.88 (0.67, 1.17)

[a] $P < 0.001$

[b] $P = 0.01$

Table 4-5: Inflammatory markers and carotid intimal medial thickness (IMT) among 608 SECURE sub-study patients.

	Baseline IMT (mm)		Slope Progression IMT (mm/year)	
	Unadjusted (SE)	Adjusted [a] (SE)	Unadjusted (SE)	Adjusted [a] (SE)
hsCRP [b]: < 1.39 mg/L	1.15 (0.02)	1.15 (0.19)	0.017 (0.003)	0.017 (0.021)
1.39-3.93	1.18 (0.03)	1.18 (0.03)	0.018 (0.004)	0.018 (0.004)
> 3.93	1.11 (0.03)	1.10 (0.03)	0.021 (0.004)	0.022 (0.004)
Fibrinogen: < 3.18 g/L	1.19 (0.02)	1.19 (0.19)	0.018 (0.003)	0.018 (0.021)
3.18-3.68	1.14 (0.03)	1.14 (0.03)	0.019 (0.004)	0.019 (0.004)
> 3.68	1.11 (0.03)	1.11 (0.03)	0.018 (0.004)	0.019 (0.004)
sICAM-1 [c]: < 256.6 µg/L	1.15 (0.02)	1.15 (0.19)	0.019 (0.003)	0.019 (0.022)
256.6-309	1.15 (0.03)	1.15 (0.03)	0.018 (0.004)	0.018 (0.004)
> 309	1.14 (0.03)	1.14 (0.03)	0.021 (0.004)	0.022 (0.004)
IL-6 [d]: < 2.31 ng/L	1.15 (0.02)	1.15 (0.19)	0.019 (0.003)	0.019 (0.022)
2.31-3.75	1.14 (0.03)	1.14 (0.03)	0.018 (0.004)	0.018 (0.004)
> 3.75	1.13 (0.03)	1.11 (0.03)	0.018 (0.004)	0.018 (0.004)

[a] Adjusted for: age, gender, ramipril (placebo, low or high dose), smoking (current, former, or never), body mass index, and past history of diabetes mellitus, hypertension, and hypercholesterolemia. P > 0.05 for all comparisons between concentrations.

[b] hsCRP: high-sensitivity C-reactive protein

[c] sICAM-1: soluble Intercellular Adhesion Molecule-1

[d] IL-6: Interleukin-6

SECTION 3:

NUCLEIC ACID MARKERS

Chapter 5:

Nucleic Acid Markers in Angiography Patients

In this section, I examine the relationship between infections and cardiovascular disease using nucleic acid detection by polymerase chain reaction in peripheral blood mononuclear cells. In chapter 5, I describe a study of *C. pneumoniae*-DNA and cytomegalovirus-DNA detection in PBMC of patients undergoing coronary angiography. In subsequent chapters, I validate three findings from this study: problems of sampling (chapter 6), association with smoking (chapter 7), and association with winter/spring season (chapters 7 and 8). This chapter was published in its current form. I have reformatted the references to the Vancouver style for consistency throughout the thesis. The full citation is:

> **Smieja M**, Chong S, Natarajan M, Petrich A, Rainen L, Mahony JB. Circulating Nucleic Acids of *Chlamydia pneumoniae* and Cytomegalovirus in Patients Undergoing Coronary Angiography. Journal of Clinical Microbiology 2001; 39: 596-600.

I presented the CMV data as a poster at the Clinical Virology Symposium in Clearwater Beach FL, April 2000, and received the Mario Escobar Award for best abstract. I presented the *C. pneumoniae* data in a poster at the Chlamydia Research Symposium, August 2000, Helsinki Finland. Clinical follow-up data were presented by Dr. Madhu Natarajan in an oral presentation at the Canadian Cardiovascular Society annual meeting, October 2000, Victoria BC.

Circulating Nucleic Acids of *Chlamydia pneumoniae* and Cytomegalovirus in Patients

Undergoing Coronary Angiography

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Running title: *Chlamydia pneumoniae* and angiography

Abstract:

Peripheral blood mononuclear cells from 208 consecutive patients undergoing elective coronary angiography or angioplasty were collected before, immediately after, and four hours after the procedure. Nucleic acids of *Chlamydia pneumoniae* and of cytomegalovirus (CMV) were detected by polymerase chain reaction, and confirmed by hybridization. Circulating *C. pneumoniae* DNA was identified in 24 patients (11.5 %), and was associated with current smoking (odds ratio {OR} = 4.5, 95 % CI: 1.6 to 12.2, P = 0.004), but not with arterial narrowing on coronary angiogram or with *C. pneumoniae* serology. Circulating CMV DNA was identified in 36 patients (17.3 %), and was associated with CMV IgG (OR = 2.7, 95 % CI: 1.2 to 6.3, P = 0.02) but not with angiographic arterial narrowing or with the need for revascularization. Neither *C. pneumoniae* nor CMV DNA detection increased after angioplasty, a procedure in which endothelium is disrupted. Larger prospective studies are needed to determine the prognostic significance of DNA detection.

Background

Previous exposure to *Chlamydia pneumoniae* (CP) and cytomegalovirus (CMV) has been associated with heart disease (1). *C. pneumoniae* antigen and DNA has been detected in coronary and carotid atheroma and in aortic aneurysms, and culture of *C. pneumoniae* from atheroma has been reported (2;3). However, recent large prospective studies have not confirmed an association between *C. pneumoniae* IgG serology and vascular events (4-6), and there was poor correlation between serology and tissue presence of *C. pneumoniae* antigen or DNA (7).

The detection of *C. pneumoniae* DNA circulating in peripheral blood mononuclear cells (PBMC) has been reported, although estimates of prevalence varied widely. In one study, 59 % of 101 heart disease patients and 46 % of 52 blood donor controls were DNA positive (8). Among 804 men undergoing coronary angiography, the prevalence of *C. pneumoniae* DNA was 8.8 % in those with heart disease versus 2.9 % in those without heart disease (9). In 41 aortic aneurysm patients, *C. pneumoniae* DNA detection in PBMCs correlated with the isolation of *C. pneumoniae* DNA from aortic aneurysms (10). Potentially, PBMC *C. pneumoniae* DNA detection could enable large scale epidemiological studies to clarify the role of *C. pneumoniae* in atherosclerotic heart disease and its complications.

CMV is associated with accelerated atherosclerosis of cardiac transplants, and may be associated with coronary artery restenosis or thrombosis after angioplasty or atherectomy (11;12). In a rat model, rat CMV increases neointimal cell proliferation after balloon injury to the carotid artery (13). A role in human disease remains unproven, and prospective studies of CMV serology have not confirmed a relationship with vascular events (5).

This study had three primary objectives: first, to determine the prevalence of

circulating *C. pneumoniae* DNA and CMV DNA in patients undergoing coronary

angiography; second, to determine if *C. pneumoniae* DNA detection increased after coronary

angioplasty, on the assumption that disrupted endothelium would release *C. pneumoniae* (but

not CMV) DNA into the bloodstream; and third, to determine whether DNA isolation was

prognostically important.

Materials and Methods

Patients: Consecutive elective outpatients were recruited from the Hamilton Regional

Angiography Suite, Hamilton Health Sciences Corporation, Hamilton ON, Canada between

February and October 1999. Information regarding age, gender, and a history of previous

cardiac disease, smoking, diabetes mellitus, hyperlipidemia, and hypertension were obtained.

Sample size calculations required 100 patients in the angioplasty stratum for an 80 %

probability of detecting an increase in DNA prevalence from 10 % to 20 %. Angiography

and angioplasty patients were entered until pre-determined strata of 100 patients each were

filled, with recruitment of angiography patients by April, 1999, and continued recruitment of

angioplasty patients until October, 1999. The angiogram report was scored by the presence

of any arterial narrowing (> 25 %) and by the number of epicardial coronary arteries with at

least 50 % narrowing in two orthogonal views or at least 70 % narrowing in one view by

visual assessment. Six month clinical outcomes (cardiac hospitalization, repeat angiogram or

angioplasty, myocardial infarction, coronary artery bypass surgery, or death) were obtained

by telephone to the patient and by hospital chart review. All clinical data were collected by

study nurses blinded to laboratory data. All participating patients gave written consent, and

the study protocol was approved by Research Ethics Boards at St. Joseph's Hospital, Hamilton Health Sciences Corporation, and McMaster University.

Blood collection: Serum was collected prior to angiography or angioplasty. Circulating peripheral blood mononuclear cells were obtained by venipuncture into an 8 mL Vacutainer CPT Cell Preparation Tube (BD Vacutainer Systems, Franklin Lakes, NJ) prior to, immediately after, and 4 hours after the procedure for a total of 3 tubes. Specimens obtained prior to the procedure were obtained in a pre-catheterization outpatient clinic days to weeks before the procedure. CPT tubes contain a blood separation media composed of a thixotropic polyester gel and a density gradient liquid solution. Laboratory personnel processed CPT tubes essentially according to the manufacturer's instructions, except for a second centrifugation. Briefly, CPT tubes were centrifuged at 1500 relative centrifugal force for 30 minutes and refrigerated. After transport to the research laboratory (generally within 24 hours), the specimens were mixed by inversion, re-centrifuged, and the mononuclear cells layer (if visible) or 1 mL of plasma directly above the gel was aspirated and frozen at $-70\ ^\circ$C. In batches, mononuclear cell preparations were thawed and 200 μL aliquots extracted using QIAamp DNA mini-kits (Qiagen, Mississauga ON) into 100 μL of elution buffer.

Detection of DNA: Laboratory staff were blind to all clinical data. A 2.5 μL aliquot was amplified by a nested polymerase chain reaction (PCR) (14), consisting of 40 amplification cycles for a 333 base pair (bp) product (external primers CP1 and CP2), and 30 cycles for a 207 bp product (internal primers CPC and CPD), followed by separation on a 2.0 % (w/v) agarose gel containing ethidium bromide, and ultraviolet light visualization. The 207 bp product was confirmed as *C. pneumoniae* by hybridization with an *in-house* specific fluorescein-

labeled oligonucleotide probe (5' TAC GGA GAC TAT GTT TTC GA 3', GenBank

Accession Number AF131889 positions 196-215). The probe detected *C. pneumoniae* VR

1310 but not *C. trachomatis* (LGV 434) or *C. psittaci* (6BC). Six controls, consisting of one

positive control (*C. pneumoniae* VR 1310), four negative water controls without DNA, and

one additional tube with master mix open to the air throughout specimen addition, were run

for every 48 specimens. PCR extraction and amplification were performed in separate

rooms. In addition, all positive samples were confirmed by re-extraction from the original

patient sample, followed by amplification in triplicate and probing. *C. pneumoniae* DNA

positive status was defined as samples which were positive initially and in at least one of the

replicates after re-extraction. Twelve amplification products from different patients were

sequenced. Oligonucleotide probe synthesis and sequencing of amplification products was

carried out at the Institute of Molecular Biology, McMaster University, Hamilton ON. PCR

positive samples were also amplified by one or more of three different non-nested PCRs,

targeting 23S rDNA (15), 16S rDNA (16), or a cloned pst1 fragment (17), with confirmation

of all positive results by specific oligonucleotide probes.

Amplification for CMV DNA was performed by a non-nested PCR procedure

targeting an immediate early gene (18), (primers P1 and P3), with confirmation by agarose

gel electrophoresis, visualization of a 123 bp product, and hybridization with a specific *in-house* oligonucleotide probe (5' TTT TGA CCT CCA TAG AAG AC 3', GenBank Accession

Number HEHCMVCG position 173700 to 173681). CMV strain AD 169 was used as a

control. All positive results were confirmed by re-extraction, repeat PCR, and probing, and

seven amplification products were sequenced at the Institute of Molecular Biology,

McMaster University, Hamilton ON.

Serology: CMV and *C. pneumoniae* serology were performed by enzyme immunoassay: *C. pneumoniae* IgG and IgA by Sero-CP (Savyon Diagnostics, Kiryat Minrav, Israel); and CMV IgG antibody by ETI-CYTOK-G Plus (DiaSorin SRL, Saluggia, Italy). *C. pneumoniae* IgG or IgA serology was defined as a cut off index of 1.1 or greater, where the index was obtained by dividing the specimen optical density by twice the mean of two negative controls, in accordance with the manufacturer's product insert. CMV IgG seropositivity was defined as a value of greater than 0.4 IU/mL, in accordance with the product insert. Serology and PCR were performed independently by different staff in separate laboratories, with blinding to other laboratory results.

Statistical analysis: Proportions were compared using Chi square or Fisher exact tests (unmatched data) or the McNemar test (matched data). Logistic regression modeling (SPSS for Windows 10.0, Chicago IL) was undertaken using DNA status as the response variable, and the following explanatory variables: serologic status, age, gender, clinical history (unstable angina, myocardial infarction, angioplasty, coronary artery bypass grafting), and clinical risk factors (angiogram score, smoking, diabetes mellitus, hypercholesterolemia, or hypertension). Six-month outcome (as the response variable) was modeled by logistic regression with CP-DNA, CMV-DNA, age, gender, clinical risk factors and angiogram score as explanatory variables. A P-value of < 0.05, two-tailed, was considered statistically significant.

Results

Description of patients

One hundred and eighteen patients undergoing angiography and 90 patients

undergoing angioplasty were enrolled. Median age was 60.4 years (min-max 21-85 years),

and 75.4 % were male. Medical history included: diabetes mellitus in 21.6 %; hypertension,

56.8 %; hypercholesterolemia, 66.1 %; current smokers, 18.1 %; former smokers, 59.9 %;

and never smokers, 22.1 %. A history of myocardial infarction was present in 46.2 %;

unstable angina in 74.1 %; previous angioplasty in 21.6 %; and coronary artery bypass

grafting in 10.1 %.

Chlamydia pneumoniae DNA detection

A total of 547 samples from 208 patients had DNA extracted and amplified for *C.*

pneumoniae, including 207 samples from before, 182 samples taken immediately after, and 158

samples taken 4 hours after the angiogram or angioplasty.

Twenty-five of 547 peripheral blood mononuclear samples (4.6 %) from 24 of 208

patients (11.5 %) were positive for *C. pneumoniae* DNA (see Table 1). In all but one patient, a

single sample from the three time periods was positive. When the positive samples were re-

extracted and PCR tested in triplicate, there were 20 replicate sets with 1 of 3 positive; 2

replicates with 2 of 3; and 3 replicates with all 3 positive (for a total of 33 positive in 75 PCR

tests). Of these original 25 *C. pneumoniae* positive samples, 24 were tested a third time, in

triplicate, and 18 of the 24 were positive. By comparison, in a random sample of 30

previously negative patient PBMC samples, 1 of 30 samples (1 of 90 PCR tests) was positive.

Among 49 plasma samples (taken from the same CPT tube from which mononuclear cell

fractions were obtained) tested in triplicate, including all 25 samples with concurrent positive

PBMC *C. pneumoniae* DNA, no *C. pneumoniae* DNA was identified (0 of 147 PCR tests). Ten

of the 25 (40.0 %) *C. pneumoniae* DNA positives were also positive by at least one of three

PCRs targeting alternative chlamydial sequences. In addition, twelve amplification products

from different patients were sequenced, and all matched *C. pneumoniae* exactly.

C. pneumoniae serology was performed in 203 of the 208 patients (see Table 2). *C.*

pneumoniae IgG serology was positive in 16 of 23 (70.0 %) PBMC *C. pneumoniae* DNA-

positive patients, versus 142 of 180 (78.9 %) who were DNA negative (OR = 0.6, 95 % CI:

0.2 to 1.8, P = 0.3). *C. pneumoniae* IgA serology was positive in 13 of 23 (56.5 %) patients

with DNA detected, versus 115 of 180 (63.9 %) DNA negative (OR = 0.7, 95 % CI: 0.3 to

1.9, P = 0.49).

By multiple logistic regression modeling, PBMC *C. pneumoniae* DNA was associated

with current smoking (OR = 4.5, 95 % CI: 1.6 to 12.2, P = 0.004), the months of February

to April compared with May to October (OR = 4.6, 95 % CI: 1.3 to 16.6, P = 0.02), and

with concurrent CMV DNA detection (OR = 3.9, 95 % CI: 1.4 to 10.6, P = 0.008).

Specifically, circulating *C. pneumoniae* DNA was detected in 9 of 37 (24.3 %) current

smokers, compared with 10 of 121 (8.3 %) former smokers and 5 of 48 (10.4 %) never

smokers. There was no association with gender, cardiac history, or other cardiovascular risk

factors. There were 21 patients with no coronary artery disease at angiography, and 13, 53,

76, and 45 with mild, 1, 2 or 3 vessel disease respectively (see Table 3). *C. pneumoniae* DNA

status was not associated with the presence (OR = 1.3, 95 % CI: 0.3 to 11.9, P = 1.00) or

degree (P = 0.56, test for trend) of coronary artery stenosis at angiography.

There was no increased detection of PBMC *C. pneumoniae* DNA in samples obtained

after coronary angiography or angioplasty (see Table 1). *C. pneumoniae* DNA was detected in

5.1 % of samples before angiography, 5.8 % immediately after, and 8.3 % four hours after

(P = 1.00 for each before/ after comparison, McNemar test). For angioplasty patients,

DNA was detected in 2.2 % of samples before the procedure, and up to 2.7 % of samples

after (P = 1.00 for each before/ after comparison).

Among the 58 patients who had the composite end-point of revascularization or a

clinical cardiac event (coronary artery bypass surgery, repeat angiogram, cardiac

hospitalization, myocardial infarction, or death) during 6 months of follow-up after the

procedure, there was no association with PBMC *C. pneumoniae* DNA status (OR = 0.8, 95 %

CI: 0.3 to 2.5, P = 0.7) (see Table 4).

Cytomegalovirus DNA detection

Forty of 559 samples (7.2 %) from 36 of 208 patients (17.3 %) were positive for

CMV DNA (see Table 1). In all but four patients, a single sample from the three time

periods was positive. Seven amplification products from different patients were sequenced,

and all matched CMV sequences exactly. In a test of 49 plasma samples (31 of which had

CMV in the corresponding mononuclear cell fraction), 9 were positive for CMV DNA. Five

of these 9 were CMV DNA positive in both plasma and mononuclear cell fraction, and 8 of

these 9 were CMV IgG positive.

CMV IgG status was positive in 124 of 207 patients (59.6 %), and in 28 of 36

patients (77.8 %) with concurrent PBMC CMV DNA (see Table 2). In a logistic regression

model, CMV DNA was associated with CMV IgG status (OR = 2.7, 95 % CI: 1.2 to 6.3,

P = 0.02) but not with age, gender, smoking, or other cardiac risk factors. CMV DNA

status was not associated with the presence (OR = 4.6, 95 % CI: 0.6 to 35.5, P = 0.14) or the

degree (P = 0.83, test for trend) of coronary artery stenosis at angiography (see Table 3).

There was no increased detection of PBMC CMV DNA following angiography or

angioplasty (see Table 1). CMV DNA was detected in 9.3 % of samples before, 8.7 %

immediately after, and 4.2 % four hours after angiography. By contrast, CMV DNA was

detected in 6.7 % before, 8.9 % immediately after, and 4.1 % four hours after angioplasty

(P = 1.0 for before/ after comparisons; P = 0.88 for angioplasty vs. angiography).

PBMC CMV DNA did not predict the 58 patients who subsequently required

revascularization or had a cardiac event (OR = 1.4, 95 % CI: 0.6 to 3.0, P = 0.42) (see Table

4). Similarly, CMV-IgG serostatus did not predict these clinical events (OR = 0.9, 95 % CI:

0.5 to 1.7, P = 0.81).

Discussion

This study addressed several issues of direct relevance for the development and

validation of molecular tests for cardiovascular studies. First, we examined the prevalence of

PBMC *C. pneumoniae* DNA in patients undergoing angiography, most of whom had

documented atherosclerotic heart disease. The estimate of 11.5 % *C. pneumoniae* DNA

prevalence was similar to one recent study (9), and may be more representative than the

higher prevalence of 59 % reported in another study (8). The discrepancy may represent

inter-laboratory variation, or a different population sampled during a time of high

community *C. pneumoniae* activity. Our laboratory used the same primers (14) as the higher

prevalence study (8), and we have previously demonstrated that this PCR was more sensitive

for the detection of *C. pneumoniae* in blood samples, despite an analytic sensitivity similar to 4

other PCRs (19). The higher prevalence in smokers and in the winter/ spring months requires confirmation in a separate study. The finding that serology was not associated with circulating *C. pneumoniae* DNA was similar to the lack of association of serology and DNA in atherosclerotic lesions (7), and may explain why serology did not predict cardiovascular events in several prospective studies (4-6). Binding of antibody in immune complexes, which have been detected in patients with atherosclerotic heart disease, is a plausible explanation for this discrepancy (20;21).

Second, we examined the reproducibility of PCR results. Despite use of a very sensitive PCR, samples were usually only positive in 1 of 3 sampled time periods, and on repeat testing were positive in only 1 of 3 replicates. Potential explanations for these results include non-specific amplification; contamination of the PCR; between-sample variation (biological sampling); and within-sample variation (statistical sampling). The amplification products were *C. pneumoniae*, as evidenced by hybridization with a specific oligonucleotide probe, and by molecular sequencing of 12 amplification products. We believe that contamination is not a likely explanation, given that all positives had been extracted on two separate occasions, 75 % were positive when tested a third time, and 40 % of the samples were confirmed by one or more PCRs targeting a different part of the genome. Furthermore, among the plasma samples and initially negative mononuclear cell samples, we detected only a single positive in 237 PCR runs. Finally, of over 200 *C. pneumoniae* negative controls run in the last one year using this PCR by our laboratory, including 40 negative controls which were open during the entire specimen addition step, there was not a single false-positive. Biological variation is plausible, with intermittent shedding of infected mononuclear cells and clearance by the reticulo-endothelial system. The most likely

explanation for intermittent positivity is statistical sampling. We have recently demonstrated that the proportion of replicates which are positive is directly related to the concentration of *C. pneumoniae* in a sample, and that a proportion positive of 1 in 3 replicates can be predicted using probit analysis for samples with low DNA concentration (Smieja et al, manuscript in preparation).

Third, this and a smaller study (10) have demonstrated that the VACUTAINER® CPT™ tube method is a simple and feasible method for obtaining mononuclear cell-associated DNA. The method obviates the need for standard Ficoll-Hypaque gradient centrifugation, and may facilitate the undertaking of similar studies by other laboratories. The finding that mononuclear cell fractions, but not the corresponding plasma fractions, were positive *for C. pneumoniae* DNA constitutes clear evidence in humans that circulating *C. pneumoniae* DNA is cell-associated and found within the mononuclear cell fraction. This corroborates the recent finding of *C. pneumoniae* antigen in circulating mononuclear cells (22).

Fourth, we demonstrated the routine detection of CMV DNA in peripheral blood of non-transplant vascular patients. To our knowledge, this has not been previously reported. CMV was found in both the mononuclear cell fraction and in plasma, although the mononuclear cell fraction detected a greater number of positives.

Fifth, we examined whether *C. pneumoniae* DNA or CMV DNA detection was augmented by coronary angioplasty. We reasoned that *C. pneumoniae* in macrophages within the atherosclerotic plaque would seed the bloodstream during and after the angioplasty, because of disruption of the endothelium and plaque. No such increase in *C. pneumoniae* DNA nor of CMV DNA was observed, either immediately after or four hours after angioplasty. Potential explanations include too low a concentration of organism in coronary

lesions; incorrect sampling time; incorrect sampling fraction (mononuclear cells); or lack of organisms in the atheroma of most patients. Further improvements in the methods of detection, and other methods of sampling such as with intra-coronary artery catheters, or before and after coronary thrombolysis, may help to resolve this issue.

Last, we examined whether detection of PBMC *C. pneumoniae* or CMV DNA had any prognostic significance. Neither CMV DNA nor *C. pneumoniae* DNA was associated prospectively with subsequent revascularization or other clinical events. To examine whether the detection of *C. pneumoniae* DNA and CMV DNA has prognostic importance, large, well-designed, prospective observational and treatment studies are needed. The measurement of PBMC DNA using the methods described may facilitate such studies, and may be a more specific measure of recent exposure than serology.

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Table 5-1: Detection of peripheral blood mononuclear cell *Chlamydia pneumoniae* and cytomegalovirus DNA by angiography or angioplasty status and by time of blood collection.

DNA detected	Procedure	Total		Positive Specimens by Time of blood collection [a]		
		Positive Patients (%)	Positive Specimens (%)	Before (%)	After (%)	4 hr. After (%)
C. pneumoniae DNA [b]	Angiogram	18 / 118 (15.3)	19 / 305 (6.2)	6 / 118 (5.1)	6 / 103 (5.8)	7 / 84 (8.3)
	Angioplasty	6 / 90 (6.7)	6 / 242 (2.5)	2 / 89 (2.2)	2 / 79 (2.5)	2 / 74 (2.7)
	Total	24 / 208 (11.5)	25 / 547 (4.6)	8 / 207 (3.9)	8 / 202 (4.0)	9 / 158 (5.7)
CMV DNA [c]	Angiogram	23 / 118 (19.5)	24 / 317 (7.6)	11 / 118 (9.3)	9 / 103 (8.7)	4 / 96 (4.2)
	Angioplasty	13 / 90 (14.4)	16 / 242 (6.6)	6 / 89 (6.7)	7 / 79 (8.9)	3 / 74 (4.1)
	Total	36 / 208 (17.3)	40 / 559 (7.2)	17 / 207 (8.2)	16 / 182 (8.8)	7 / 170 (4.1)

[a] Before, After and 4 Hours After indicate time of blood collection in reference to the time of angiography or angioplasty. Statistical testing by Fisher exact (angiography vs. angioplasty) or McNemar test (Before vs. After, Before vs. After, Before vs. Four hours after). No comparisons were significant at P < 0.05, 2-tailed.

[b] One angiogram patient was positive for *C. pneumoniae* DNA in two time periods (Before and After, but not Four Hours After). All other positive patients were positive in only one of the three time periods.

[c] One angiography patient was positive for CMV DNA in two time periods (After and 4 Hours After), and three angioplasty patients were positive for CMV DNA in two time periods (two were positive Before and After, and one was positive Before and 4 Hours After). All other positive patients were positive in only one of the three time periods.

Table 5-2: Detection of peripheral blood mononuclear cell *Chlamydia pneumoniae* and cytomegalovirus DNA by serologic status.

Serology	Total Seroprevalence (%)	Seroprevalence among DNA positive		OR (95 % CI)	P-Value[a]
		C. pneumoniae DNA (%)	CMV DNA (%)		
C. pneumoniae IgG	158 / 203 (77.8)	16 / 23 (70.0)[b]	-	0.6 (0.2 to 1.8)	0.31
C. pneumoniae IgA	128 / 203 (63.1)	13 / 23 (56.5)	-	0.7 (0.3 to 1.9)	0.49
CMV IgG	124 / 207 (59.9)	-	28 / 36 (77.8)	2.7 (1.2 to 6.3)	0.02

[a] Statistical testing by Chi square test for comparison of seroprevalence in DNA positive versus DNA negative patients.

[b] Serum was not available for one patient in whom *C. pneumoniae* DNA was detected in the mononuclear cell fraction.

Table 5-3: Detection of peripheral blood mononuclear cell *Chlamydia pneumoniae* and cytomegalovirus DNA by extent of disease at coronary angiography.

DNA detected	Severity of disease [a]					Odds Ratio	P-Value [c]
	Normal (%)	Mild (%)	1 vessel (%)	2 vessel (%)	3 vessel (%)	(95% CI)	
CP DNA [b]	2 / 21 (9.5)	3 / 13 (23.1)	4 / 53 (7.5)	8 / 76 (10.5)	7 / 45 (15.6)	1.6 (0.5 to 4.7)	1.00
CMV DNA	1 / 21 (4.8)	4 / 13 (30.8)	12 / 53 (22.6)	12 / 76 (15.8)	7 / 45 (15.6)	4.6 (0.6 to 35.5)	0.14

[a] Severity of disease assessed by coronary angiogram visual inspection. Normal- < 25% narrowing of epicardial coronary arteries; 1, 2 or 3 vessel narrowing for number of epicardial arteries with ≥ 50% narrowing in two views or ≥ 70% narrowing in 1 view; mild disease if ≥ 25 % but did not reach criteria for significant narrowing.

[b] CP, *Chlamydia pneumoniae*.

[c] Statistical testing of Normal vs. Any disease by Fisher exact test, 2-tailed.

Table 5-4: Association of peripheral blood mononuclear cell *Chlamydia pneumoniae* and cytomegalovirus DNA with subsequent cardiac procedures and events.

Procedure	Total	Six-Month Clinical End-points[a,b]				
		Revascularization[a]	Other Events[b]	Patients with End-points[c]	Percentage with CP-DNA (%)[d]	Percentage with CMV-DNA (%)
Angiogram	118	48 (40.7 %)	6 (5.1 %)	52 (44.1 %)	7 / 52 (13.5 %)	11 / 52 (21.2 %)
Angioplasty	90	5 (5.6 %)	3 (3.3 %)	6 (6.7 %)	1 / 6 (16.7 %)	1 / 6 (16.7 %)
Total	208	53 (25.5 %)	9 (4.3 %)	58 (27.9 %)	8 / 58 (13.8 %)[e]	12 / 58 (20.7 %)[f]

[a] Revascularization: coronary artery bypass graft surgery or coronary angioplasty.

[b] Other events: repeat angiogram or angioplasty, myocardial infarction, or death.

[c] Patients reaching six-month end-point of revascularizaiton or clinical event.

[d] CP, *Chlamydia pneumoniae*.

[e] CP-DNA association with six-month clinical end-points: OR=0.8, 95% CI=0.3 to 2.5, P=0.7 by logistic regression (SPSS).

[f] CMV-DNA association with six-month clinical end-points: OR=1.4, 95% CI=0.6 to 3.0, P=0.42 by logistic regression.

Chapter 6

Probit Regression Models and Sampling

In Chapter 6, I develop a probit regression model to examine the influence of

sampling and low DNA copy number. The paper refers to "nested" and "non-nested"

polymerase chain reactions. The non-nested assay consists of a set of two primers, which

amplify a given DNA fragment. The nested assay adds an additional step, in which a second

set of two primers for a sequence within the initial DNA fragment is further amplified. This

yields an assay of higher sensitivity and potentially of higher specificity.

This chapter presents the published manuscript as it appeared, with reformatting of

the references to Vancouver style for consistency throughout the thesis. The full citation is:

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> 1801.

I presented the results as a poster at the 40th Interscience Conference on

Antimicrobials Agents and Chemotherapy (ICAAC), September 17-20 2000, Toronto ON.

Replicate PCR Testing and Probit Analysis to Detect and Quantitate

Chlamydia pneumoniae in Clinical Specimens.

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Acknowledgements

Abstract:

Nucleic acid amplification of clinical specimens with low target concentration has variable sensitivity. We examined whether testing multiple aliquots of extracted DNA increased the sensitivity and reproducibility of *Chlamydia pneumoniae* detection by PCR. Nested and non-nested *C. pneumoniae* PCR assays were compared using 10 replicates of 16 serial dilutions of *C. pneumoniae* ATCC VR-1310. The proportion positive versus *C. pneumoniae* concentration was modeled by probit regression analysis. To validate the model, 10 replicates of 26 previously positive patient specimens of peripheral blood mononuclear cells (PBMC), sputum, or nasopharyngeal swabs (NPS) were tested. The proportion of replicates that were positive varied with the concentration of *C. pneumoniae* in the sample. At concentrations above 5 infection forming units (IFU)/mL, both nested and non-nested PCR assay sensitivities were 90 % or greater. The nested PCR was more sensitive (median detection 0.35 vs. 0.61 IFU/mL, relative median detection = 0.58, 95 % CI: 0.31 to 0.99, $P = 0.04$). In clinical specimens, replicate PCR detected 15/26 (nested) vs. 1/26 (non-nested, $P < 0.001$). For PBMC specimens, testing 1, 3 or 5 replicates detected 3, 5 or 9 of 10 positive specimens, respectively. Median *C. pneumoniae* concentrations were estimated at 0.07 IFU/mL for PBMC and < 0.03 IFU/mL for NPS specimens. We conclude that performing 5- or 10-replicates considerably increased the sensitivity and reproducibility of *C. pneumoniae* PCR and enabled quantitation for clinical specimens. Due to sampling variability, PCR tests done without replication may miss a large proportion of positive specimens, particularly for specimens with low amounts of target *C. pneumoniae* DNA present.

Introduction

Controversy surrounds the association of *C. pneumoniae* with atherosclerotic heart disease (1-3), asthma (4,5), multiple sclerosis (6-8), and Alzheimer's disease (9-11), primarily because of the lack of a definitive test for detecting *C. pneumoniae*. Culture is performed successfully by few laboratories, and was much less sensitive than PCR for detection in vascular tissue (12). Serology has been considered the "gold standard" for diagnosis of infection (13), but did not correlate with the presence of *C. pneumoniae* DNA or antigen in tissue (12,14,15). A superior marker of current or recent infection is required to clarify the clinical importance of *C. pneumoniae* infection in chronic diseases such as atherosclerosis.

Nucleic acid amplification tests such as PCR enable detection of low concentrations of organism in clinical specimens. However, great variability of detection has been reported. For atherosclerotic tissue, reports of between 0 and 100 % detection have been published, as recently summarized (12,16). Similarly, prevalence of *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMCs) varied between 9 % (17) and 59 % (18) among patients with proven atherosclerotic heart disease. Some of the discrepancy may be attributable to differences between assays, but sampling variability is an alternative explanation. Whether replicate testing improves sensitivity or reproducibility has not, to our knowledge, been systematically examined for *C. pneumoniae* nucleic acid amplification tests.

In a previous study, we compared five *C. pneumoniae* PCRs for both analytical and clinical sensitivity (19), and noted a major discrepancy between tests. Despite relatively similar analytical test sensitivity, only the nested PCR based on the *ompA* gene (20) routinely detected a number of PBMC positives. We hypothesized that sampling variability as well as

differences in PCR performance explained the results, and inferred that clinical specimens

had low concentrations of *C. pneumoniae* DNA.

In this paper, we test these hypotheses with probit regression analysis. Specifically,

we sought to determine: 1. Can replicate *C. pneumoniae* PCR increase test sensitivity over

testing a single time (analytical sensitivity)? 2. Does replicate testing increase *C. pneumoniae*

detection in clinical specimens (clinical sensitivity)? 3. Can probit analysis quantitate *C.*

pneumoniae in clinical specimens?

Methods

PCR methods

comparison of five PCRs for *C. pneumoniae* was previously described (15), and two of

these PCRs--a nested (20) and a non-nested procedure (21)--were used in this study. 200 µL

samples of laboratory cultured strains or clinical specimens (see details below) were extracted

using QIAamp DNA Mini-Kits (Qiagen, Mississauga ON) following a tissue or blood

extraction protocol, and eluted in 100 µL of buffer. PCR was performed on 2.5 µL purified

DNA samples in a total volume of 25 µL. Components of the reaction mix and

thermocycling conditions were previously described by Campbell (21) for the non-nested

PCR and by Tong and Sillis (20) for the nested PCR. The non-nested procedure consisted

of 40 rounds and amplified a 437 base pair (bp) cloned *PstI* fragment. The nested PCR

consisted of 40 rounds of amplification of a 333 bp fragment of the *ompA* gene, followed by

30 rounds of amplification of a 207 bp internal fragment. AmpliTaq Gold (Perkin-Elmer,

Branchburg, NJ) was used for all amplifications. All amplification products were analyzed

by 2 % (w/v) agarose gel electrophoresis followed by ethidium bromide staining. Stringent

procedures to minimize or detect contamination included: extraction and amplification in

separate rooms after changing gloves and lab coats, use of plugged pipette tips and positive

displacement pipettors, and insertion of at least one blank every five to ten tubes. Every

fifth blank was left open during specimen addition to detect aerosol contamination.

Dilution series of C. pneumoniae

C. *pneumoniae* ATCC VR-1310 was cultured in U-937 human mononuclear cells for

40-48 hours, then spiked into a repeatedly negative volunteer-derived PBMC fraction (CPT

tube, BD Vacutainer Systems, Franklin Lakes NJ). In PCR replicates of 2, serial 10-fold

dilutions were tested to establish an upper threshold (all tests positive) and lower thresholds

(all tests negative) of test sensitivity as 4 inclusion forming units (IFU)/mL (0.01 IFU/2.5

μL PCR) and 0.04 IFU/mL (0.0001 IFU/2.5 μL PCR) respectively. For each of the non-

nested and nested PCRs, we performed 10 replicates of 6 dilutions between 4 IFU/mL and

0.04 IFU/mL, and, at a separate time, 10 replicates of 10 dilutions between 4 IFU/mL and

0.008 IFU/mL. The final probit regression model included data from both of these two

dilution series (total 160 tests for each of nested and non-nested PCRs).

Clinical validation

For the clinical validation set, 26 clinical specimens (each from unique patients) were

tested in 10 replicates by both PCR assays (total 520 PCR tests): 10 PBMC specimens from

patients undergoing elective coronary angiography, 6 pediatric and 8 adult nasopharyngeal

specimens (NPS) from patients with acute respiratory symptoms, and 2 sputum specimens

from adults with chronic airways limitation. A patient's specimen was considered positive if

one or more of the 10 individual determinations were positive. All specimens were

previously C. *pneumoniae* positive in at least 1 of 3 replicates using the nested PCR, and

confirmed by Southern blotting and oligonucleotide hybridization with *C. pneumoniae*-specific

probe.

Statistical methods

The relationship between the proportion positive from each replicate of 10, and the

corresponding log concentrations of *C. pneumoniae*, was examined using probit regression

analysis (SPSS for Windows 10.0, SPSS Inc., Chicago IL). Using the probit model, the two

tests were compared, median detection concentration of *C. pneumoniae* was estimated, and the

concentrations corresponding to probits of 0.01 to 0.99 were calculated. . Proportions were

tested with StatXact version 3.02 (Cytel Software Corp., Cambridge MA). A P value of

< 0.05 was taken as statistically significant, 2-tailed. The relationship between replicate

number and test sensitivity was calculated in Excel 97 (Microsoft Corp., Redmond WA),

using the calculated probit at a given concentration and the formula: Probability of at least

one positive in "n" replicates = $1 - (1\text{-probit})^n$. To estimate median *C. pneumoniae*

concentration in clinical specimens, the concentration corresponding to the observed

proportion positive of 10 replicates was interpolated from the nested PCR probit analysis

table.

Results:

Constructing probit regression models

Separate probit regression curves were constructed for the non-nested and nested

PCR assays using data from 10 replicates each of 16 dilutions of culture-grown *C. pneumoniae*

ATCC VR-1310. Figure 1 shows the relationships between the number of positives at each

dilution and the concentration of *C. pneumoniae* for the non-nested and nested PCRs. The

circles (non-nested, panel A) or triangles (nested, panel B) represent the number of positive

results, from the 10 replicates, at each concentration of *C. pneumoniae*, and the solid line represents the fitted probit regression line for the assay. At a concentration of 4 IFU/mL, both PCRs detected all 20 replicates as positive. At a concentration of 1 IFU/mL, the non-nested PCR detected 5/10 vs. 8/10 for the nested PCR; and at a concentration of 0.1 IFU/mL, the non-nested PCR detected 0/10 vs. 1/10 for the nested PCR.

The SPSS statistical program generated the probit (predicted proportion positive) versus *C. pneumoniae* concentration with 95 % confidence intervals shown in Table 1. For example, for the nested PCR, a concentration of 0.15 IFU/mL was associated with a probit of 0.30. Thus, repeated enough times, a positive result would be obtained in 30 % of replicates. Conversely, at a concentration of 5 IFU/mL or greater, 9 of 10 replicates would be expected to be positive with either PCR, and a single PCR determination would be positive 90 to 95 % of the time.

In Table 1 and Figure 2, the probit models for the non-nested and nested *C. pneumoniae* PCRs were directly compared. The nested PCR regression line (triangles) is shifted up and to the left of the non-nested PCR line (circles). For any concentration of *C. pneumoniae*, the probability of detection was greater with the nested PCR, and the nested PCR regression curve was statistically significantly different from the non-nested PCR curve. The predicted median detected concentrations (probit = 0.50) were 0.35 IFU/mL (nested) and 0.61 IFU/mL (non-nested), for a relative median detection of 0.58 (95 % CI: 0.31 to 0.99, P = 0.04). Model fit was assessed and adequate (Pearson goodness of fit $X^2 (29) = 34.7$, P = 0.22; parallelism test $X^2 (1) < 0.001$, P = 1.00).

Relationship between detection and number of replicates

For a given probit and its corresponding *C. pneumoniae* concentration, the sensitivity of replicate testing and the number of replicates are related. The number of replicates needed for various probits of 0.01 to 0.99, to achieve an over-all test sensitivity of 50, 80, 90 or 95 %, were calculated using the nested *C. pneumoniae* PCR data (see Table 2). For example, for a *C. pneumoniae* concentration of 0.35 IFU/mL (probit of 0.50), a single PCR determination has 50 % sensitivity, a 3-replicate assay would detect at least one positive with 80 % sensitivity; a 4-replicate assay with 90 % sensitivity; and a 5-replicate assay with 95 % sensitivity. At lower concentrations, more replicates are required for a given over-all test sensitivity. At 0.05 IFU/mL (probit 0.10), a single test achieves 10 % sensitivity, compared with 7 replicates (50 % sensitivity), 16 replicates (80 % sensitivity), or 29 replicates (95 % sensitivity). Conversely, above 5 IFU/mL, a single PCR determination is 95 % sensitive for detecting *C. pneumoniae*, and replicates would not increase sensitivity further.

In Figure 3, the relationship between increasing number of replicates and over-all test sensitivity is presented in graphical form, with the curve for the 10 replicate PCR curve on the far left and that for singlicate PCR on the far right. At higher *C. pneumoniae* concentrations, repeating the sample increases test sensitivity very little. At a lower concentration of 0.1 IFU/mL, singlicate PCR is 20 % sensitive, compared with 40 % for duplicate PCR, 50 % for replicates of 3, 70 % for replicates of 5, and 90 % for replicates of 10.

From the probit model, we predicted that the nested PCR would have better sensitivity than the non-nested PCR for detection of *C. pneumoniae* in clinical specimens, particularly at lower concentrations of target DNA, and at these concentrations replicates of

3, 5 or 10 would identify increasingly more specimens as positive compared with singlicate PCR.

Detection of C. pneumoniae in clinical specimens

We next compared the performance of non-nested and nested PCR assays using clinical specimens. For 10 previously-positive PBMC specimens from coronary angiography patients, the non-nested PCR detected a single positive only on the tenth repeat of that specimen (data not shown). In the first 1, 3, or 5 replicates, no positives were detected. For the 14 nasopharyngeal and 2 sputum specimens, no positives were detected in any of 10 replicates. In summary, the non-nested PCR identified 1 of 26 patient specimens, and 1 of 260 PCR tests, as positive.

With the nested PCR, all 10 PBMC specimens were identified as positive (Table 3). In the first 1, 3 or 5 replicates, 3, 5 and 9 of the 10 specimens were positive, respectively. By comparison, for a *C. pneumoniae* concentration of approximately 0.1 IFU/mL, the probit model predicted 2, 5 and 7 positives in 1, 3 or 5 replicates, respectively (Figure 3). One of the 2 sputa was positive, in 2/10 replicates, and the second sputum was negative in all 10 replicates. The nested PCR detected 4/8 adult NPS specimens (Table 3, specimens Resp A164, A190, A192, and A269), but did not detect any *C. pneumoniae* in 6 pediatric NPS specimens. In summary, the nested PCR detected 15/26 clinical specimens or 34/260 individual PCR determinations, and was superior to the non-nested for the detection of *C. pneumoniae* (15/26 vs. 1/26, P < 0.001). Had only 3 replicates been done per specimen, and defining 1/3 or more as *C. pneumoniae* positive, 0 of 26 non-nested and 6/26 nested specimens were positive (P = 0.03).

Quantitation of C. pneumoniae in clinical specimens

By interpolation from the probit regression analysis for the nested PCR (Table 1), estimates of *C. pneumoniae* in clinical specimens were made (Table 3). For the ten PBMC specimens, median probit was estimated at 0.15, for an interpolated concentration of 0.07 IFU/mL (95 % CI = 0.04 to 0.11). For specific patient specimens, probits varied between 0.1 and 0.9, or 0.05 to 2.7 IFU/mL. The single positive sputum was positive in 2/10 replicates, for a probit of 0.2 (0.09 IFU/mL, 95 % CI: 0.06 to 0.14). In 4/8 adult NPS specimens, 1/10 replicates was positive (probit of 0.10), for an interpolated concentration of 0.05 IFU/mL (95 % CI: 0.02 to 0.08). In the remaining 4 adult, and all pediatric NPS specimens, none of the 10 replicates detected *C. pneumoniae* (< 0.03 IFU/mL). At a probit of < 0.05, the median concentration for the nasopharyngeal specimens was < 0.03 IFU/mL (95 % CI: 0.00 to 0.05).

Discussion

In this paper, we demonstrated that replicate *C. pneumoniae* PCR markedly increased analytical sensitivity compared with performing a single PCR test. We validated the model by demonstrating that replicate testing increased *C. pneumoniae* detection in clinical specimens, particularly with the nested PCR, and that the sensitivity in analytical and clinical samples were consistent with the probit analysis predictions. We then used probit analysis to quantitate *C. pneumoniae* in clinical specimens, and inferred a higher concentration of *C. pneumoniae* in PBMCs compared with NPS.

The interpretation of replicate testing was facilitated by probit regression analysis, which has been utilized in particular for toxicology studies. In microbiology, probit analysis has been used very rarely: we found only four references in a MEDLINE search of the

literature between 1967 and 2000. Vrielink and colleagues used probit analysis to compare the diagnostic sensitivity of enzyme immunoassays for HTLV-I, II, or HCV (22-24), and Saldanha used this regression technique to quantitate HCV genome and compare PCR sensitivity (25).

The sensitivity of a diagnostic test is often considered a constant property, apart from some variation due to laboratory technique or specimen type. However, interpreting the probit as the test sensitivity, we demonstrated that PCR sensitivity varied between 0 and 100 %, depending on *C. pneumoniae* concentration. The finding that PCR was approximately 100 % sensitive above a certain threshold and 0 % sensitive below a certain threshold is not surprising. What is surprising is the 100-fold interval of concentrations (between 4 IFU/mL and 0.04 IFU/mL) in which PCR results were intermittently positive. Within this interval, repeat testing and probit modeling could be exploited for detection and quantitation.

Our findings may have important implications for the routine detection of *C. pneumoniae* in clinical specimens such as blood or respiratory specimens. Conversely, lack of recognition of these concentrations of intermittently positive values may yield unreliable results.

In an excellent review of the molecular diagnosis of *C. pneumoniae*, Boman, Gaydos and Quinn discuss specimen collection, preparation of nucleic acid from samples, choice of gene target and primer selection, optimal amplification conditions, and detection of the amplification product (16). They briefly review sampling variation as a cause of false-negative results, and discuss increasing sample volume as a possible strategy to increase sensitivity, while acknowledging that this strategy may cause an unacceptable increase in the level of PCR inhibitors. We suggest adding the issue of PCR replicates to their list of areas

where standardization is required. Readers need to know how many PCR replicates were done by a laboratory, and how a positive specimen was operationally defined.

We acknowledge two potentially serious limitations of our study: face validity and feasibility. By face validity, we refer to whether most readers or laboratory directors would have confidence that a single positive in 5 or 10 PCR determinations represented a true positive. At a more stringent requirement of 2 PCR positives per 10 replicates, 0/26 clinical specimens were positive by the non-nested PCR, and 6/26 specimens by the nested PCR (P = 0.03). A single positive PCR determination may represent contamination, a non-specific reaction, or a true positive. We demonstrated that lower analytical concentrations were only intermittently PCR positive, and this relationship was predictable from a statistical viewpoint. These results are not likely to be due to contamination, which would not have varied predictably with concentration. In addition, 0/200 negative controls tested with this assay by our laboratory have been positive. To assure specificity of the reaction, all first-time positive PCR clinical specimens were confirmed with Southern blotting and oligonucleotide hybridization, and 12 specimens had DNA sequencing of PCR product. The results all confirmed a *C. pneumoniae*-specific amplification product. If contamination and non-specificity are ruled out, the results are true positives. Nevertheless, we would not consider a single 1 of 10 samples positive as a "confirmed" *C. pneumoniae* positive. In our PBMC study, we verified positive specimens by independent re-extraction, followed by PCR in triplicate (26). As more sensitive assays are developed, confirmation by amplifying a different target will be preferable (27).

Regarding feasibility, we acknowledge that a trade-off may be required in determining the optimal number of replicates for different specimen types. For the

detection of *C. pneumoniae* DNA in clinical specimens, replicates of 2 or 3, depending on the specimen type, may be adequate if the laboratory can demonstrate high reproducibility. Larger number of replicates will likely not be feasible, but the laboratory may wish to test a small number of positives in 5 or 10 replicates to examine reproducibility. In a research setting, replicates of up to 10 may be desirable for specimen types in which *C. pneumoniae* concentration is likely to be low. We currently test blood and respiratory specimens in replicates of 3, but have increased both the concentration (DNA eluted in 50 μL rather than 100 μL) and the sample size (5 μL per 50 μL PCR rather than 2.5 μL per 25 μL PCR) to approximate the same sensitivity as previously achieved with 10 replicates. Nevertheless, we suggest replicate PCR as a "reference standard" only until methods of extraction and detection are improved to the point where single or duplicate PCR will provide comparable sensitivity and reliability. Methods to concentrate target, such as monocytes enrichment using CD14 antibodies (28), or nucleic acid molecule selection using capture probes, may obviate the need for replication altogether.

We conclude that repeat testing of the same specimen markedly increases the sensitivity and reliability of a PCR assay, particularly for clinical specimens with low *C. pneumoniae* concentration. Replicate testing may improve development and comparison of PCRs, and provide more precise estimates of organism prevalence in various chronic disease states. Conversely, failure to recognize the low sensitivity of a single PCR determination may cause frustration when positive results cannot be reliably confirmed. Further validation is needed with other *C. pneumoniae* assays and other clinical specimens.

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Table 6-1. Predicted proportion of replicates positive versus *C. pneumoniae* concentration for non-nested and nested *C. pneumoniae* PCR tests.

Probit[a]	*C. pneumoniae* IFU/mL (95 % CI)	
	Non-nested PCR[b]	Nested PCR[c]
0.01	0.02 (0.01, 0.03)	0.01 (0.003, 0.02)
0.05	0.05 (0.02, 0.08)	0.03 (0.01, 0.05)
0.10	0.08 (0.04, 0.13)	0.05 (0.02, 0.08)
0.15	0.12 (0.07, 0.18)	0.07 (0.04, 0.11)
0.20	0.16 (0.10, 0.24)	0.09 (0.06, 0.14)
0.30	0.27 (0.17, 0.40)	0.15 (0.10, 0.23)
0.40	0.41 (0.28, 0.61)	0.24 (0.16, 0.35)
0.50	0.61 (0.42, 0.92)	0.35 (0.24, 0.52)
0.60	0.92 (0.62, 1.42)	0.53 (0.36, 0.80)
0.70	1.41 (0.94, 2.29)	0.81 (0.55, 1.28)
0.80	2.34 (1.51, 4.08)	1.34 (0.88, 2.23)
0.90	4.69 (2.82, 9.25)	2.70 (1.66, 5.12)
0.95	8.34 (4.68, 18.4)	4.79 (2.77, 10.2)
0.99	24.5 (11.9, 67.8)	14.1 (7.07, 37.3)

[a] Probit, predicted proportion of replicates positive from probit regression analysis (SPSS), based on 10 replicates of 16 dilutions of *C. pneumoniae* ATCC VR-1310 (see text).

[b] Non-nested PCR, Campbell (1992).

[c] Nested PCR, Tong and Sillis (1993).

Table 6-2: Relationship between predicted number PCR replicates to achieve various test sensitivities and *C. pneumoniae* concentration.

Probit[a]	CP conc.[b]	Replicates to achieve sensitivity of[c]			
		50 %	80 %	90 %	95 %
0.01	0.01	69	161	230	299
0.05	0.03	14	32	45	59
0.10	0.05	7	16	22	29
0.20	0.09	4	8	11	14
0.30	0.15	2	5	7	9
0.40	0.24	2	4	5	6
0.50	0.35	1	3	4	5
0.60	0.53	1	2	3	4
0.70	0.81	1	2	2	3
0.80	1.34	1	1	2	2
0.90	2.70	1	1	1	2
0.95	4.79	1	1	1	1
0.99	14.1	1	1	1	1

[a] Probit, predicted proportion of replicates positive from probit regression analysis (SPSS), based on 10 replicates of 16 dilutions of *C. pneumoniae* ATCC VR-1310 (see text).

[b] *C. pneumoniae* ATCC VR-1310 concentration (IFU/mL), calculated from probit analysis.

[c] Replicates calculated in Microsoft Excel spreadsheet from the equation: Probability (at least 1 positive in n replicates) $= 1-(1-\text{probit})^{n}$

Table 6-3. Nested PCR detection and quantitation of *C. pneumoniae* in individual clinical

specimens by number of replicates.

ID	Specimen	Number of PCR replicates				Proportion positive[a]	CP[b] (95 %CI)
		1	3	5	10		
Angio 202B	PBMC[c]	0	0	1	1	0.10	0.05 (0.02, 0.08)
Angio 208B	PBMC	1	1	1	2	0.20	0.09 (0.06, 0.14)
Angio 257B	PBMC	0	0	0	1	0.10	0.05 (0.02, 0.08)
Angio 287A	PBMC	0	0	1	2	0.20	0.09 (0.06, 0.14)
Angio 244B	PBMC	1	2	5	9	0.90	2.70 (1.66, 5.12)
Angio 246C	PBMC	0	1	2	4	0.40	0.24 (0.16, 0.35)
Angio 276A	PBMC	0	0	1	1	0.10	0.05 (0.02, 0.08)
Angio 291B	PBMC	0	0	1	1	0.10	0.05 (0.02, 0.08)
Angio 321B	PBMC	0	1	1	1	0.10	0.05 (0.02, 0.08)
Angio 337C	PBMC	1	3	5	6	0.60	0.53 (0.36, 0,80)
Bronch 10	Sputum[d]	0	1	1	2	0.20	0.09 (0.06, 0.14)
Resp A164	NPS[e]	1	0	0	0	0.10	0.05 (0.02,0.08)
Resp A190	NPS	0	0	0	1	0.10	0.05 (0.02,0.08)
Resp A192	NPS	0	0	0	1	0.10	0.05 (0.02,0.08)
Resp A269	NPS	0	0	0	1	0.10	0.05 (0.02,0.08)

[a] Proportion positive of 10 PCR replicates. All 26 patient samples previously positive at least

1/3 by Tong and Sillis (1993) PCR.

[b] CP: *C. pneumoniae* concentration (IFU/mL) interpolated from probit model of nested PCR

of ATCC VR-1310 dilution series (Probit regression analysis, SPSS). For details see text.

[c] PBMC, peripheral blood mononuclear cells from patients undergoing coronary

angiography.

[d] Induced sputum from patients with chronic airflow limitation. One additional sputum was

negative in all PCR replicates.

[e] NPS: nasopharyngeal swabs from adult with acute respiratory symptoms. An additional 4

adult and 6 pediatric NPS were negative in all PCR replicates.
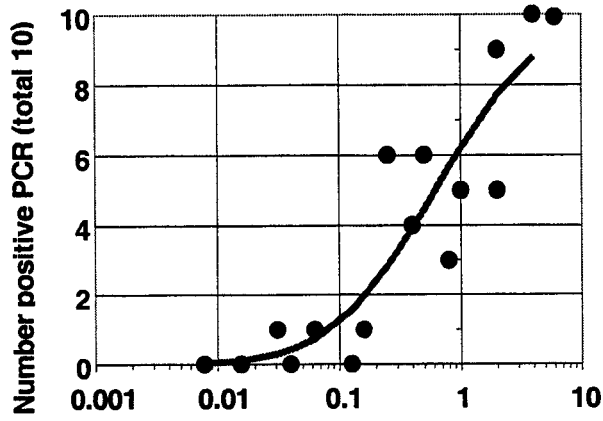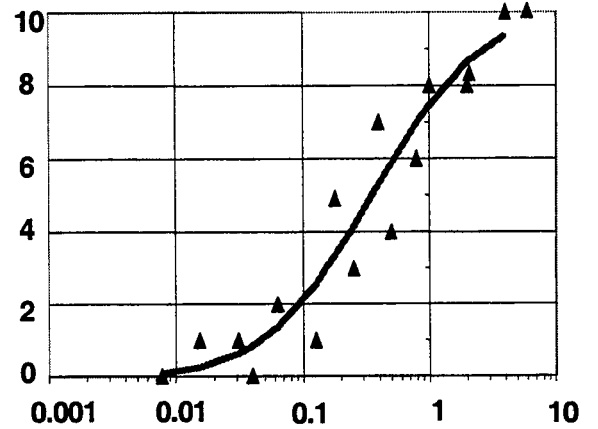
Figure 6-1. Number of positives per 10 replicates vs. concentration of *C. pneumoniae*

ATCC VR-1310 for non-nested PCR (circles, panel A) and nested PCR (triangles, panel

B), and regression curve by probit regression analysis (SPSS).
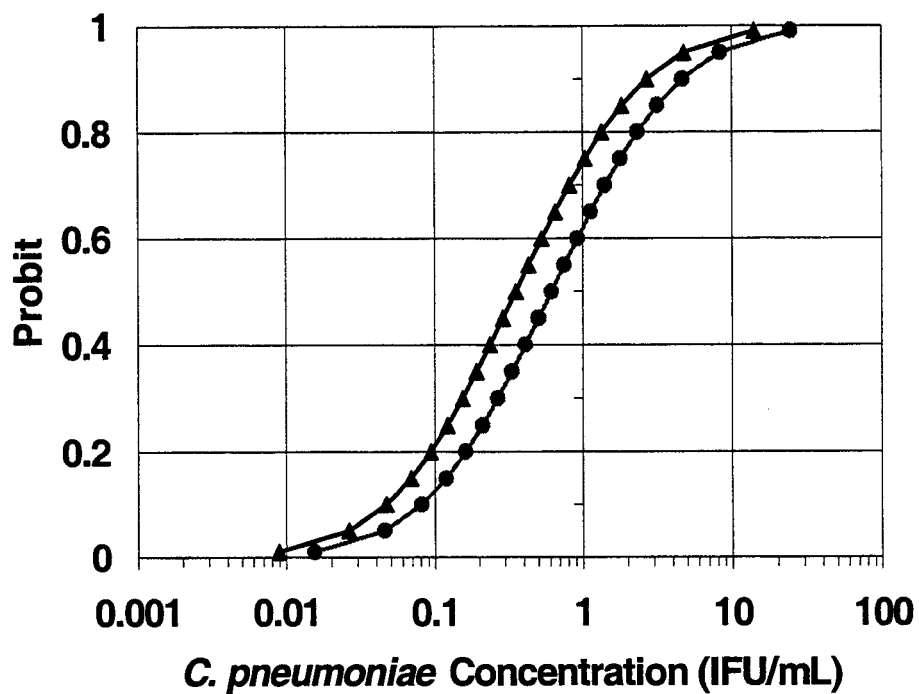
Figure 6-2: Comparison of probit regression curves for a non-nested (circles) and nested

(triangles) *C. pneumoniae* PCR (SPSS). The probit (predicted proportion of replicates

positive) vs. *C. pneumoniae* ATCC VR-1310 concentration (IFU/mL) was obtained from 10

replicates of 16 dilutions (see text).
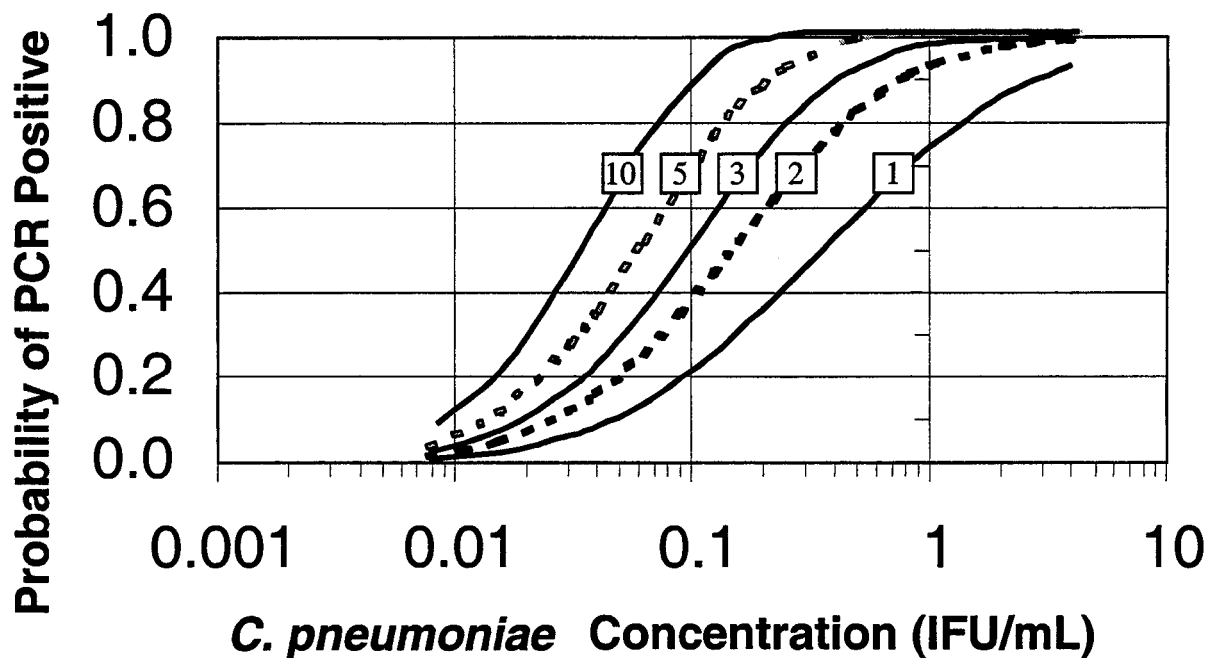
Figure 6-3. Predicted probability of PCR positive test vs. *C. pneumoniae* concentration for

nested PCR by number of replicates. Replicates of 10, 5, 3, or 2 vs. singlicate PCR illustrated

from left to right. Singlicate PCR curve obtained from probit regression analysis of 10

replicates of 16 dilutions of *C. pneumoniae* ATCC VR-1310 (see text). Remaining four curves

calculated in spreadsheet program (Microsoft Excel), using the formula: Probability (at least

1 positive in n replicates) = $1-(1-\text{probit})^{n}$.

Chapter 7

Association with Smoking and Season

In Chapter 7, I present results of a study examining *C. pnuemoniae* DNA detection in patients with chronic obstructive pulmonary disease (COPD). Few of these patients had known cardiovascular disease, and one reason for choosing this study group was to examine C. pneumoniae prevalence in a non-cardiac group of patients.

This study validated the previous observation (Chapter 5) of a relationship between current smoking status and *C. pneumoniae* DNA detection, and between winter/ spring months and DNA detection. We also show that *C. pneumoniae* prevalence is apparently higher in COPD patients than in angiography patients, after adjusting for season and smoking. We further examine the issue of *C. pneumoniae* prevalence in cardiovascular patients compared with other controls in Chapter 8 and 9.

This study was submitted to BMC Infectious Diseases in May 2002. The full citation is:

> **Smieja M**, Leigh R, Petrich A, Chong S, Kamada D, Hargreave FE, Goldsmith CH, Chernesky M, Mahony JB. Detection of *Chlamydia pneumoniae* DNA in clinically stable patients with chronic obstructive pulmonary disease: association with smoking and season. Submitted to BMC Infectious Diseases, May 2002.

This study was presented in part by Dr. Richard Leigh at the American Thoracic Association meeting, Orlando FL, May 2001, and by Dr. Astrid Petrich at the Canadian Association for Clinical Microbiology and Infectious Diseases, Victoria BC, November 2001.

Detection of *Chlamydia pneumoniae* DNA in Clinically Stable Patients with Chronic

Obstructive Pulmonary Disease: Association with Smoking and Season

Running title: *Chlamydia pneumoniae* and COPD

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Abstract:

The role of *Chlamydia pneumoniae* in chronic obstructive pulmonary disease (COPD) remains unclear. Peripheral blood mononuclear cells were obtained from 100 outpatients with smoking-related, clinically stable COPD, and induced sputum was obtained in 62 patients. Patients had mean age (standard deviation) of 65.8 (10.7) years, mean forced expiratory volume in one second of 1.34 (0.61) L, and 61 (61.0 %) were male. *Chlamydia pneumoniae* nucleic acids were detected by nested polymerase chain reaction in 27 (27.0 %). Current smoking or season (November to April) predicted *Chlamydia pneumoniae* DNA (adjusted odds ratio{OR} = 11.1, 95 % confidence interval {CI}: 2.4, 50.5, P = 0.002), and prevalence was higher among COPD patients than among previously-studied patients undergoing coronary angiography (adjusted OR = 2.9, 95 % CI: 1.5, 5.7, P = 0.002). Prospective studies are needed to examine the role of *Chlamydia pneumoniae* nucleic acid detection in COPD disease progression.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and

mortality throughout the world (1). By 2020, the World Health Organization predicts that

COPD will become the 5th (currently 12th) most prevalent disease worldwide, and the 3rd

(currently 6th) most common cause of death (2). Smoking is the major identified risk factor,

and approximately 15 % of smokers develop COPD (1). Smoking cessation reduces the rate

of decline in forced expiratory volumes, but no pharmacological intervention has been

shown to modify the progression of disease. Bacterial and viral infections of the respiratory

tract trigger acute exacerbations of chronic bronchitis, and may contribute to disease

progression (3).

A role for *Chlamydia pneumoniae* in patients with COPD remains unclear. *Chlamydia*

pneumoniae infection, diagnosed serologically, caused 5 to 10 % of acute exacerbations in

patients with chronic bronchitis (4,5) The seroprevalence and geometric mean titres of

Chlamydia pneumoniae IgG (5,6) and IgA (7) antibodies were higher in COPD patients than

controls, irrespective of exacerbation status. Von Hertzen et al measured serum IgG and

IgA, salivary secretory IgA and circulating immune complexes, and found evidence of

"chronic" *Chlamydia pneumoniae* infection in 65 % of COPD patients (8), and recently

Lieberman et al showed an association between *Chlamydia pneumoniae* serology, determined by

a commercial immunoassay, and COPD exacerbation (9). However, the reproducibility and

validity of serologic testing remain uncertain (10).

Given these limitations of serology, the direct detection of *Chlamydia pneumoniae*

nucleic acids may facilitate studies of its role in chronic disease etiology. The presence of

Chlamydia pneumoniae has been directly demonstrated in lung tissue (11), and correlated with

detection of *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells (PBMC) by

PCR (Blasi et al, Proceedings of the Fourth Meeting of the European Society for Chlamydia

Research, Helsinki, Finland, 2000). However, there are limited data regarding the

prevalence of *Chlamydia pneumoniae* DNA in PBMC of COPD patients.

In a previous study, we found that PBMC *Chlamydia pneumoniae* DNA detection

among cardiac patients (12) was associated with current smoking and winter/spring season.

The primary objective of this study was to examine the prevalence and clinical determinants

of *Chlamydia pneumoniae* DNA in COPD patients, and in particular to validate the observed

association with current smoking and season. Secondary objectives were to explore whether

Chlamydia pneumoniae detection was associated with unique symptoms, pulmonary function,

or sputum cell counts, and to contrast the prevalence among respirology and cardiology

patients.

Materials and Methods

Subjects

One hundred consecutive consenting outpatients were recruited from the Firestone

Institute for Respiratory Health at St. Joseph's Healthcare between March 2000 and October

2001. The study protocol was approved by the hospital's Research Ethics Board, and all

subjects gave written consent. Patient characteristics are summarized in Table 1. Patients

were current or former smokers who had a previous diagnosis of smoking-related chronic

airflow limitation, with best forced expiratory volume in one second (FEV_1) of < 70 %

predicted and FEV_1/ Vital Capacity < 70 %, were clinically stable, and had not received

antibiotics in the prior month. Spirometry was obtained before and 10 minutes after

inhalation of 200 µg inhaled salbutamol. Detailed smoking history, symptoms, use of

corticosteroids, and past history of pneumonia, diabetes mellitus, angina pectoris, and myocardial infarction were obtained. Clinical and laboratory data were collected independently by blinded study personnel. Seven non-smoking patients were enrolled, and their spirometry data and sputum cell counts were excluded from the analyses.

Sample Size

A sample size of 100 produces a 95 % confidence interval equal to the sample proportion ± 0.08 when the estimated proportion is 0.20. For an estimated 50 % prevalence of each of current smoking and season, assuming independence, the study had 64 % power to detect an odds ratio (OR) of 5.0 or higher, and 40 % power to detect an odds ratio of 3.0 or higher, for each of these factors as predictors of Chlamydia pneumonia DNA detection, using X^2 test with continuity correction and with a significance level of 0.05, two-tailed (Power Analysis and Sample Size for Windows 2000, NCSS, Kaysville, UT, USA).

Sputum Induction and Examination

Sputum induction and processing was performed as described by Pizzichini et al(13) for the first 73 patients. Sputum induction was discontinued based on low prevalence of Chlamydia *pneumoniae* DNA and to increase enrollment. A differential count of 400 non-squamous cells was performed by experienced laboratory technologists, and a sample of sputum was frozen at –70 °C for PCR testing in 62 patients. Insufficient sputum was available on the remaining 11 patients after performance of sputum cytology.

Blood collection:

Peripheral blood mononuclear cells (PBMC) were obtained by venipuncture into an 8 mL cell preparation tube (CPT, BD Vacutainer Systems, Franklin Lakes, NJ, USA). Within 2 hours of venipuncture, specimens were mixed by inversion and centrifuged at 1500

x g for 30 minutes. The mononuclear cells layer was aspirated and frozen at –70 °C.

Samples were thawed in batches, 200-500 μL aliquots were pelleted and extracted using

QIAamp DNA mini-kits (Qiagen, Mississauga ON) into 50-100 μL of elution buffer.

Detection of Chlamydia pneumoniae DNA:

A 5-10 μL aliquot was amplified in triplicate by a nested polymerase chain reaction

(PCR) targeting the *ompA* gene (14), consisting of 40 amplification cycles for a 333 base pair

product and 30 cycles for a 207 bp product, followed by separation on a 2.0 %

(weight/volume) TAE agarose gel containing ethidium bromide, with ultraviolet light

visualization. The 207 base pair product was confirmed as *Chlamydia pneumoniae* by

hybridization with a specific fluorescein-labeled oligonucleotide probe (ECL, Amersham). A

probe-confirmed positive in any of the three reactions was considered a positive, as

previously described (12, 15). Eight controls, consisting of one low strength positive control

and one intermediate strength positive controls, six negative controls without DNA, and

one additional tube with master mix open to the air throughout specimen addition, were run

for every 13 patient specimens. PCR extraction and amplification were performed in

separate rooms.

Nicotine metabolites assay:

Using the plasma fraction from the citrated CPT tube, nicotine metabolites were

assayed in 73 patients by enzyme immunoassay (Immulite Nicotine Metabolite detection kit,

Diagnostic Products Corporation, Los Angeles CA, USA). The Immulite nicotine metabolite

assay has a calibration range of 10 to 500 μg/L, and non-smokers are defined by a nicotine

metabolite level of < 25 μg/mL.

Statistical analysis:

Continuous variables were tested by unpaired *t*-test or Mann Whitney U test, and proportions were compared using X^2 or Fisher exact tests (unmatched data) or the McNemar test (matched data). Multiple logistic regression modeling (SPSS for Windows 10.0, SPSS Inc., Chicago IL) was undertaken using DNA status (in blood or sputum) as the response variable. *A priori*, an association with current smoking and with winter/spring season was expected. All associations with clinical history, spirometry and cell counts were considered hypothesis-generating. To examine the effect of missing data, smoking and season were analysed after imputation of *Chlamydia pneumoniae* DNA status for PBMC negative subjects in whom sputum data was missing. Imputation was performed based on proportion sputum DNA positive among subjects who were DNA negative in PBMC (SPSS).

Prevalence of PBMC *Chlamydia pneumoniae* DNA in COPD patients was compared with our coronary angiography study(12) using gender, smoking and season-stratified Maentel-Haentzel X^2. Predictors of FEV_1 were modeled by linear regression, for the entire study group and separately by age $\leq$ 65 years or > 65 years. A P-value of < 0.05, two-tailed, was considered statistically significant for the primary hypothesis of an association with current smoking or season, and a P-value of < 0.01, two-tailed, was considered significant for all secondary analyses.

Results

Chlamydia pneumoniae DNA prevalence was 24 of 100 (24.0 %) in PBMC and 7 of 62 (11.3 %) in induced sputum, for a total of 27 of 100 (27.0 %) patients positive in at least

one specimen type. Among 62 subjects from whom both PBMC and sputum were obtained, PBMC detected 21 of 24 (87.5 %) positives, whereas sputum detected 7 of 24 (29.2 %) positives (P = 0.003, McNemar test).

Chlamydia pneumoniae DNA detection in PBMC or sputum by categorical clinical characteristics is tabulated in Table 1, with unadjusted odds ratios, and by continuous clinical and laboratory characteristics in Table 2.

Current smoking (OR = 3.5, 95 % CI: 1.2, 9.9, P = 0.02) and season (November to April, OR = 3.9, 95 % CI: 1.4, 10.9, P = 0.008) were associated with DNA detection, independent of age and gender, whereas male gender was not (OR = 2.2, 95 % CI: 0.8, 6.3, P = 0.14). A month-by-month breakdown for *Chlamydia pneumoniae* DNA positive PBMC is illustrated in Figure 1. The composite of current smoking or season identified 25 of 27 (92.6 %) *Chlamydia pneumoniae* positives, and was strongly associated with DNA detection (age and gender adjusted OR = 11.1, 95 % CI: 2.4, 50.5, P = 0.002). The composite of smoking or season was also associated with DNA detection in PBMC alone (adjusted OR = 9.0, 95 % CI: 2.0, 41.4, P = 0.005).

To further explore the relationship between current smoking status and the detection of *Chlamydia pneumoniae* DNA, we measured plasma nicotine metabolites in 73 subjects. Levels ≥ 25 μg/L indicate current smoking status. At this threshold, seven former smokers were reclassified as current smokers, and one current smoker as a former smoker. Nicotine metabolites ≥ 25 μg/L were associated with *Chlamydia pneumoniae* DNA with OR of 2.1 (95 % CI: 0.8, 5.7, P = 0.13). There was no relationship between higher plasma nicotine levels and the probability of detecting *Chlamydia pneumoniae* DNA (see Figure 2.)

To assess the influence of missing sputum *Chlamydia pneumoniae* DNA status of sputum, imputation of one, three or seven additional sputum positives was performed, corresponding to the proportion of observed sputum DNA positives with 95 % confidence interval among subjects who were PBMC DNA negative. Imputation was performed without regard to smoking or season. The OR associated with season was 3.7 in the non-imputed model, and 3.4, 3.1, and 2.4 respectively for one, three or seven imputed positives, in a model adjusted for smoking, age and gender. The OR for smoking in these models was 3.7, 3.4, 2.6, and 2.0, for the zero, one, three or seven imputed positives, respectively.

Self-reported chronic sputum production was associated with *Chlamydia pneumoniae* DNA detection (age, gender, smoking and season-adjusted OR = 4.2, 95 % CI: 1.1, 16.0, P = 0.04), whereas self-reported chronic cough, use of corticosteroids, or a history of coronary artery disease or diabetes mellitus were not (see Table 1). Chronic sputum production identified 24 of 27 (88.9 %) of patients with *Chlamydia pneumoniae* DNA detected. In a forward selection model including sputum production, smoking, season, gender and age, only sputum production (OR = 5.3, 95 % CI: 1.4, 19.6, P = 0.01) and season (OR = 2.8, 95 % CI: 1.1, 7.5, P = 0.04) were independent predictors. Two-way interactions were not statistically significant.

We examined the association between *Chlamydia pneumoniae* DNA and measures of pulmonary function (Table 2), and sputum cell counts. Pulmonary function was moderately to severely impaired, and no relationship was found with *Chlamydia pneumoniae* DNA detection. Sputum neutrophil, macrophage, and eosinophil counts were not associated with *Chlamydia pneumoniae* detection.

We also examined the association between *Chlamydia pneumoniae* DNA and spirometry by linear regression modeling of FEV_1. Age, gender, current smoking, *Chlamydia pneumoniae* DNA status, and season were included in the model (Table 3). In the entire study group, age and male gender were predictors of FEV_1. We then examined subjects aged 65 years or younger, and those older than 65 years, separately. In the younger age group, age predicted FEV_1, whereas current smoking, male gender and *Chlamydia pneumoniae* DNA status were associated at $P < 0.10$. In the older age group, male gender was associated with FEV_1, but age, current smoking, season or DNA status were not.

PBMC results from the COPD patients in this study were compared with patients from the coronary angiography study reported previously (12). COPD patients were more likely to have *Chlamydia pneumoniae* DNA in PBMC than patients undergoing coronary angiography (24 of 100 versus 24 of 208, adjusted OR = 2.9, 95 % CI: 2.4, 9.9, $P < 0.001$), independent of current smoking status, gender, or season. The highest monthly prevalence was found in March and April, irrespective of year or study group. Among cardiology patients, the *Chlamydia pneumoniae* prevalence in March and April 1999 was 16 % (20 of 122), compared with a prevalence of 5 % (3 of 60) in the months of May to October. Similarly, in COPD patients studied in 2000 and 2001, March and April prevalence was 50 % (9 of 18), compared with 15 % (6 of 41) in the months of May to October. In the combined dataset, season (November to April OR = 4.3, 95 % CI: 2.0, 9.2, $P < 0.001$) and smoking (OR = 2.7, 95 % CI: 1.3, 5.4, $P = 0.005$) predicted *Chlamydia pneumoniae* DNA detection in PBMC, whereas male gender did not (OR = 1.5, 95 % CI: 0.7, 3.2, $P = 0.28$). The combined measure of current smoking or season was associated with DNA detection (OR = 10.1, 95 % CI: 3.0, 33.8, $P < 0.001$).

Discussion

Among clinically stable patients with smoking-related COPD and moderate to severe pulmonary function impairment, we detected a high prevalence of *Chlamydia pneumoniae* DNA. Peripheral blood (PBMC) specimens were superior to induced sputa, and an association between *Chlamydia pneumoniae* DNA detection, current smoking and winter/spring months was demonstrated. Chronic sputum production was associated with *Chlamydia pneumoniae*, but we found no clear association with other clinical symptoms, measures of lung function, or sputum cell counts.

Data on the prevalence of *Chlamydia pneumoniae* DNA in PMBC of COPD patients have been published solely in abstract form. Black et al found a prevalence of 14 of 20 (70.0 %) Australian patients, using whole blood or PBMC (Black et al, Proceedings of the Fourth Meeting of the European Society for Chlamydia Research, Helsinki, Finland, 2000). Blasi et al found a prevalence of 10 among 21 (47.6 %) Italian patients, compared with 11 of 21 (52.4 %) who had *Chlamydia pneumoniae* DNA in trans-bronchial lung biopsy specimens (Blasi et al, Proceedings of the Fourth Meeting of the European Society for Chlamydia Research, Helsinki, Finland, 2000). Our prevalence of 24.0 % in PBMC of Canadian COPD patients is within the confidence intervals of the previous estimates.

We found a low prevalence (11.3 %) of *Chlamydia pneumoniae* DNA in induced sputum among patients with chronic stable COPD. By contrast, Von Hertzen et al detected DNA in spontaneous sputum of 59.0 % of patients with severe COPD (8), and Black et al detected DNA in 94.0 % of patients hospitalized for COPD exacerbation (Black et al, Proceedings of the Fourth Meeting of the European Society for Chlamydia Research, Helsinki, Finland, 2000). We obtained induced sputum to measure sputum cell counts by a

validated methodology, but spontaneous sputum may be superior for routine *Chlamydia pneumoniae* detection. Conversely, sputum positivity may be higher during acute exacerbations, and our study intentionally excluded such patients. Our data support the utility of PBMC in preference to sputum for *Chlamydia pneumoniae* DNA detection.

A clearer relationship between *Chlamydia pneumoniae* and smoking is emerging. Hahn first proposed that smoking was a potential confounder of the association between *Chlamydia pneumoniae* antibody levels and cardiovascular disease (16), and Von Hertzen et al showed that smoking was associated with higher *Chlamydia pneumoniae* IgA levels in smoking-discordant twins (17). This study has validated our previous observation that current smoking was associated with a higher prevalence of *Chlamydia pneumoniae* nucleic acid detection (12). Whether *Chlamydia pneumoniae* associations with chronic diseases such as COPD or coronary artery disease are confounded by inaccurate ascertainment of smoking status, or whether infection is directly involved in the causal pathway for smoking-associated diseases, will require careful investigation.

Annual *Chlamydia pneumoniae* seasonal cycles have not, to our knowledge, been previously reported. Among both cardiology and respirology patients, we found peak prevalence in the winter/spring months, particularly in March and April, over three consecutive winters. Whether similar seasonal variation is present in other geographic areas is not known.

We identified no clear relationship between FEV_1 and *Chlamydia pneumoniae* DNA detection. In the sub-group of patients aged 65 years or younger, current smoking and *Chlamydia pneumoniae* DNA status were each associated with a reduced of FEV_1 approximately 400 mL, although neither was nominally significant at a level of $P < 0.05$. A

larger sample size may allow more precise estimation of this relationship, although only a prospective study design will adequately determine whether DNA detection is associated with greater decline in FEV_1. Our data suggest that a study enrolling subjects 65 years or younger may have greater power for examining whether *Chlamydia pneumoniae* is associated with lung function decline independently of smoking status.

We identified self-reported chronic sputum production as a strong risk factor for *Chlamydia pneumoniae* DNA detection. However, this association requires validation, and was not significant at $P < 0.01$ as pre-specified for secondary analyses. Nevertheless, *Chlamydia pneumoniae* DNA detection in stable COPD patients may be associated with respiratory symptoms.

In conclusion, *Chlamydia pneumoniae* was prevalent in patients with COPD and associated with current smoking and winter/spring season, and may be associated with chronic sputum production. *Chlamydia pneumoniae* DNA detection in PBMC may facilitate the undertaking of large prospective epidemiologic studies examining the role of *Chlamydia pneumoniae* in COPD etiology and progression.

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Table 7-1. Patient characteristics by *Chlamydia pneumoniae* DNA detection status.

	Entire Study Group (%)	DNA [a] Present (%)	DNA Absent (%)	Unadjusted OR [b] (95 % CI)
Number	100	27 (27.0)	73 (73.0)	
Male	61 / 100 (61.0)	20 / 27 (74.1)	41 / 73 (56.2)	2.2 (0.8, 5.9)
Current smoking	32 / 100 (32.0)	13 / 27 (48.1)	19 / 73 (26.0)	2.6 (1.1, 6.6)
Season (Nov. to April)	44 / 100 (44.0)	18 / 27 (66.7)	26 / 73 (35.6)	3.6 (1.4, 9.2)
Smoker or Season	63 / 100 (63.0)	25 / 27 (92.6)	38 / 73 (52.1)	11.5 (2.5, 52.2)
Chronic cough	50 / 98 (51.0)	16 / 26 (61.5)	34 / 72 (47.2)	1.8 (0.7, 4.5)
Chronic sputum	64 / 99 (64.6)	24 / 27 (88.9)	40 / 72 (55.6)	6.4 (1.8, 23.2)
Pneumonia in 1 year	9 / 98 (9.2)	2 / 26 (7.7)	7 / 72 (9.7)	0.8 (0.2, 4.0)
Prednisone	18 / 98 (18.4)	3 / 27 (11.1)	15 / 71 (21.1)	0.5 (0.1, 1.8)
Inhaled corticosteroids	67 / 98 (68.4)	19 / 27 (70.4)	48 / 71 (67.6)	1.1 (0.4, 3.0)
Coronary artery disease	10 / 92 (10.9)	2 / 27 (7.4)	8 / 65 (12.3)	0.6 (0.1, 2.9)
Diabetes mellitus	5 / 92 (5.4)	2 / 27 (7.4)	3 / 65 (4.6)	1.7 (0.3, 10.5)

[a] *Chlamydia pneumoniae* DNA detected by nested polymerase chain reaction (14).

[b] Unadjusted odds ratios (OR) with 95 % confidence intervals (SPSS).

Table 7-2: Spirometry[a] and sputum cell counts by *Chlamydia pneumoniae* DNA detection status among 92 patients with chronic obstructive pulmonary disease.

	Number of Subjects	Entire Study Group	DNA[b] Present	DNA Absent	P-value (t-test)
Age in years (SD)	93	65.8 (10.7)	66.9 (11.9)	65.3 (10.3)	0.53
Smoking pack-years (SD)	93	43.1 (25.1)	48.9 (29.3)	41.8 (24.1)	0.22
FEV$_1$[c] (SD), L	93	1.35 (0.61)	1.34 (0.64)	1.36 (0.61)	0.88
FEV$_1$ % predicted (SD)	93	46.9 (16.8)	44.3 (15.1)	48.0 (17.5)	0.33
FEV$_1$/VC[d] % (SD)	93	45.7 (15.4)	42.6 (15.8)	47.0 (15.2)	0.22
Total cell count, 10^6/g (SD)	73	10.9 (15.4)	9.9 (12.5)	11.4 (16.8)	0.70
Viability, % (SD)	73	61.5 (24.2)	60.4 (22.7)	62.0 (25.0)	0.79
Squamous Epithelial, % (SD)	73	3.8 (8.6)	3.7 (5.3)	3.9 (10.0)	0.92
Neutrophils, % (SD)	73	63.6 (25.3)	60.6 (27.2)	65.2 (24.5)	0.46
Macrophages, % (SD)	73	25.6 (19.2)	26.4 (21.2)	25.2 (18.3)	0.78
Eosinophils, median % (Inter-quartile range)[e]	73	1.2 (3.0)	1.2 (3.7)	1.0 (2.9)	0.71[e]

[a] All spirometry after inhalation of 200 μg salbutamol.

[b] *Chlamydia pneumoniae* DNA detected in peripheral blood mononuclear cells or induced sputum by nested polymerase chain reaction (14)

[c] FEV$_1$: forced expiratory volume in 1 second

[d] VC: vital capacity

[e] Comparison by Mann Whitney U test (SPSS).

Table 7-3: Clinical predictors of forced expiratory volume in one second among 93 patients with chronic obstructive pulmonary disease.

	Beta Coefficient (95 % Confidence Interval)[a]		
Risk Factor	Entire Study Group	Age ≤ 65 years (n = 34)	Age > 65 years (n = 59)
Age (L/year)	-0.03 (-0.04, -0.01)[b]	-0.05 (-0.08, -0.02)[b]	-0.01 (-0.04, 0.02)
Male	0.43 (0.21, 0.66)[b]	0.35 (-0.03, 0.74)[d]	0.41 (0.12, 0.70)[c]
Current Smoker	-0.17 (-0.41, 0.08)	-0.40 (-0.80, 0.00)[d]	-0.05 (-0.36, 0.27)
Chlamydia pneumoniae DNA[e]	-0.16 (-0.41, 0.10)	-0.40 (-0.86, 0.06)[d]	-0.10 (-0.43, 0.23)
November to April	0.19 (-0.04, 0.41)	0.19 (-0.20, 0.57)	0.17 (-0.12, 0.47)

[a] Multiple linear regression beta coefficient (litres) associated with the presence of clinical variables for prediction of FEV_1 (SPSS). Age in years, all other variables coded as present (1) or absent (0).

[b] $P < 0.001$

[c] $P < 0.05$

[d] $P < 0.10$

[e] *Chlamydia pneumoniae* DNA detected in peripheral blood mononuclear cells or induced sputum by nested polymerase chain reaction (14)
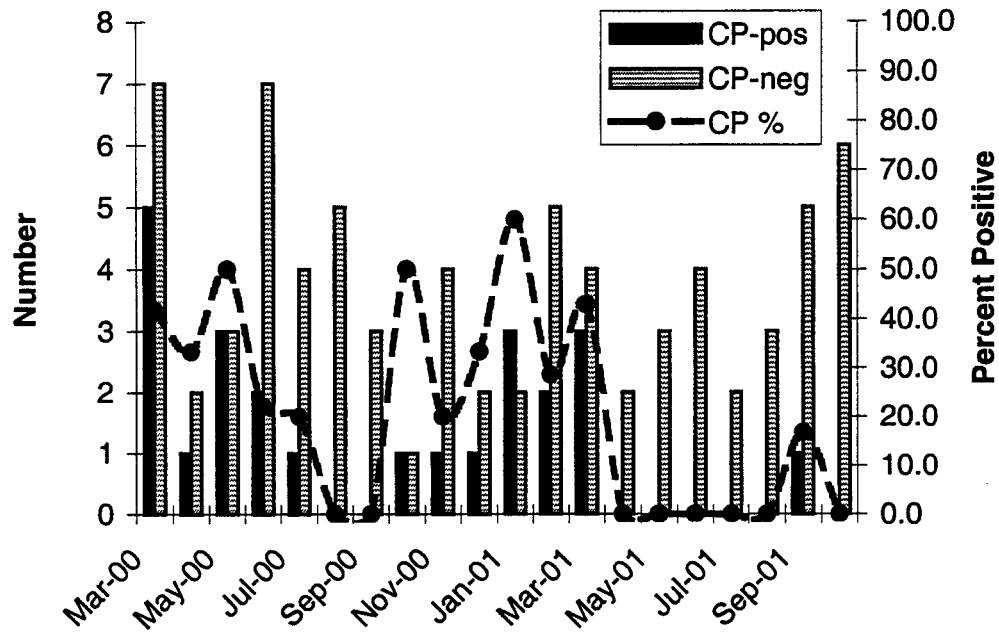
Figure 7-1. Relation of *Chlamydia pneumoniae* (CP) DNA prevalence in blood peripheral mononuclear

cells versus month of study visit among 100 patients presenting to a respirology referral clinic. CP-

DNA positive (solid), CP-DNA negative (thatched) or proportion CP-DNA positive (line).
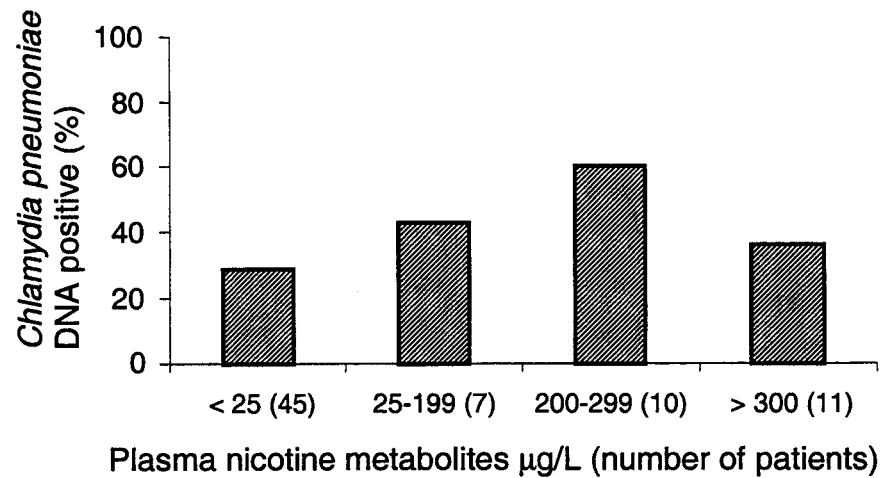
Figure 7-2. Relation of *Chlamydia pneumoniae* DNA prevalence in blood or sputum versus plasma

nicotine metabolite levels among 73 patients with stable chronic obstructive pulmonary disease.

Nicotine metabolites of < 25 µg/L indicate no recent exposure to smoking. For nicotine

metabolites ≥ 25 µg/L, odds ratio = 2.1 (95 % CI: 0.8, 5.7, P = 0.13) for detection of *Chlamydia*

pneumoniae DNA (SPSS).

Chapter 8

Angioplasty Patients *versus* Controls

In Chapter 5 and 7, we demonstrated that *C. pneumoniae* DNA detection was associated with current smoking and winter/spring season. In chapter 7, we found the prevalence among COPD patients to higher than among coronary angiography patients, in a non-concurrent ananlysis adjusted for age, gender, smoking and month of enrollment. Thus, *C. pneumoniae* prevalence was not apparently associated with cardiovascular disease per se, but rather with smoking. In this chapter, we further explore the question of whether circulating *C. pneumonaie* DNA prevalence in higher in patients with established coronary artery disease, compared with family practice controls.

We found a very low prevalence of circulating *C. pneumoniae* DNA among coronary angioplasty patients compared with family practice controls. The current chapter will be split eventually into two separate manuscripts: one comparing the angioplasty patients with family practice controls recruited concurrently, and a second paper analyzing the prevalence and determinants of *C. pneumoniae* DNA detection in 275 family practice patients (to be completed by July, 2002). The former will be submitted to Circulation or the American Heart Journal, with M. Smieja as first author, and the latter to an undetermined infectious diseases journal, with either M. Smieja or J. Munoz as first author. The family practice study was presented in part by Dr. Juan Munoz at the North American Primary Care Research Group 29th Annual Meeting, October 2001, Halifax NS.

Prevalence of Circulating *Chlamydia pneumoniae*-DNA and Cytomegalovirus-DNA in

Patients Undergoing Coronary Angioplasty versus Family Practice Controls

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Abstract:	240
Background:	176
Methods:	921
Results:	537
Discussion:	794
TOTAL Text:	2668

Abstract:

Background: Serologic tests have not established a clear role for infections in atherosclerotic heart disease, whereas preliminary data indicate that direct detection of nucleic acids may be associated with disease. Our objective was to determine whether circulating *Chlamydia pneumoniae* (CP)-DNA and cytomegalovirus (CMV)-DNA were more common among patients undergoing coronary angioplasty, compared with family practice controls.

Methods and Results: Peripheral blood mononuclear cells were obtained from 310 consecutive patients undergoing elective coronary angioplasty, and from 103 family practice controls obtained by stratified sampling. Nucleic acids of CP and CMV were detected by polymerase chain reaction, and confirmed by hybridization. CP-DNA prevalence was 13 of 310 (4.2 %) among coronary angioplasty patients versus 31 of 103 (30.1 %) among family practice controls (age, gender, smoking and season-adjusted odds ratio {OR} = 0.1, 95 % confidence interval {CI}: 0.04, 0.2, P < 0.001). CP-DNA was associated with season (February to April, OR = 6.2, 95 % CI: 2.9, 13.3, P < 0.001), and inversely associated with the use of anti-platelet and lipid lowering agents (OR = 0.4 per agent, 95 % CI: 0.1, 0.9, P = 0.03). Circulating CMV-DNA was detected in 18 of 310 (5.8 %) of angioplasty patients versus 13 of 103 (12.6 %) family practice patients (adjusted OR = 0.4, 95 % CI: 0.2, 0.9, P = 0.03).

Conclusions: Circulating CP-DNA and CMV-DNA were less common among coronary angioplasty patients compared with family practice controls. While these data suggest that these infections are not associated with established cardiovascular disease, a potential protective role of anti-platelet and lipid-lowering drugs requires further investigation.

Key words: Infection, detection, polymerase chain reaction, coronary artery disease,

angioplasty

Background

A role for infections in human atherosclerotic heart disease remains unproven, in part due to the limitations of using sero-epidemiology for the investigation of prognosis and attributable risk (1;2). Molecular epidemiologic tools such as the detection of circulating *Chlamydia pneumoniae* (CP) or cytomegalovirus (CMV) nucleic acids, hold the promise of clarifying the relationship between infections and heart disease, but the methods are technically difficult and few published studies have appeared to date (3-7).

We recently demonstrated a prevalence of 11.3 % and 17.3 % for CP-DNA and CMV-DNA, respectively, among 208 patients undergoing coronary angiography or angioplasty (6). In that cross-sectional study, the presence of CP-DNA was associated with season (February to April compared with May to October) and current smoking status, but no relationship was found with presence or extent of coronary atherosclerosis.

The primary objective of this study was to determine whether the prevalence of circulating CP-DNA and CMV-DNA is higher in patients undergoing coronary angioplasty, compared with family practice controls. Secondary objectives were to examine the relationship between DNA detection and cardiovascular risk factors.

Materials and Methods

Coronary Angioplasty Patients: Consecutive elective outpatients scheduled for coronary angioplasty at the Hamilton Regional Angiography Suite, Hamilton Health Sciences Corporation, Hamilton ON, Canada between December 2001 and July 2002 were approached for participation in a phase 2, randomized controlled trial of dalteparin (Fragmin) versus unfractionated heparin peri-procedurally. Details of the study design, patient characteristics, and study outcome are described separately (Natarajan M et al,

manuscript in preparation). Of 322 patients in the randomized study, blood samples were obtained on 310 (96.3 %) and form the basis of the current study. Study nurses obtained information regarding age, gender, and a history of previous cardiac disease, smoking, diabetes mellitus, hyperlipidemia, hypertension and cardiac medications (anti-platelet agents and lipid-lowering agents) from patient interview and from the medical record. All clinical data were collected by study nurses blinded to laboratory data (see Table 1). Briefly, patients had mean (SD) age of 63.0 (11.3) years, 216 (69.7 %) were men, 66 (21.3 %) were current smokers, 77 (24.8 %) had a history of diabetes mellitus, 209 (67.4 %) a history of hypertension, and 275 (88.7 %) gave a history of dyslipidemia. All participating patients gave written consent, and the study protocol was approved by Research Ethics Board at the Hamilton Health Sciences. A study size of 300 cardiology and 100 family practice patients was designed to detect an odds ratio of 3.0, assuming 25 % CP-DNA prevalence among cardiology patients, with power of 90.1 % and alpha of 0.05, two-tailed. For an OR of 2.0, power was estimated at 59 %.

Family Practice Patients: Consecutive family practice patients aged 40 years and older attending one of two family practitioners affiliated with McMaster University were approached to participate in a circulating *C. pneumoniae* DNA prevalence study. Eligible patients were stratified according to smoking status: from the daily list of patient visits, we approached every 2nd eligible smoking and every 3rd non-smoking patient presenting for annual general physical examination, or for any symptomatic or follow-up visit unrelated to acute cardiorespiratory symptoms. Enrollment started in December 2000, concurrent with the Angioplasty study described previously, and continued to May 2002, with a total of 275 patients enrolled. The 103 patients who were enrolled between December 2000 and July

2001 are included in this study, to enable direct comparison with cardiology patients.

Limited demographic and health status data were obtained, including age, gender, smoking,

alcohol use, cardiac and respiratory history, and self-assessed health status. Briefly, patients

had mean (SD) age of 59.7 (9.0) years, 54 (52.4 %) were men, 28 (27.2 %) were current

smokers, and 10 (9.7 %) had a history of myocardial infarction or angina pectoris (see Table

1). To examine the relationship between infection and general health, patients were asked to

grade their health status, compared to their same-aged peers, as excellent, very good, good,

fair or poor.

Blood collection: Circulating peripheral blood mononuclear cells were obtained by

venipuncture into an 8 mL Vacutainer Cell Preparation Tube (CPT, BD Vacutainer Systems,

Franklin Lakes, NJ). Laboratory personnel processed CPT tubes generally within 2-4 hours

of venipuncture, according to the manufacturer's instructions. Briefly, CPT tubes were

centrifuged at 1500 *g* for 30 minutes, and the 1 mL of buffy coat divided into two aliquots of

500 μL. The first of these two aliquots was pelleted and resuspended in phosphate-buffer-

saline (PBS), the second resuspended in DMSO-fetal calf serum. Samples were frozen at −70

°C. In batches, mononuclear cell preparations were thawed and 200 μL aliquots extracted

using QIAamp DNA mini-kits (Qiagen, Mississauga ON) into 100 μL of elution buffer.

Detection of CP-DNA: Laboratory staff were blinded to all clinical data. Three separate 10 μL

aliquots were amplified by a nested polymerase chain reaction (PCR) (8), consisting of 40

amplification cycles for a 333 base pair (bp) product and 30 cycles for a 207 bp product,

followed by separation on a 2.0 % (w/v) agarose gel containing ethidium bromide, and

ultraviolet light visualization. The 207 bp product was confirmed as *C. pneumoniae* by

hybridization with a specific fluorescein-labeled oligonucleotide probe. Any of three was

considered a true positive, confirmed by hybridization, to account for sampling differences at low levels of DNA, as described previously (6;9). Six controls, consisting of one positive control, four negative controls without DNA, and one additional tube with master mix open to the air throughout specimen addition, were run for every 48 specimens. PCR extraction and amplification were performed in separate rooms, with standard precautions for prevention of amplicon contamination.

Detection of CMV-DNA: Amplification for CMV DNA was performed by a non-nested PCR procedure targeting an immediate early gene (10), using a single 10 µL aliquot of the extracted DNA, and confirmed by agarose gel electrophoresis, visualization of a 123 bp product, and hybridization with a specific oligonucleotide probe.

Statistical analysis: Proportions were compared using X^2 and continuous data with Student's *t*-test. Logistic regression modeling (SPSS for Windows 10.0, Chicago IL) was undertaken using DNA status as the response variable. The pre-specified model examined the following explanatory variables: age, gender, smoking, and season (dichotomized as February to April versus all other months). In addition, cardiovascular risk factors and medications which were potentially statistically significant in unadjusted analyses ($P < 0.10$) were examined in a model adjusted for age, gender, smoking and season. To further explore the relationship between medications and *C. pneumoniae* DNA prevalence, a score of the total number of anti-platelet or lipid lowering agents (0-3 points) was modeled as an independent ordinal variable. A P-value of < 0.05, two-tailed, was considered statistically significant.

Results

Patient characteristics

A comparison of patient characteristics is summarized in Table 1. Angioplasty patients were older than family practice controls, a higher proportion were male, and fewer were current smokers. A greater proportion of family practice patients were enrolled between February and April. Data on diabetes mellitus, hypertension, body mass index, dyslipidemia, and use of lipid lowering or anti-platelet drugs were obtained for angioplasty, but not for family practice, patients.

Chlamydia pneumoniae DNA detection

CP-DNA was detected by PCR in peripheral blood mononuclear cells of 13 of 310 (4.2 %) of angioplasty patients, compared with 31 of 103 (30.1 %) family practice controls (unadjusted odds ratio {OR} = 0.1, 95 % CI: 0.05, 0.2, $P < 0.001$, see Table 1). Table 2 details the pre-specified model combining angioplasty and family practice patients, adjusted for age, gender, current smoking status and season (February-April versus all other months), with adjusted OR of 0.1 (95 % CI: 0.04, 0.2, $P < 0.001$) for the association of angioplasty patient status with CP-DNA detection. Season, but not current smoking, was a strong predictor of CP-DNA detection (OR = 5.6, 95 % CI: 2.5, 12.7, $P < 0.001$).

CP-DNA determinants were also modeled separately for angioplasty (Table 3) and family practice subjects (Table 4). Among angioplasty patients, season alone was an independent predictor of CP-DNA detection. Anti-platelet agents and lipid lowering agents were associated with a point estimate of a lower CP-DNA prevalence, and a score of the total number of anti-platelet or lipid lowering agents (0-3 points) was inversely associated

with CP-DNA detection (OR = 0.4 per agent, 95 % CI: 0.1, 0.9, P = 0.03). On adjustment

for age, gender, smoking and season, total anti-platelet had an OR of 0.4 (0.1, 1.1, P = 0.06).

Among family practice patients, cardiovascular risk factors were not obtained. A

history of angina pectoris (n = 10) or previous myocardial infarction (n = 4) was obtained in

10 patients, with an OR of 1.6 (95 % CI: 0.4, 6.2, P = 0.48) for the association with CP-

DNA in unadjusted analyses (data not shown). Season (OR=6.0) and poor self-assessed

health status (OR=2.7, assessed as fair or poor versus other categories) were associated with

CP-DNA detection in unadjusted analyses. Alcohol and smoking were modeled with an

interaction term, which indicated that interaction was plausible. In the full model (Table 4),

season (OR=15.3), cardiac history (OR=8.3), and poor health (OR=6.6) were all associated

with increased odds of CP-DNA detection. The smoking-by-alcohol interaction indicated

that alcohol may be protective of CP-DNA in the absence of smoking.

Cytomegalovirus DNA detection

Circulating peripheral blood mononuclear cell CMV-DNA was detected by PCR in

18 of 310 (5.8 %) angioplasty patients versus 13 of 103 (12.6 %) of family practice controls

(unadjusted OR = 0.4, 95 % CI: 0.2, 0.9, P = 0.03, see Table 1). In the model adjusted for

age, gender, smoking and season, angioplasty status remained inversely associated with

CMV-DNA detection (OR = 0.5, 95 % CI: 0.2, 0.98, P = 0.049, see Table 2). Age, gender,

smoking and season were not associated with CMV-DNA detection. No other

cardiovascular risk factors were associated with CMV-DNA detection in angioplasty

patients, apart from a potential inverse relationship with dyslipidemia (Table 3).

Discussion

In this cross-sectional comparative study, we found that circulating peripheral blood mononuclear cell DNA detection of two micro-organisms putatively associated with cardiovascular disease, *Chlamydia pneumoniae* and cytomegalovirus, were less prevalent among coronary angioplasty patients than among family practice controls.

The detection of CP-DNA circulating in peripheral blood mononuclear cells was first reported from Sweden in 1998 by Boman and colleagues, who found that 59 % of 101 heart disease patients and 46 % of 52 blood donor controls were CP-DNA positive (3). Wong and colleagues examined over 1,000 patients undergoing coronary angiography, and found an association among men but not among women (5). Among 804 men undergoing coronary angiography, the prevalence of CP-DNA was 9 % in those with heart disease versus 3 % in those without heart disease. The low prevalence found in the current study is similar to that in Wong's study (5)and in our previous study (6). The much lower prevalence among cardiac patients compared with family practice controls appears to contradict the infectious theory of a possible role of *C. pneumoniae* in human atherosclerosis, and possible explanations are required.

The suppression of *C. pneumoniae* infection by cardiac medications is an intriguing explanation for the low observed CP-DNA. *In vitro* data showed that statin drugs modify the inflammatory response to *C. pneumoniae* infection by macrophages and endothelial cells (11), and platelets may be activated by *C. pneumoniae* (12). Whether statin drugs or anti-platelet agents affect chlamydial growth has not been investigated. In the current study, all patients were on at least one anti-platelet agent, and 79 % were on statin drugs. A score based on the number of anti-platelet and lipid lowering agents was inversely associated with CP-DNA

detection, but not with CMV-DNA detection. This relationship was of borderline statistical significance after adjustment for potential confounders. This finding should be interpreted as hypothesis generating, and requires validation in other study groups. It is also possible that these drugs may interfere with nucleic acid testing, and we will examine this in future. Regardless of whether these drugs modify *C. pneumoniae* prevalence, prospective observational studies are needed to determine whether *C. pneumoniae* detection has clinical importance. The current study group will be followed for 30 day and 1 year clinical outcome, and a future analysis will examine whether CP-DNA and CMV-DNA detection is of any prognostic importance.

We have previously shown that season (winter/spring months) and current smoking were strongly associated with CP-DNA detection in both cardiac (6) and respirology study groups (13). In the current study, season remained strongly associated but current smoking status was not. We hypothesize that winter/spring detection represents new acquisition of infection (incident cases), whereas out-of-season detection represents prevalent cases, and that only the latter are associated with smoking. In the current study, very few out-of-season cases were detected, and the study lacked the power to show an association with smoking.

Among family practice patients, we had the opportunity to study two other factors potentially associated with CP-DNA detection. We examined whether alcohol protected against CP-DNA detection, theorizing that upper respiratory tract colonization would be inhibited by alcohol consumption. An interaction with smoking was found, which we interpret as follows. Smoking overpowers any protective effect of alcohol, and smoking remains a risk factor for CP-DNA detection. In the absence of smoking, alcohol was inversely associated with CP-DNA detection (OR = 0.2, 95 %: CI: 0.0, 0.8, P = 0.02),

validating our hypothesis of protection against pharyngeal colonization. We will continue recruiting family practice patients to a total sample size of 200, and will then have adequate power to address the smoking and alcohol interactions more precisely. A second novel association found in the family practice patients was an association with worse self-assessed health status. This health status measure is a single item from the SF-36 questionnaire, which has been previously found to be a strong predictor of all-cause mortality (14;15). In the current study, patients who categorized their health as fair or poor were much more likely to have CP-DNA detected in their bloodstream. No association was found between self-assessed health status and CMV-DNA (data not shown).

CMV-DNA detection has, to our knowledge, been studied among non-immunocompromised cardiac patients only in the single published study by our group (6), where we found a prevalence of 17.3 %. In the current study, the lower CMV-DNA prevalence may be due to sampling blood once, rather than the three blood samples in the previous study. The current study provides important information by providing control patients in whom CMV-DNA was determined by identical means. We found that CMV-DNA detection was no higher, and may be lower, among cardiology patients. The only clinical correlate of CMV-DNA detection was a history of dyslipidemia, but this may represent a chance finding.

In summary, neither CP-DNA nor CMV-DNA detection in peripheral blood was associated with coronary atherosclerosis. We are currently studying whether CP-DNA or CMV-DNA detection affects clinical cardiovascular outcomes.

Acknowledgements:

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Table 8-1. Patient characteristics among coronary angioplasty and family practice patients.

	Coronary Angioplasty	Family Practice	P-value[a]
Number	310	103[b]	
Age, mean (SD), years	63.0 (11.3)	59.7 (9.0)	0.01
Male	216 (69.7 %)	54 (52.4 %)	0.002
Current smoker	66 (21.3 %)	28 (27.2 %)	0.24
Former smoker	136 (43.9 %)	-[c]	-
February-April	123 (39.7 %)	54 (52.4 %)	0.02
History of angina or MI	310 (100 %)	10 (9.7 %)	< 0.001
Diabetes mellitus	77 (24.8 %)	-	-
Hypertension	209 (67.4 %)	-	-
Dyslipidemia	275 (88.7 %)	-	-
Statin drug use	246 (79.4 %)	-	-
ASA	296 (95.5 %)	-	-
Ticlopidine/clopidogrel	277 (89.4 %)	-	-
CP-DNA[d]	13 (4.2 %)	31 (29.8 %)	< 0.001
CMV-DNA[d]	18 (5.8 %)	13 (12.6 %)	< 0.05

[a] Comparison by t-test or X^2 (SPSS).

[b] Clinical data were missing for one patient.

[c] These factors were not coded for family practice patients.

[d] Detection of Chlamydia pneumoniae (CP)-DNA or cytomegalovirus (CMV)-DNA by polymerase chain reaction in peripheral blood mononuclear cells of 103 patients (see text).

Table 8-2. Determinants of circulating CP-DNA and CMV-DNA detection in 310

angioplasty and 103 family practice patients

	Odds Ratio (95 % Confidence Intervals)			
	CP-DNA[a]		CMV-DNA[a]	
	Unadjusted	Adjusted [b]	Unadjusted	Adjusted [b]
Angioplasty	0.1 (0.05, 0.2)[c]	0.1 (0.04, 0.2)[c]	0.4 (0.2, 0.9)[d]	0.5 (0.2, 0.98)[d]
Age (per year)	1.00 (0.97, 1.03)	1.02 (0.98, 1.06)	0.98 (0.95, 1.02)	0.98 (0.95, 1.02)
Male	0.8 (0.4, 1.5)	1.4 (0.6, 2.9)	1.0 (0.5, 2.1)	1.1 (0.5, 2.5)
Smoking	1.3 (0.7, 2.7)	1.4 (0.6, 3.3)	0.6 (0.2, 1.7)	0.6 (0.2, 1.5)
February-April	6.2 (2.9, 13.3)[c]	5.6 (2.5, 12.7)[c]	1.9 (0.9, 4.1)	1.8 (0.8, 3.8)

[a] Adjustment by multiple logistic regression (SPSS).

[b] Detection of *Chlamydia pneumoniae* (CP)-DNA or cytomegalovirus (CMV)-DNA by

polymerase chain reaction in peripheral blood mononuclear cells (see text).

[c] $P < 0.001$

[d] $P < 0.05$

Table 8-3. Determinants of CP-DNA and CMV-DNA among coronary angioplasty patients

	Odds Ratio (95 % Confidence Intervals)			
	CP-DNA[a] (n = 13)		CMV-DNA[a] (n = 18)	
	Univariable	Multivariable[b]	Univariable	Multivariable[b]
Age (per year)	1.02 (0.97, 1.07)	1.01 (0.96, 1.07)	0.98 (0.94, 1.02)	0.97 (0.93, 1.01)
Male	1.0 (0.3, 3.3)	0.9 (0.3, 3.0)	1.6 (0.5, 4.9)	1.4 (0.5, 4.8)
Smoking	1.1 (0.3, 4.2)	1.4 (0.3, 5.9)	0.4 (0.1, 2.0)	0.3 (0.1, 1.6)
February-April	5.4 (1.5, 20.1)[c]	5.4 (1.4, 20.2)[c]	0.7 (0.3, 2.0)	0.7 (0.3, 2.0)
Diabetes mellitus	2.0 (0.6, 6.2)	-	0.4 (0.2, 2.1)	-
Hypertension	0.5 (0.2, 1.7)	-	1.7 (0.6, 5.4)	-
Body mass index >27	0.9 (0.3, 2.7)	-	0.5 (0.2, 1.4)	-
Dyslipidemia	0.4 (0.1, 1.5)	-	0.3 (0.1, 0.9)	0.3 (0.1, 0.8)[c]
ASA	0.4 (0.1, 3.6)	-	0.6 (0.1, 5.0)	-
Clopidogrel / ticlopidine	0.3 (0.1, 1.3)	-	1.9 (0.2, 14.8)	-
Lipid lowering	0.5 (0.1, 1.6)	-	1.1 (0.3, 4.0)	-
Total number anti-platelet/lipid drugs	0.4 (0.1, 0.9)[c]	0.4 (0.1, 1.1)	1.2 (0.4, 3.2)	-

[a] Detection of *Chlamydia pneumoniae* (CP)-DNA or cytomegalovirus (CMV)-DNA by polymerase chain reaction in peripheral blood mononuclear cells (see text).

[b] Adjustment by multiple logistic regression (SPSS).

[c] $P < 0.05$

Table 8-4. Determinants of peripheral blood *C. pneumoniae*-DNA detection among family practice patients[a]

	Odds ratio (95 % CI) for *Chlamydia pneumoniae*-DNA[b]		
	Unadjusted	Interaction	Adjusted
Age (per year)	1.03 (0.98, 1.08)		1.03 (0.96, 1.10)
Male	1.3 (0.5, 3.0)		1.9 (0.6, 6.0)
February-April	6.0 (2.2, 16.6)		15.5 (3.9, 61.5)
Smoking	1.2 (0.5, 3.0)	0.6 (0.1, 2.4)	0.5 (0.1, 2.9)
Alcohol	0.5 (0.2, 1.2)	0.3 (0.1, 0.9)[c]	0.2 (0.0, 0.8)[c]
Smoke-by-alcohol	-	4.8 (0.7, 34.7)	28.2 (1.9, 421.9)[c]
Angina or MI	1.6 (0.4, 6.2)		8.3 (2.2, 31.2)[c]
Poor health[d]	2.7 (1.1, 6.4)[c]		6.6 (1.1, 40.2)[c]

[a] Stonechurch and McMaster University Medical Centre sites were pooled for these analyses (OR between sites = 1.06 (95 % CI: 0.4, 2.5, P = 0.90).

[b] Detection of *Chlamydia pneumoniae* (CP)-DNA by polymerase chain reaction in peripheral blood mononuclear cells (see text).

[c] P < 0.05

[d] Score of 4 (fair) or 5 (poor) versus 1-3 by self-assessed health status

Chapter 9

Systematic Review of Nucleic Acid Markers

In this chapter, I conduct a systematic review of studies of circulating *C. pneumoniae* DNA detection. In particular, I summarize prevalence among cardiovascular patients compared with controls. While recognizing the limitations of performing meta-analysis of observational data, I combine these results to demonstrate that there is no clear association between. *C. pneumoniae* DNA detection and cardiovascular disease.

I also examine the determinants of high *C. pneumoniae* DNA prevalence. I separately examine clinical determinants, such as age, gender, smoking and season, and technique-related determinants.

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These results have not been presented at any conference.

Circulating *Chlamydia pneumoniae* DNA as a Molecular Epidemiologic Tool for Cardiovascular

Research: A Systematic Review.

Running title: *Chlamydia pneumoniae* Review

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Abstract:

Extensive data from a number of laboratories support the findings that *Chlamydia pneumoniae* antigens, nucleic acids, or intact organisms are present in human atheroma. Yet, no consistent prospective association has been found between *Chlamydia pneumoniae* IgG or IgA antibody, and subsequent cardiovascular events. To better define the attributable risk of *Chlamydia pneumoniae* infection in human atherosclerotic cardiovascular disease, studies are required using well-validated markers that correlate with bacterial presence in plaque. In this systematic review, we summarize the data on *Chlamydia pneumoniae* DNA detection in peripheral blood mononuclear cells by polymerase chain reaction in 19 identified studies. One of two studies found a relationship between *Chlamydia pneumoniae* DNA from concurrent blood and atheroma specimens. In 10 cardiovascular studies with control patients, the prevalence of circulating *Chlamydia pneumoniae* DNA was 4.2 % to 59 % in cardiovascular patients and 0 % to 46 % in controls, for a pooled odds ratio of 1.6 (95 % CI: 0.7, 3.5, P = 0.22). Studies were highly heterogeneous (P < 0.001). High prevalence (> 40 %) was found in patients with cardiac disease, peripheral vascular disease, chronic obstructive pulmonary disease, or renal dialysis, as well as in apparently healthy community controls. We discuss differences between studies in methods of sampling, extraction, and PCR targets, and outline some of the challenges that need to be overcome for molecular detection to become a feasible molecular epidemiologic tool.

Key Words: *Chlamydia pneumoniae*, atherosclerosis, DNA detection, polymerase chain reaction, systematic review

Introduction:

Since Fabricant et al demonstrated infection-induced atherosclerosis in chickens in 1978(1), a number of viruses and bacteria have been investigated for a possible association with human atherosclerosis. The majority of investigators have focused on the putative role of the gram-negative, intracellular bacterium *Chlamydia pneumoniae* and atherosclerosis(2-4).

Indirect evidence of a possible association between *Chlamydia pneumoniae* and atherosclerosis was first found by Saikku et al in 1988, who measured circulating *Chlamydia pneumoniae*-specific IgG antibody by micro-immunofluorescence in patients with acute or chronic heart disease(5). In a review of the existing literature as of 1997, Danesh et al found a relationship between *Chlamydia pneumoniae* IgG seroprevalence and heart disease, primarily based on cross-sectional studies(6). In a subsequent meta-analysis that pooled 15 prospective studies, Danesh et al found no over-all relationship between *Chlamydia pneumoniae* IgG seroprevalence and subsequent cardiovascular events after adjusting for age, gender, smoking, and socio-economic status(7). Similarly, in 3,168 patients enrolled in the Heart Outcomes Prevention and Evaluation study, we found no consistent relationship between *Chlamydia pneumoniae* IgG or IgA positivity and subsequent cardiovascular events during 4.5 years of follow-up (8). In aggregate, these sero-epidemiologic data suggest that *Chlamydia pneumoniae* is not an important cause of cardiovascular disease.

Pathological studies of arterial wall provide more direct evidence for an association between *Chlamydia pneumoniae* and human atherosclerosis, albeit with marked variability between laboratories (9). Using electron microscopy, chlamydia-like elementary bodies have been observed in atheroma(10). Using immunohistochemistry with specific monoclonal antibodies, *Chlamydia pneumoniae* antigen has been detected in atheroma and localized to macrophages, endothelial cells, and smooth muscle cells *in vitro* and *in vivo*(11-13). Polymerase chain reaction (PCR) has been used

to amplify *Chlamydia pneumoniae*-specific nucleic acids within atheroma, and localization to abnormal but not to normal arterial wall has been demonstrated(14). Fifteen arterial specimens were analyzed by nine laboratories using 16 PCR techniques, with the detection of *Chlamydia pneumoniae* DNA in at least one specimen by six of nine laboratories(15). Over-all, only 9 % of specimens were positive, suggesting low copy number and large inter-laboratory variation in extraction efficiency, sensitivity, or specificity of the PCR methods. Using reverse transcriptase (RT)-PCR, *Chlamydia pneumoniae*-specific RNA has been demonstrated in lesions implying that the bacteria are viable and transcribing specific RNA(16). Finally, in a small number of patients, viable *Chlamydia pneumoniae* have been cultured from cardiac(17) and carotid vessels (18;19)and from aortic aneurysms(20). In aggregate, these molecular data demonstrate that *Chlamydia pneumoniae* or its antigens and nucleic acids are commonly found in human atheroma.

Thus, a major discrepancy exists between the widespread detection of *Chlamydia pneumoniae* in atheroma, and the lack of attributable risk in sero-epidemiologic studies. A key question is whether *Chlamydia pneumoniae* IgG or IgA correlates with the presence of *Chlamydia pneumoniae* in plaque. Campbell found no correlation between *Chlamydia pneumoniae* IgG by micro-immunofluorescence and the presence of *Chlamydia pneumoniae* in plaque. Similarly, Maass(21) and Berger(22) found no correlation between serology and DNA in plaque. Thus, *Chlamydia pneumoniae* serology may be a poor predictor for *Chlamydia pneumoniae* in plaque, and the lack of a prospective association between serology and cardiovascular events may therefore not be relevant. Hence, the question of whether *Chlamydia pneumoniae* in plaque contributes to atherosclerotic progression or to clinical events remains unanswered.

In this paper, we review studies of *Chlamydia pneumoniae* DNA detection in PBMCs, to

determine whether this would be a superior epidemiologic tool for prospective studies assessing the

contribution of infection to human cardiovascular disease.

Methods:

We performed a MEDLINE search (1980-2001 inclusive) using the terms *"Chlamydia*

pneumoniae", "atherosclerosis" as major headings and "nucleic acid" or "polymerase chain reaction"

as text words. We sought human studies in which nucleic acids, antigen, or intact *Chlamydia*

pneumoniae were detected in peripheral blood. We also sought studies in which these were correlated

with the tissue presence of *Chlamydia pneumoniae* by antigen detection, nucleic acid amplification, or

culture. For sentinel papers, we searched for citations in the Science Citation Index to identify recent

papers using similar technologies. We searched through the citations of found manuscripts as well

as searching the abstracts of infectious diseases, microbiology and cardiology meetings between 1998

and 2001. Last, we wrote to investigators in the field to identify unpublished data. Studies with

control patients were entered into Review Manager 4.04 and a pooled odds ratio was calculated

using a random effects model.

Results

We identified 19 studies in which circulating blood *Chlamydia pneumoniae* DNA detection was

undertaken, as summarized in Tables 1 and 2. The majority of these involved cardiovascular

patients, and the remainder examined respirology or nephrology patients or blood donors. One

unpublished study examined family practice controls.

Prevalence in cardiovascular studies with control groups

Boman and colleagues, working in Sweden, first demonstrated the detection of *Chlamydia*

pneumoniae DNA in PBMC of cardiac patients(23). They found a prevalence of 59 % of patients

undergoing coronary angiography, using a nested PCR targeting an outer membrane protein (*ompI*) gene. Prevalence was also high in middle-aged blood donors (46 %), with a higher prevalence in cases than controls.

A number of investigators have corroborated Boman's observation of circulating *Chlamydia pneumoniae* DNA in peripheral blood of cardiac patients, albeit with widely varying estimates of prevalence (Table 1). We identified nine other studies, including five published manuscripts (24;25), three abstracts (Da Costa et al, 2000; Kaul et al, 2000; and Sessa et al, 2000; Proceedings of the Fourth Meeting of the European Society for Chlamydia Research, Helsinki, Finland, 2000), and one unpublished study (26) in which cardiovascular patients were compared with controls. These included patients undergoing coronary angiography or angioplasty, vascular patients, patients with acute myocardial infarction or unstable angina, and patients with carotid atherosclerosis at ultrasonography. Controls consisted of blood donors, medical students, apparently healthy controls, or patients with normal coronary angiograms.

The largest of these ten studies was a British study of 1,205 British patients undergoing coronary angiography conducted by Wong et al (24). They found a prevalence of 8.7 % in patients with angiographically-proven coronary artery disease, versus 7.2 % in those with normal coronary arteries(27). In sub-group analysis, they found an association between coronary atherosclerosis and *Chlamydia pneumoniae* DNA detection in men but not in women. However, the prevalence of smoking was higher in male cases (78 %) than in controls (67 %), whereas prevalence was similar in female cases (53 %) and controls (51 %).

We examined the ten studies by meta-analysis (Figure 1), and found a pooled prevalence of 265 of 2,055 (12.9 %) in cardiovascular disease patients versus 105 of 884 (11.9 %) in controls. The results were highly heterogeneous (Breslow-Day X^2 = 62.2 on 9 degrees of freedom, P < 0.001).

The pooled odds ratio, using a random effects model, was 1.6 (95 % CI: 0.7, 3.5, P = 0.22), as

illustrated in Figure 1. A funnel plot of the odds ratio versus study precision is illustrated in Figure

2. In both of these figures, one study (26)emerges as a significant outlier. Excluding this study, the

remaining nine studies found *Chlamydia pneumoniae* DNA prevalence of 252 of 1,745 (14.4 %) in

patients with cardiovascular disease and 74 of 780 (9.5 %) in controls. No statistical heterogeneity

between studies was found between these nine studies (Breslow-Day $X^2 = 8.4$ on 8 degrees of

freedom, P = 0.40). We calculated a pooled odds ratio of 1.8 (95 % confidence interval: 1.3, 2.5,

P < 0.001), using a random effects model. Thus, with exclusion of one outlier study, cardiovascular

patients had a higher over-all prevalence of circulating *Chlamydia pneumoniae* DNA than controls.

Prevalence in other studies

In Table 2, we summarize nine other identified studies which examined the prevalence of

Chlamydia pneumoniae DNA in PBMC. In three studies of cardiovascular disease (28-30), prevalence

of 27.3 % to 47.0 % was observed. In two studies of chronic obstructive pulmonary disease,

prevalence of 24.0 % (31)and 47.6 % (Blasi et al, Proceedings of the Fourth Meeting of the

European Society for Chlamydia Research, Helsinki, Finland, 2000) were observed. The highest

prevalence, 60.0 %, was found in a small study of patients undergoing peritoneal dialysis (32).

Among blood donors, prevalence was 16.7 % in Australia (33), 41.5 % in Italy (34), and 8.9 % in the

United States (35). The studies by Bodetti and Timms (33), and by Haranaga et al (35), both

corroborated PCR results by detection of *Chlamydia pnuemoniae*-specific antigen in circulating

mononuclear cells.

Correlation of Chlamydia pneumoniae DNA detection in PBMC and in atheroma

We identified two studies in which DNA was sought concurrently in atheroma and in PBMC

(22;29). Blasi et al examined 41 patients with aortic aneurysms resected at surgery (29). Sixteen

patients had *Chlamydia pneumoniae* DNA detected in the resected aneurysmal tissue and in PBMC,

three in PBMC alone, and one in arterial wall alone. Thus, 16 of 17 (94.0 %) of patients with

Chlamydia pneumoniae in arterial wall were identified by PCR of PMBC.

Berger et al examined PBMC and arterial tissue in 60 patients with vascular disease resected

at surgery, including carotid and peripheral arteries and aortic aneurysms (22). Among sixty patients

with concurrent aneurysmal tissue and PBMC, DNA was detected in 12 plaques. Five of these 12

(42.0 %) were also PBMC DNA positive. Conversely, 5 of 12 (42.0 %) PBMC DNA positive

patients were also DNA positive in plaques. No statistically significant correlation between

Chlamydia pneumoniae DNA presence in plaque or PBMC was found. Furthermore, there was also no

correlation between the amount of *Chlamydia pneumoniae* DNA in plaques and in PBMC.

Relation to age and gender

A putative relationship between age and PBMC DNA prevalence was found by Bodetti and

Timms (33), with highest prevalence in patients < 35 years (5 of 22, 23 %) and > 50 years (3 of 13,

23 %) compared with those aged 35 to 50 (2 of 25, 8 %), but the sample sizes are too small to allow

meaningful inference. Berger et al (22) found that younger patients (mean 59 versus 68 years, P <

0.05) were more likely to have *Chlamydia pneumoniae* DNA in PBMC. By contrast, Iliescu et al (32)

found an association between older age and *Chlamydia pneumoniae* DNA detection (62.5 versus 51.9

years, P = 0.01), whereas Haranaga et al (35) found a lower prevalence among older blood donors.

No clear relationship with gender was identified in any study.

Relationship to smoking

A relationship between smoking and *Chlamydia pneumoniae* IgG or IgA seropositivity or titre

level has been previously demonstrated (36;37). Boman et al found an increased prevalence of

smoking among patients with *Chlamydia pneumoniae* DNA in PBMC, but the association was not

statistically significant (23). Smieja et al found a strong and statistically-significant relationship

between *Chlamydia pneumoniae* DNA in PBMC and smoking, with 10.0 % prevalence in non-smokers,

9.0 % in former smokers, and 25.0 % prevalence in current smokers (25). Current smoking was

associated with CP-DNA positivity independent of age, gender, and season (OR = 4.5, P = 0.004).

This relationship was recently confirmed in COPD patients, in whom current smoking was

associated with *Chlamydia pneumoniae* DNA detection in PBMC or sputum (OR = 2.6, P = 0.04)(31).

Of interest, Berger et al found a similar relationship between current smoking and the presence of

chlamydial DNA in atherosclerotic plaque (22).

Relationship to Season

Smieja et al found a strong relationship between *Chlamydia pneumonia* DNA detection and

season: in cardiology, respirology and family practice patients in studies spanning three separate

Canadian winter seasons (25;26;31), seasonal variation in prevalence was seen with highest

prevalence between February and April (OR = 3.6 to 6.2), and lowest prevalence between June and

October. No other reported study has adequately examined seasonal prevalence, but potential inter-

month differences were noted in three studies. Rassu et al (34) reported higher prevalence among

Italian blood donors in February (57.6 %) compared with October (37.9 %) . Haranaga et al (35)

found a lower prevalence in October and November (0 of 137, 0.0 %) compared with August or

September (21 of 100, 21.0 %). Berger et al reported that aneurysmal plaque was DNA positive

more often in May (25.0 %) or June (50.0 %) than in July to October (0.0 to 8.0 %, P < 0.05).

Blood sampling, extraction, and amplification

Blood sampling, extraction, and PCR targets are summarized in Tables 1 and 2. Generally,

Ficoll-hypaque centrifugation of 5 to 10 mL of EDTA blood was used to isolate PBMC. In five

studies by two research groups, cell preparation tubes (CPT, BD Vacutainer Systems, Franklin Lakes

NJ) were used. No studies were found for a direct comparison between Ficoll-hypaque and CPT methods.

Study investigators sampled 200-1000 μL of PBMC, extracted into a volume of 40-200 μL, and used 3-50 μL for the PCR reaction. Thus, between 0.15 and 2.50 mL of whole blood was sampled in the various studies. Among eight studies in which sufficient details were given to calculate the total blood sampled, higher DNA prevalence was associated with greater sampled volume (X^2 for linear trend $= 55.0$, $P < 0.001$).

Two methods were used for DNA extraction from PBMC: phenol-chloroform or Qiagen QIAamp DNA mini-kits. Examining only cardiovascular patients to enable valid comparison, prevalence was 188 of 1195 (15.7 %) extracted with phenol-chloroform, compared with 67 of 818 (8.2 %) extracted with Qiagen columns (OR $= 2.1$, 95 % CI: 1.6, 2.8, $P < 0.001$).

Ten of 19 studies used a nested PCR based on the *ompl* gene of the major outer membrane protein (MOMP) (38), and a variety of nested and non-nested PCRs targeting 16sDNA, heat shock proteins, or other targets were used in the remaining studies. Examining only cardiovascular patients, prevalence was higher using the nested MOMP PCR (132 of 901, or 14.7 %) than with the remaining assays (109 of 1061, or 10.3 %, OR $= 1.5$, 95 % CI: 1.1, 2.0, $P = 0.003$). However, the latter group was heavily influenced by the low prevalence of 79 of 913 (8.7 %) in one study (24).

Discussion

Epidemiologic issues

Two studies examined whether *Chlamydia pneumoniae* DNA detection in PBMC correlated with its presence in atherosclerotic plaque, and reached different conclusions. Further studies are

required to validate circulating DNA detection as a surrogate for bacterial presence within atherosclerotic plaque.

The prevalence of *Chlamydia pneumoniae* DNA detection in PBMC was found to vary widely: between 0.0 and 46.0 % among controls, and between 4.2 % and 59.4 % in patients with cardiovascular disease. Using meta-analysis, we demonstrated that circulating DNA prevalence was no higher among cardiovascular patients than among controls, although exclusion of a single study yielded a relatively strong, highly statistically significant difference in the prevalence among cardiovascular cases compared with controls.

We caution that meta-analysis of non-randomized data may be quite misleading due to heterogeneity of the control groups. Possible explanations for the found association include selection bias, publication bias, confounding by other cardiovascular risk factors, or a true association (whether causal or innocent) between *Chlamydia pneumoniae* and atherosclerosis. Selection bias may be acting in the selection of cases (which represent primarily patients with prevalent, chronic disease and not incident cases), or in the selection of controls. The use of blood donors as controls selects a group in whom many cardiovascular risk factors are less prevalent than in the general population. A second possible bias is of publication bias, in that positive results may have been more likely to get published. This is suggested by examination of a funnel plot (Figure 2), in which the estimated odds ratio of the association between *Chlamydia pneumoniae* DNA detection and vascular disease is graphed against the precision of the estimate. Ordinarily, a funnel shaped with the apex in the middle of the funnel should be seen, as indicated on the diagram (39). The lack of symmetry in the graph suggests that there may be a number of unpublished studies in existence, or that bias in the selection of cases or controls may be playing a role.

Confounding may be playing an important role in any found association with cardiovascular disease. We demonstrated a strong association between PBMC *Chlamydia pneumoniae* DNA detection and current smoking status (25), and Berger et al (22) demonstrated that current smokers are more likely to have *Chlamydia pneumoniae* DNA in atherosclerotic plaque. Future studies of circulating *Chlamydia pneumononiae* DNA detection need to be carefully controlled, as a minimum, for current smoking status. A more detailed smoking history, or laboratory measures of smoking metabolites such as cotinine, may be useful to better control for confounding by smoking status.

We also found a strong association with Canadian winter or spring season in three of our studies (25;26;31), and the studies of Rassu et al(34) and Berger et al(22) suggest similar seasonal patterns in Italy and Germany, respectively. Most studies did not recruit for sufficiently long to observe seasonal patterns. Of interest, Boman et al's original study(23) recruited patients between April and May 1998. Similarly, other high prevalence studies ($\geq$ 40.0 %) such as Iliescu et al(32) (April to May, 1999), and Blasi et al(29) (December 1998 to February 1999) recruited during times these "high prevalence" months. Conversely, Wong et al(27) recruited between August 1998 and March 1999, and found prevalence of only 8.7 %.

Important implications of this seasonal hypothesis are that prevalence will be lowest between June and October, and that controls for patients need to be carefully recruited with matching by month. Berger et al's observation of a peak in *Chlamydia pneumoniae* DNA in atheroma during June(22), and of a lack of correlation with concurrent PBMC DNA, suggests that there may be some delay between PBMC DNA presence and its presence in atheroma. However, Berger et al did not sample between January and April, months during which *Chlamydia pneumoniae* DNA prevalence may have been higher in both PBMC and in atheroma, and during which time a better correlation might have been found between the two sites.

In summary, these data suggest that *Chlamydia pneumoniae* DNA detection in PBMC should be performed, as a minimum, during winter and spring months, and controlled for smoking status. For prospective studies, it would be desirable to know not only whether a patient had infection during a given year, but also the total duration of circulating *Chlamydia pneumoniae* DNA.

Laboratory Issues

Boman and colleagues have reviewed many of the technical aspects of *Chlamydia pneumoniae* DNA amplification in general(9;40), and of PBMC DNA detection in specific(41). In this review, we examined four important differences in technique which require further investigation: the volume of blood sampled, type of blood specimen used, DNA extraction methods, and PCR primers.

We found that the total volume of blood sampled in the PCR reaction was related to a higher detection. We previously demonstrated the relationship between low copy numbers and the proportion of PCR replicates which were positive(42). Solutions to this sampling problem including extracting a large volume of blood, and doing replicate PCRs to maximize detection. However, with larger volumes, inhibitors will also be more concentrated. This may be overcome by testing different concentrations.

Commercial products are now available to simplify and standardize two aspects of testing: BD Vacutainer CPT tubes for blood collection as an alternative to ficoll-hypaque gradient centrifugation, and Qiagen QIAamp DNA mini-kits as an alternative to phenol-chloroform extraction. The commercial products save labour time but are more expensive. Our review suggests that phenol-chloroform extraction may have a higher yield than Qiagen columns, but inter-study comparisons may be misleading and a direct comparison is needed to validate this finding with paired samples. Perhaps more importantly, there are still no published studies of a commercial PCR

assay that would enable meaningful comparison between centres, although we have been recently

involved in such a study (43).

The MOMP nested PCR was used in half of the identified studies. Somewhat higher

prevalence was seen with this PCR than with other primers, but no firm conclusions should be

based on a comparison between studies that used heterogeneous populations. However, they are in

accord with our comparison of five in-house published PCRs, in which we found that the Tong and

Sillis nested PCR had superior clinical sensitivity for *Chlamydia pneumoniae* in PBMC despite similar

analytical sensitivity(44).

Confirming the Presence of Chlamydia pneumoniae in PBMC

Regardless of the best "screening" test for detecting *Chlamydia pneumoniae* DNA in PBMC,

additional methods of confirming the identification of *Chlamydia pneumoniae* are desirable(41). Such

confirmation would ensure the early identification of tests false-positives, and ensure valid

comparisons between tests. However, simply confirming that circulating *Chlamydia pneumoniae*

nucleic acids are present does not imply that their detection is clinically relevant. Rather,

confirmation is required to find the appropriate molecular tools for prospective clinical studies.

There is good evidence that *Chlamydia pneumoniae* circulates in the bloodstream as a cell-

associated infection. We (25) and others(33) have demonstrated that the plasma fractions of PBMC-

positive patients were uniformly negative for *Chlamydia pneumoniae* DNA. However, it remains

unclear which cells within the peripheral blood mononuclear cell layer contain *Chlamydia pneumoniae*.

Peripheral blood cells contain monocytes, dendritic cells, and lymphocytes, and may be

contaminated with polymorphonuclear cells or platelets. Using CD14 monoclonal selection, Maass

et al(28)demonstrated a prevalence of 27.0 % *Chlamydia pneumoniae* DNA positivity among patients

presenting to hospital with acute coronary syndromes. What is unclear is whether this prevalence

was higher than in whole blood or buffy coat alone. Kaul et al(45) used adherence to select CD14 cells and found that the CD3+ lymphocytes fraction was positive more often (10 of 28 patients, 35.7 %) than the "adherent" fraction of CD14+ cells (3 of 28, 10.7 %). Further work is required to determine which cells are infected, and whether cell enrichment would improve detection compared with mononuclear cell preparations.

Potential methods for confirming whether *Chlamydia pneumoniae* is present in PBMC include use of a second DNA-based amplification test, use of RNA-based methods, detection of antigen, or culture of intact organism. These will be discussed in the same order.

As more sensitive methods are developed, an alternate DNA-based nucleic acid amplification test with a different amplification target may be used to confirm a specimen as a true positive. However, due to low copy number and sampling error, confirmation of only 50-80 % of such samples should be expected(15;42). Use of higher blood volumes and extraction of a greater proportion of infected cells may improve initial detection as well as confirmation. Development of methods of quantitating *Chlamydia pneumoniae* in whole blood and PBMC have been developed using TaqMan or LightCycler real-time PCR detection technologies, and Berger et al used a quantitative assay *Chlamydia pneumoniae* DNA in PBMC and atherosclerotic plaque(22). Three replicates were run per specimen, and the number of negative runs was not reported. In our experience, only one of three replicates are positive in the majority of patients, indicating very low copy number(25;42). For such patients, quantitative methods are unlikely to improve detection, although studies of quantitation versus clinical importance may be informative.

Alternatively, RNA targets may be used for confirmation of *Chlamydia pneumoniae* PBMC positivity. Gieffers et al(46) reported the use of a reverse transcriptase PCR (RT-PCR) targeting 16S RNA transcript, and demonstrated active RNA production by infected PBMC. In volunteers who

took antibiotics prior to blood sampling, RNA was still detected, indicating that viable chlamydia were present and that antibiotics had not suppressed infected PBMC. Conversely, as with DNA-based validation, RNA-based confirmation is likely subject to the same sampling problems.

An attractive alternative would be to use a different technology for detection of *Chlamydia pneumoniae* in PBMC. Bodetti and Timms(33) described the detection of circulating *Chlamydia pneumoniae* antigen in PBMC using a specific monoclonal antibody. Ten of 10 PCR positive PBMC specimens were also antigen positive, compared with zero of ten PCR negative specimens. Antigenemia was used also used in a recent paper by Haranaga et al(35). Wider experience with the sensitivity and specificity of this technique is required, and while it may emerge as a confirmatory test, the method is technically difficult and not suitable for a screening test.

Conclusions

Epidemiologists and other researchers need a sensitive and valid tool which correlates with the endovascular presence of *Chlamydia pneumoniae* DNA. *Chlamydia pneumoniae* DNA detection within PBMC is emerging as a potential candidate for molecular epidemiologic studies, but issues such as appropriate sampling, extraction, and validation of a reproducible test, need to be resolved. Investigators need to consider potential seasonal changes in prevalence, to sample repeatedly, and to carefully control for smoking status. Using the nested MOMP PCR or a validated commercial nucleic acid amplification test, when available, may enable prospective epidemiologic studies to investigate the attributable risk of *Chlamydia pneumoniae* in cardiovascular disease progression and complications.

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Table 9-1: Studies with controls of *Chlamydia pneumoniae* DNA detection in peripheral blood mononuclear cells.

Reference	Country	Cases	Prevalence (%)	Controls	Prevalence (%)	Blood sampling	DNA Extraction	PCR[a] target
Boman, 1998	Sweden	Coronary Angiography	60 of 101 (59.4)	Blood donors	24 of 52 (46.1)	10 mL EDTA[b] blood	Phenol-chloroform	Nested MOMP[c]
Wong, 1999	United Kingdom	Coronary Angiography	79 of 913 (8.7)	Normal angiogram	21 of 292 (7.2)	4-5 mL EDTA blood	Phenol-chloroform	Cunningham Eur Resp J 1998; 11:345.
Kaul, 2000	United States	Coronary artery disease	13 of 28 (46.4)	Blood donors	5 of 19 (26.3)	20-30 mL EDTA, CD3+ cell selection	SDS-proteinase K	Nested MOMP HSP[d]-60
Berger, 2000	Germany	Vascular surgery	12 of 60 (20.0)	Not stated	7 of 51 (13.7)	4 mL EDTA blood	Phenol-chloroform	Quantitative 16S rDNA
Da Costa, 2000 (abstract)	Germany	Coronary Angioplasty and antibiotics	14 of 233 (6.0)	Blood donors	5 of 200 (2.5)	Not stated	QIAamp or phenol-chloroform	Nested
Freidank, 2000 (abstract)	Germany	Carotid atherosclerosis	10 of 42 (23.8)	Medical students	0 of 15 (0.0)	10 mL whole blood	Not stated	Nested PCR HL-1, HR-1, HM-1, HR-2
Sessa, 2000 (abstract)	Italy	Unstable angina, myocardial infarction	24 of 93 (25.8)	Healthy controls	2 of 42 (4.8)	EDTA blood	Phenol-chloroform	Not stated
Maraha, 2001	Netherlands	Aortic aneurysm	18 of 88 (20.5)	Healthy controls	8 of 88 (9.1)	EDTA blood	QIAamp	16s rDNA
Smieja, 2001	Canada	Coronary Angiography or angioplasty	22 of 187 (11.8)	Normal angiogram	2 of 21 (9.5)	8 mL BD CPT[e] tube	QIAamp	Nested MOMP
Smieja, 2002	Canada	Coronary angioplasty	13 of 310 (4.2)	Family practice	31 of 104 (29.8)	8 mL BD CPT tube	QIAamp	Nested MOMP

[a] PCR, polymerase chain reaction; [b] EDTA, ethylene diamine tetra-acetic acid; [c] MOMP, major outer membrane protein

[d] HSP, heat shock protein; [e] CPT, BD cell preparation tube (BD Vacutainer Systems, Franklin Lakes NJ, USA)

Table 9-2: Studies without controls of *Chlamydia pneumoniae* DNA detection in peripheral blood mononuclear cells.

Reference	Country	Study group	Prevalence (%)	Blood sampling	DNA Extraction	PCR target
Maass, 2000	Germany	Unstable angina, myocardial infarction	65 of 238 (27.3)	8 mL EDTA[a], CD14+ cell selection	QIAamp DNA extraction	HL-1/HR-1 (Campbell)
Blasi, 2000	Italy	Aortic aneurysm	19 of 41 (46.3)	8 mL CPT[b] tube	Phenol-chloroform	Nested MOMP[c]
Iliescu, 2000	Canada	Dialysis	33 of 55 (60.0)	10 mL heparin	QIAamp DNA extraction	53 kDa protein (Kubota)
Bodetti 2000	Australia	Blood donors	10 of 60 (16.7)	9 mL EDTA	Not stated	Nested MOMP
Taylor-Robinson, 2000	United Kingdom	Peripheral vascular disease	Not stated (47.0)	Not stated	Not stated	Not stated
Blasi, 2001	Italy	COPD[d]	10 of 21 (47.6)	8 mL CPT tube	Not stated	
Smieja, 2001	Canada	COPD	24 of 100 (24.0)	8 mL CPT tube	QIAamp DNA extraction	Nested MOMP
Rassu, 2001	Italy	Blood donors	78 of 169 (43.2)	8 mL EDTA	Phenol-chloroform	Nested MOMP
Haranaga, 2001	USA	Blood donors	21 of 237 (8.9)	5 mL EDTA	QIAamp DNA extraction	Touchdown 16S (Madico)

PCR, polymerase chain reaction. HSP, heat shock protein.

[a] EDTA, ethylene diamine tetra-acetic acid

[b] CPT, BD cell preparation tube (BD Vacutainer Systems, Franklin Lakes NJ, USA)

[c] MOMP, major outer membrane protein

[d] COPD, chronic obstructive pulmonary disease

Comparison: 01 C. pneumoniae DNA
Outcome: 01 C. pneumoniae Prevalence Among Cardiovascular Patients and Controls

Study	Cardiovascular n/N	Control n/N	OR (95%CI Random)	Weight %	OR (95%CI Random)
Berger	12 / 60	7 / 51		10.6	1.57[0.57,4.35]
Boman	60 / 101	24 / 52		11.8	1.71[0.87,3.35]
Da Costa	14 / 233	5 / 200		10.5	2.49[0.88,7.05]
Freidank	10 / 42	0 / 15		4.8	10.02[0.55,182.18]
Kaul	13 / 28	5 / 19		9.7	2.43[0.69,8.58]
Maraha	18 / 88	8 / 88		11.1	2.57[1.05,6.28]
Sessa	24 / 93	2 / 42		8.8	6.96[1.56,31.00]
Smieja	22 / 187	2 / 21		8.7	1.27[0.28,5.81]
Smieja-2	13 / 310	31 / 104		11.7	0.10[0.05,0.21]
Wong	79 / 913	21 / 292		12.2	1.22[0.74,2.02]
Total(95%CI)	265 / 2055	105 / 884		100.0	1.58[0.71,3.51]

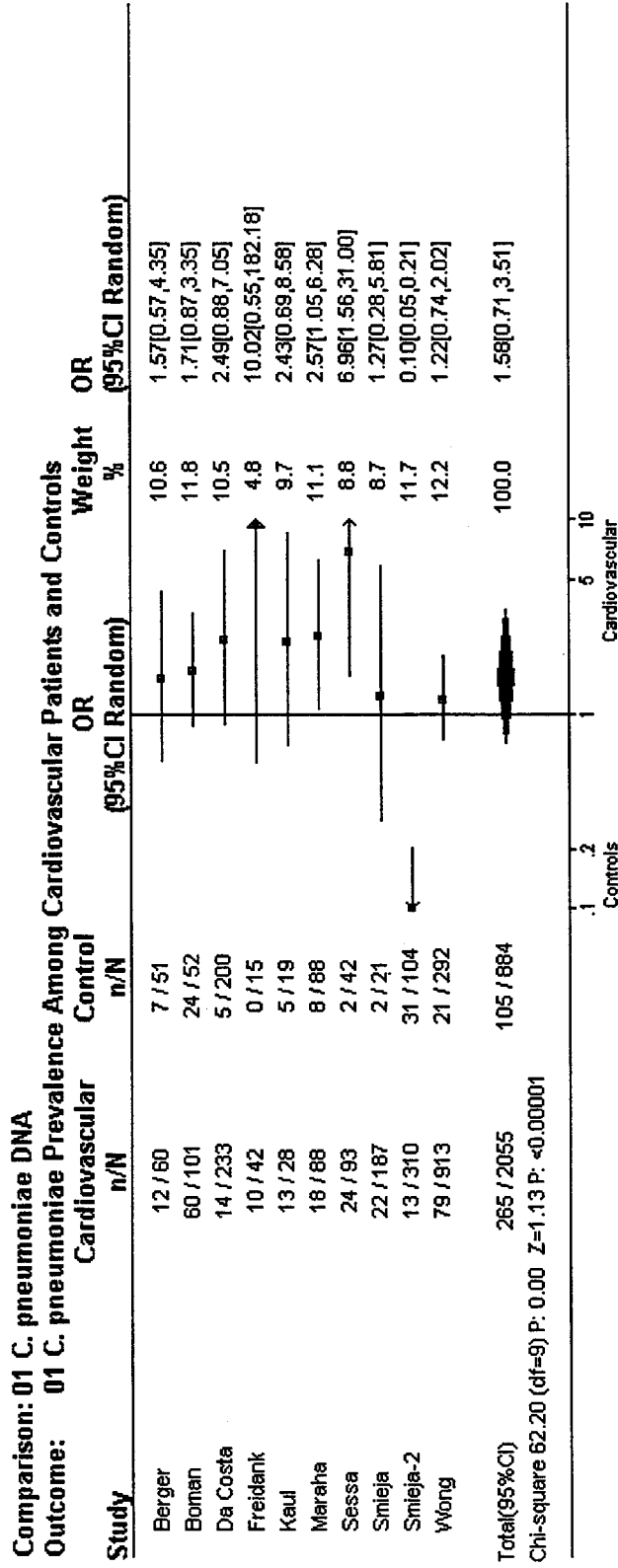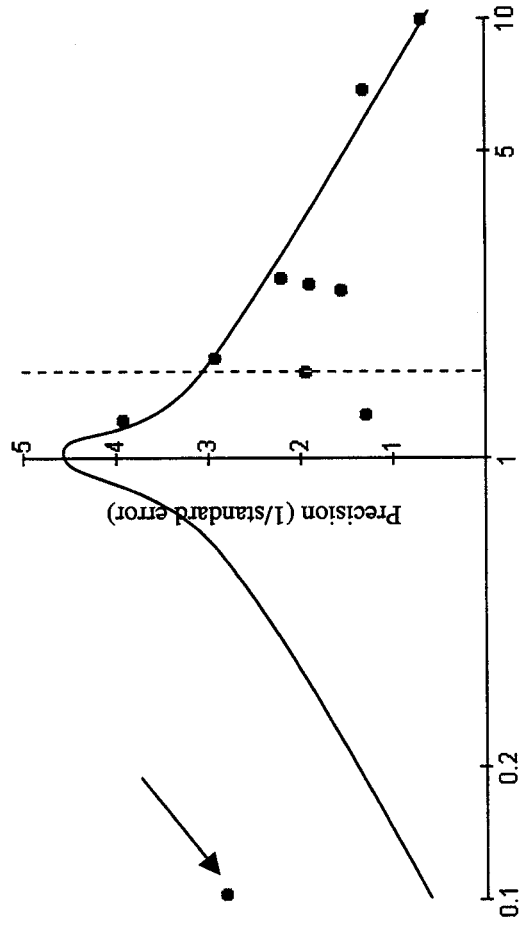Chi-square 62.20 (df=9) P: 0.00 Z=1.13 P: <0.00001

Figure 9-1. Meta-analysis of circulating *Chlamydia pneumoniae* DNA detection in cardiovascular studies with controls. Odds ratios with 95 % confidence intervals and pooled odds ratios calculated under a random effects model (Review Manager 4.04).

Odds Ratio for *Chlamydia pneumoniae* DNA and Cardiovascular Disease

Figure 9-2. Funnel plot of *Chlamydia pneumoniae* DNA detection (Review Manager 4.04). Precision of estimate (reciprocal of standard error) versus odds ratio for nine studies of circulating *C. pneumoniae* DNA detection by cardiovascular disease status compared with disease-free controls. One study was a significant outlier (arrow). The curve illustrates a funnel plot, in which an equal number of studies are expected in the left and right tails.

SECTION 4:

CONCLUSIONS

Chapter 10.

Conclusions and Future Studies

It might be said of me
that here I have merely
made up a bunch of other men's flowers,
and provided nothing of my own
but the string to tie them together.

--Michel De Montaigne, 1533-1592.
Essays, I, 39

Summary

In this chapter, I summarize the individual chapters and put them in context of the

remainder of the thesis and of new developments in the field. I expand in some detail upon

the relationship between infection, inflammation, and smoking. I examine separately the

two proposed causal domains: proving association between infections and heart disease, and

demonstrating experimental modification.

Next, to guide in the design and interpretation of etiologic studies, I propose four

phases of risk marker development studies as direct counter-parts to the four phases of drug

development studies: 1. Cross-sectional and prospective validation of the putative risk

marker, 2. Treatment studies with risk markers as surrogate outcomes, 3. Treatment studies

with clinical outcomes alongside risk marker outcomes, and 4. Ongoing "post-marketing"

validation studies. I conclude with a discussion of potential future studies into the

contribution of infectious diseases to the etiology and progression of human atherosclerosis.

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Section 1: Introduction.

One of the goals of this thesis was to validate the McMaster Causality Scale, a measure of the strength of evidence for a causal association of a putative infection/disease pair. In the first chapter, a number of validation criteria were examined, including: face, content, construct and criterion validity. The McMaster Causality Scale was compared with the results of Expert Opinion, and found to correlate well. By contrast, neither Koch's postulates nor Bradford Hill's guidelines consistently correlated with Expert Opinion. Thus, a simple measure entailing two domains—association and experimental modification— shows promise as a scale for measuring causal associations. Assessing the *Chlamydia pneumoniae*/coronary artery disease association using this scale, it scored a "possibly causal" ranking. By contrast, most of Koch's and Bradford Hill's criteria were fulfilled, suggesting a "probably causal" ranking. Expert Opinion concurs with the McMaster Causal Scale ranking of "possibly causal". In future, wider application to putative infection/disease pairs, examination of inter-observer reproducibility of score assignment, and assessing the scaling properties of the score will be required.

Section 2: Serologic and inflammatory markers

Chapter 2 describes a case-control study conducted in Hamilton, a small study that provided useful background data for the design of the Inflammatory and Infectious Markers in the HOPE Study. We found an association between chlamydial serology and heart disease, and no association with four other infectious markers. However, by using anonymous laboratory controls, we could not measure potentially confounding cardiovascular risk factors. It is likely that smoking was more common among cases than controls, and confounding by smoking status is the most likely explanation for the

discrepancy between the case-control study and the subsequent prospective studies. A strength of this small study was the investigation of multiple infections, to determine the specificity of any found association. At the time, there were very few studies that examined multiple serologies concurrently. One incidental finding, which may be worthwhile investigating in future, was a possible association between seropositivity to adenovirus and a history of hypertension.

Chapter 3 describes a study designed to prospectively validate the findings of the case-control study. We examined four infections (*C. pneumoniae*, *H. pylori*, CMV, and HAV) for an association with cardiovascular events and atherosclerotic disease progression in a large cohort of HOPE study patients.

The study was designed to demonstrate an association with *C. pneumoniae*, but not with other infections. It did not verify this association. While the study was ongoing, other prospective studies, primarily nested case-control studies of primary prevention cohorts, came to similar conclusions (1). As the study was finishing, publication of a study of total pathogen burden by Zhu and colleagues (2) inspired examination of the same concept in the HOPE study patients. This concept relates cardiovascular disease to the sum exposure of a number of infections, rather than to *C. pneumoniae* alone. Rupprecht et al (3) also found an association between total pathogen burden and cardiovascular mortality after coronary angiography. In this, the third prospective study of total pathogen burden, we did not find a prognostic role for total pathogen burden. It will be important to determine whether the addition of other serologies, such as the herpesviruses, account for the difference between studies. If verified, such markers may serve as "risk markers", but alone will not prove

whether infections play a direct role in atherogenesis, or are reactivated by the same inflammatory process that drives atherosclerosis and its complications.

A potential issue is whether HOPE study patients were the best group in whom to study the contribution of infections to cardiovascular events and anatomical disease progression. Older patients with multiple contributing causes, and on multiple partially-effective drugs, may be a difficult population in which to disentangle webs of causation. One study of carotid artery atherosclerosis showed a relationship between infection and disease in younger, but not older, patients (4).

In Chapter 4, we demonstrated that high-sensitivity C-reactive protein (hsCRP), fibrinogen, soluble intercellular adhesion molecule-1 (sICAM-1), and interleukin-6 were associated with cardiovascular outcome. Inflammatory markers such as CRP and fibrinogen have been previously well-validated as cardiovascular risk markers (5;6), and they were included to allow appropriate correlation with infectious markers, and to compare between inflammatory markers. The greater utility of fibrinogen and sICAM-1, compared to hsCRP, is noteworthy.

Infections, Inflammation and Smoking

Let us examine in more detail the relationship between infections, inflammation, and smoking. Within the IIM-HOPE study, an association between serologic evidence of previous exposure to the four studied infections—*Chlamydia pneumoniae*, *Helicobacter pylori*, cytomegalovirus, and hepatitis A virus—and the log-transformed concentration of inflammatory markers, were sought using Student's t-tests. These data were not included in the manuscripts due to lack of space. *C. pneumoniae* IgG $\geq$ 1:32 was associated with hsCRP (P = 0.02) and fibrinogen (P = 0.01), *C. pneumoniae* IgA $\geq$ 1:16 with sICAM-1 (P = 0.04) and

IL-6 (P = 0.003), and the composite measure of IgG ≥ 1:512 or IgA ≥ 1:64 with IL-6 alone

(P = 0.0002). The presence of cytomegalovirus-IgG antibody or *Helicobacter pylori*-IgG

antibody was not associated with hsCRP, fibrinogen, sICAM-1 or IL-6. Hepatitis A virus-

IgG was associated with hsCRP (P=0.02), sICAM-1 (P=0.02), and with IL-6 (P=0.01).

These associations, although statistically significant, were small in magnitude. *A priori*, an

association was expected between *C. pneumoniae* and inflammatory measures such as CRP or

fibrinogen that correlated with cardiovascular outcome. While the inflammatory markers

were predictive of future cardiovascular events, *C. pneumoniae* serology was not.

Individually, all inflammatory markers—hsCRP, fibrinogen, sICAM-1, and IL-6—

were strongly associated with current or former smoking status. Odds ratios of 2.5 to 3.3 for

current smoking, and 1.3 to 1.7 for former smoking, were found (see Table 4-3). The cause

of ongoing inflammation in current and former smokers needs further investigation, and

may provide therapeutic insights into the process of inflammation as it relates to

cardiovascular disease.

Similarly, two of the four infections—*C. pneumoniae*, whether measured by IgG, IgA,

or the composite of the two, and *H. pylori*—were strongly associated with current or former

smoking status. However, these serologic markers were not independently associated with

cardiovascular outcome. The study identified a potential interaction between smoking

status, chlamydia serology, and clinical outcome: chlamydial serology predicted future

cardiac events among former or never smokers, but not among current smokers. One

interpretation of the data—as discussed in Chapter 3—is that *C. pneumoniae* status may be a

measure of recent smoking cessation. The smoking/chlamydial relationship is explored in

more detail in chapters 5, 7, 8 and 9: smoking and *C. pneumoniae* are associated, but whether

the association is one of confounding or part of a causal pathway remains to be studied. These relationships will be re-examined in future studies, using DNA evidence of infection instead of serologic markers.

Unlike *C. pneumoniae*, CMV—the only infection associated with an excess of cardiovascular events in the HOPE study—was *not* related to smoking. Furthermore, the hazard ratio associated with CMV was independent of the risk associated with inflammatory markers. There may, however, be an interaction between CMV and inflammatory markers. An analysis of CMV risk association, divided by CRP tertiles, found a higher adjusted HR for incident MI, stroke, or CV death associated with the lower concentrations: covariate-adjusted HRs of 1.68 (1.08 to 2.61), 1.32 (0.90, 1.92) and 1.11 (0.79, 1.58) were found for the lowest, middle, and upper thirds respectively. The CMV-by-CRP interaction term was statistically significant (P=0.04), and a similar pattern was seen for CMV by fibrinogen concentrations. This inverse relationship between serostatus and inflammatory marker levels is counter-intuitive and was not a planned analysis: this was done at the request of the journal reviewers. Nevertheless, interactions between inflammatory and infectious markers will be sought in future studies, and need to be eventually incorporated into our understanding of the link between the two. While continued study and development of inflammatory risk markers will contribute to targeting and developing appropriate interventions, these markers can also serve as a "surrogate" end-points for studies of infections and atherosclerosis. Infections that affect the inflammatory state, and whose eradication improves that inflammatory state, could then be investigated prospectively in observational and eventually treatment studies to determine whether they are contributing causes.

Section 3: Nucleic acid markers

Whereas Section 2 explored the application of well-developed infectious and inflammatory markers to studies of prognosis, the chapters in Section 3 represent the development of new infectious markers using DNA amplification technologies. This work was based on the assumption that the detection of circulating bacterial or viral DNA would likely be a more valid measure of infected atheroma than serology, although that assumption will require thorough testing in future.

The fifth chapter, and the first in this section, was our group's first study examining *C. pneumoniae* -DNA detection in peripheral blood mononuclear cells. This was a cross-sectional study of the prevalence of *C. pneumoniae* and CMV DNA in peripheral blood of angiography patients, with a limited prospective component. We made three noteworthy discoveries in the angiography study: we found that *C. pneumoniae* -DNA detection was associated with sampling problems, current smoking, and winter/spring season. We validate these sequentially in chapters 6 to 8.

In chapter 6, probit regression analysis was used to explore the relationship between low copy numbers and the probability of detecting a positive. The inspiration for this analysis was a simple observation: in the original angiography study (chapter 5): among 23 of 24 patients in whom *C. pneumoniae* DNA was detected, only one of three blood samples taken from each patient was positive. Despite extensive analytical work, we were concerned that our assay may be performing unreliably. Yet, we had shown that the assay was performing better than four other published assays (8). Communication by laboratory members with other chlamydia research laboratories revealed similar problems at those sites (S. Chong, personal communication 1999). The hypothesis that low reproducibility was a

function of sampling due to low DNA copy number, rather than of poor assay sensitivity, was a critical step toward improving test performance. I developed the probit regression model at the suggestion of Dr. Goldsmith, my thesis supervisor, to examine cumulative distribution functions. I was able to find a statistical basis for the test's performance with clinical samples, and to use this model as the basis for designing testing strategies for improving sensitivity. Thus, we needed to improve our sampling of blood, but not the molecular assay we were using—a simple insight in retrospect. One of the practical applications of this probit model was to compare different tests in replicates at a concentration at which 50 % of samples would be expected to contain DNA. In a multi-centre diagnostic study, we recently examined this concept in a comparison between five North American laboratories (9). In a validation of the probit model, we showed a clear relationship between volume of extracted DNA sampled and analytic sensitivity (9;10).

In chapter 7, we validated the association between current smoking and chlamydial infection with a new study group: patients with stable chronic obstructive pulmonary disease (COPD). Together, chapters 5 and 7 help establish *C. pneumoniae* as a smoking-related infection, and not one specifically associated with cardiac disease. Recently, an association between smoking and chlamydial DNA detection in atheroma has been reported (7).

The relationship between infections, inflammation, and smoking has been discussed previously for serology, and an association found between chlamydial infection and smoking. However, if smoking correlates with *C. pneumoniae* IgG or IgA serology (chapter 3), and with *C. pneumoniae* DNA detection in PBMC (chapter 6 and 7) or atheroma (7), why has serology not proven to be a good marker for DNA detection in either circulating monocytes or within atheroma? I believe the answer will be found by conducting the appropriate

prospective studies, in which serology and molecular detection are measured concurrently throughout the year to study their relationship. I hypothesize that serologic titres will increase only after clearance of *C. pneumoniae* DNA, and this temporal sequence has major implications for prognostic studies. If true, patients with low or absent *C. pneumoniae* IgG or IgA titres represent not only those who have not been recently exposed, and therefore are at low risk, but also those who are currently exposed, and therefore at highest risk. In support of this hypothesis, the *C. pneumoniae* IgG and IgA titres we measured in the angiography study were somewhat lower among those with DNA detected compared to those without DNA. Similarly, high titres—rather than representing "chronic infection"—may represent recent clearance of infection. Hence, they may not be associated with ongoing risk. In support of this, the highest *C. pneumoniae* IgG titres had the lowest event rates during the first 400 days of the HOPE study (Figure 3-1A). Two other studies have found no relationship between chlamydial serology and the presence of chlamydial DNA in PBMC (11;12). Thus, *if serology does not correlate with chlamydial presence within atheroma, then serology provides no information with which to prove or disprove a role for C. pneumoniae in heart disease.*

I concluded then, and reiterate here: the association between *C. pneumoniae* and smoking may be confounded or part of a causal pathway. Thus, while *C. pneumoniae* may not be an independent risk factor for cardiovascular disease, it may be the mechanism through which smoking causes smoking-associated diseases. While the major focus of the thesis is on infections and cardiovascular disease, I also conclude that *C. pneumoniae* be investigated as part of the causal pathway of other smoking-associated diseases such as chronic lung disease, as suggested by von Hertzen (13).

I suggest on the basis of the data in chapters 5, 7 and 8 that infection is common in winter/spring months regardless of smoking status, but that persistence is common only in smokers. This hypothesis challenges the concept of "chronic" *C. pneumoniae* infection developed by investigators using serology or detecting immune complexes. It implies that clearance and re-infection may happen very often, perhaps annually in many patients. To study whether *C. pneumoniae* is associated with chronic disease, we require a measure that can incorporate such data. With a risk factor such as smoking, the relevant measure is not simply whether someone smoked on a certain day in May, but whether they smoked throughout the year. To study and quantitate chlamydial persistence, we need prospective studies and repeated measurements of circulating *C. pneumoniae* DNA. As a minimum, I propose measuring *C. pneumonia* DNA every three months, and studying the clinical consequences both of detection, and of persistence, of *C. pneumoniae* DNA.

In chapter 8, we asked whether *C. pneumoniae* -DNA or CMV-DNA detection was more common among coronary artery disease patients than in family practice controls, controlling for age, gender, smoking and month of enrollment. In both study groups, highest prevalence of *C. pneumoniae* -DNA was observed between February and April, in agreement with the angiography (chapter 5) and COPD (chapter 7) studies. Surprisingly, we found that both infections were more prevalent among family practice controls. This apparent protection from infection among angioplasty patients is puzzling. I speculate that cardiac drugs such as statins or anti-platelet agents may have a protective role, and will design new studies to investigate these possibilities. Conversely, if patients awaiting angiography dramatically curtail their social activity, it is possible that the reduced prevalence reflects

fewer contacts with infected individuals. Nevertheless, these data do not support an increased prevalence of these infections among cardiac patients.

In chapter 9, I summarized 19 studies of *C. pneumoniae* DNA detection in PBMC, including 10 studies in which prevalence among cardiovascular and control patients was reported. I found no association with disease over-all. While many if not all of these studies had imperfect controls, and most were not adjusted for clinical co-variates, this evidence suggests that DNA detection is not specific for cardiovascular disease. Whether *C. pneumoniae* DNA detection has any prognostic implications remains to be determined.

The McMaster Causality Scale: Evidence of Association

In the introductory chapter, I summarized the evidence for an association between *C. pneumoniae* and atherosclerotic heart disease: pathologic evidence of chlamydial antigen, DNA, or intact organism within atheroma; animal models of infection-induced atherosclerosis; and sero-epidemiologic and molecular epidemiologic data of association.

In this thesis, only one domain of the proposed McMaster Causality Scale—proving "association"—was explored. Chapters 2 to 4 examined serologic and inflammatory markers and cardiovascular disease, whereas chapters 5 to 9 examined nucleic acid (DNA) markers and cardiorespiratory diseases. These studies, in aggregate, do not support a causal association between *C. pneumoniae* and cardiovascular disease. *C. pneumoniae*, *H. pylori*, and HAV exposure, as assessed by serology, were clearly not associated with cardiovascular outcome, and the association with CMV was of borderline clinical importance or statistical significance. Thus, one would conclude that there is no epidemiologic evidence of any association. However, the pathologic data of an association cannot be ignored, and I believe the problem lies in the choice of serology as surrogate marker. As Huebner writes, in his

"Bill to prevent the ascribing of causality to innocent viruses", there must be sufficient money to study an association before declaring that one does or does not exist (14). Similarly, without sufficient investment in the appropriate tools and studies to examine for a causal association, *C. pneumonia*'s role cannot be definitively determined. Further molecular epidemiologic studies are required, recognizing that *C. pneumoniae* may act in concert with other recognized cardiovascular risk factors such as smoking, and that its effects may be short term and cumulative.

The McMaster Causality Scale: Evidence of Experimental Modification

The studies described in this thesis did not address the domain of demonstrating experimental modification. In Chapter 1, I discussed animal models of infection and atherosclerosis. However, animal models will never prove the relevance of infections to human disease, and human clinical trials are required to show causation. The emphasis in the Infectious Diseases and Cardiology community—awaiting the results of large randomized clinical trials—demonstrates the importance of this interventional methodology in proving causation. While the first two studies published were both positive, only a small number of events occurred in those studies.

In the Weekly Intervention with Zithromax [Azithromycin] for Atherosclerosis and Its Related Disorders (WIZARD) study (15), 7,700 post-MI patients received 3 months of weekly azithromycin or placebo. Patients were followed for recurrent cardiac events, hospitalization for unstable angina, or revascularization procedures. The results were a 7 %, non-statistically significant reduction in the primary end-point (M. Dunne, Infection and Atherosclerosis Symposium, April 29-30, 2002, Washington DC). An early treatment effect was confined to the first two months, and sub-group efficacy was seen among smokers,

men, and patients with diabetes mellitus. The majority of patients (67%) received statin drugs. The Cedars Sinai trial of Azithromycin in Acute Coronary Syndromes (AZACS) study randomized 1,439 patients with unstable angina to azithromycin or placebo, and found similar events rates in the two arms (P.K. Shah, Infection and Atherosclerosis Symposium, April 29-30, 2002, Washington DC.), where 70% of study patients were taking concurrent statin therapy. The AZACS study, in agreement with the WIZARD study, found an early benefit which was not sustained by 3 months of follow-up.

A key question is whether the chlamydial hypothesis is wrong, or whether the study design was unable to test it appropriately. While lending credence to the former interpretation, these studies cannot disprove involvement of *C. pneumoniae* in heart disease. Had the investigators been able to demonstrate the presence of infection, and clearance with antibiotic, the conclusions would likely be much more robust in terms of disproving the infectious hypothesis. With the current study design, a lack of efficacy of antibiotic for endovascular infection remains a plausible explanation. For future studies, the measurement of appropriate surrogate markers of infection will be necessary to determine whether the intervention clears infection, and to correlate infection and its clearance with cardiovascular prognosis. I explore this concept further in a proposal for risk marker development in the paragraphs which follow.

Four phases of risk marker development: A Proposal

My four-year experience with helping develop molecular tests for detecting *C. pneumoniae* in PBMC has led to examining the entire process whereby risk markers are developed and validated.

Let us briefly consider the chronology of *C. pneumoniae* investigations in cardiovascular disease. The first studies were of serologic and pathologic association, followed by studies of animal and small human therapeutic studies, culminating in large cardiovascular studies of drug treatment. While these follow Koch's postulates, there were no appropriate prospective studies to validate serology as an appropriate risk marker, or attempts to estimate attributable risk. There are now some 16,000 patients randomized to antibiotics or placebo to investigate this putative association (16;17).

Based on our molecular data, I predicted that these large intervention studies would not demonstrate that antibiotics improve prognosis after myocardial infarction. If *C. pneumoniae* has a role in precipitating cardiovascular events, an analysis confined to patients treated between January and April may show some reduction in clinical events, whereas treatment at other times of the year would not. The twelve month course used in the NIH-sponsored study is more likely to demonstrate antibiotic efficacy (17), but probably only during the year that the antibiotics are taken, and the study may be under-powered to detect such results. Results are expected in 2004. Furthermore, data exist to suggest that *C. pneumoniae* circulating in mononuclear cells may not be amenable to antibiotic therapy (18), and our data in Chapter 8 suggest that statin and other cardiovascular drugs may be associated with a lower prevalence of chlamydial infection.

I now consider a framework of developing risk markers studies and applying them to causation research, based on epidemiologic principles rather than on Koch's postulates.

The development of a new drug therapy follows four well-described phases of human studies. A pre-clinical phase based on animal studies initiates their application in human clinical studies. In phase 1, a therapy is given to volunteers to ascertain safety. In

phase 2, dose finding is done in a limited number of patients. In phase 3, a large randomized trial is conducted to prove efficacy. In phase 4, ongoing surveillance after a drug comes to market is used to ascertain the occurrence of rare side effects, and, increasingly, to determine whether efficacy in the "real world" corresponds to that found in clinical trials.

I suggest an analogous process for developing risk markers correlating to a putative causal agent. The pre-clinical phase would consist of test development and demonstration of reproducibility and analytic sensitivity and specificity in the laboratory. In phase 1, markers would be shown to be associated with a given illness in cross-sectional (1A) and prospective (1B) studies. In phase 2, these markers would be used as surrogate markers in treatment studies: thus, a short-term treatment study would determine whether antibiotics suppress or clear *C. pneumoniae* DNA, before embarking on a longer-term study looking at clinical events alongside surrogate markers (phase 3). Finally, in phase 4, the proven risk markers would be continually re-evaluated to see whether they remain predictors of risk. Using the example of CRP, this was well validated in cross-sectional and prospective studies (6), and shown to be a surrogate marker for improved outcome in studies of ASA and statins (19;20) as well as of adverse outcome with estrogen(21). The HOPE study may illustrate the "post-marketing" fourth phase of the development of such risk markers: we demonstrated that in patients on multiple cardiac medications, the marker may no longer predict excess risk.

If we apply these development guidelines to studies of *C. pneumonia* and cardiovascular disease, we find data for Phase 1A (cross-sectional), negative studies for Phase 1B (prospective), a paucity of data for Phase 2 (effect of therapy on surrogate markers). Initial reports of large therapeutic studies (Phase 3) are essentially negative, but, without appropriate surrogate markers of persistence of infection, we do not know whether a

negative study corresponds to lack of antibiotic efficacy against the infection, or lack of association between the infection and disease.

There are numerous infection/chronic disease associations emerging, and establishment of such phases may allow planned and efficient assessment of promising associations through sequential stages, and for unlikely associations to be abandoned at an early stage.

Future Studies

In keeping with the proposed McMaster Causality Scale domains, two categories of future studies are envisioned: ones studying an association of infection with cardiovascular disease, and ones to experimentally modify disease.

Association of cardiovascular disease with inflammatory and infectious markers

In collaboration with Dr. Stefan Blankenberg, a cardiologist from Mainz Germany, we are re-examining the HOPE study cohort for novel inflammatory and infectious markers. The primary rationale is to study markers, such as sVCAM-1, which have been validated in other prospective studies (22), but were not included in the IIM-HOPE study. Our primary criterion for selection of inflammatory markers in the IIM-HOPE grant proposal was the existence of published prospective association with cardiovascular events. Additionally, these markers will be assessed as effect modifiers of the efficacy of ramipril and vitamin E— which may eventually lead to a better understanding of the determinants (infectious, immune, and genetic) of that inflammatory state. For infectious diseases research, better definition of a validated inflammatory score may also serve as an important surrogate measure for studies screening various putative infectious markers.

Rupprecht, Blankenberg and colleauges (3) recently demonstrated that multiple infections—particularly intra-cellular viruses that may be re-activated or contribute to ongoing inflammation—predict cardiovascular events and mortality after coronary angioplasty. We will study the HOPE cohort with these assays to determine whether an expanded total pathogen burden predicts cardiovascular events.

Association of cardiovascular disease with dental pathogens

Dental organisms are an important group of organisms have been associated with cardiovascular disease, but were not studied in this thesis. Infections such as *Porphyromonas gingivalis* and *Streptococcus sanguis* will be sought by serological or molecular methods in future studies, and their relationship to smoking and vascular disease ascertained in cross-sectional studies using carotid intimal medial thickness as a surrogate outcome.

Further molecular epidemiologic studies of Chlamydia pneumoniaee

Prospective studies in Cardiology, Respirology, and Family Practice are needed to better define the epidemiology of acute and persistent *C. pneumoniae* infection—its incidence, prevalence, and association with inflammatory markers.

As *C. pneumoniae* DNA is cell-associated, banks of stored serum or plasma cannot be used to measure *C. pneumoniae* DNA. Once the molecular assays are sufficiently validated, I will endeavor to find collaborations in which *C. pneumoniae* DNA can be measured in buffy coat. One such study group would be Inter-HEART (23), a large international case-control study examining factors associated with first-time myocardial infarction. Of particular interest would be to examine strata of young patients and smokers, in whom smoking is the main known cardiac risk factor. I hypothesize that *C. pneumoniae* is most likely to be associated with myocardial infarction in such patients. An important feasibility issue for any

of these studies would be validating the sensitivity of the buffy coat obtained in these

studies, versus the BD Vacutainer Cell Preparation Tubes that we have used in our

molecular studies. Selection of a small number of Inter-HEART patients and controls

would allow testing two hypotheses:

1. Does the presence of *C. pneumoniae* DNA account for some of the seasonal excess of cardiovascular events; and
2. Does the presence of *C. pneumoniae* DNA account for some of the excess in cardiovascular events associated with smoking.

Experimental modification: determining whether antibiotics affect surrogate end-points

We have designed a prospective study of 200 respirology and family practice COPD

patients (Smieja and Petrich, co-P.I.s). The study entails both an observational component

and a treatment component. In the second year of the study, between January and March of

2003, we will treat patients with 3 months of azithromycin or placebo to determine whether

antibiotics prior to expected infection decrease subsequent *C. pneumoniae* -DNA detection.

The study will better define the relationship between smoking and *C. pneumoniae* -DNA

detection, determine whether antibiotics given "pre-emptively" affect the incidence and

prevalence of infection, and may establish a relationship between *C. pneumoniae* -DNA

detection and subsequent lung function decline.

The study results will have implications for the design of cardiovascular studies using

similar surrogate end-points of *C. pneumoniae* DNA in peripheral blood. The impact on

inflammatory markers will also be measured. Furthermore, a study of lung function decline

in parallel with carotid intimal medial thickness changes could examine the interaction

between smoking, inflammation, and infection on both lung decline and vascular changes

simultaneously. If similar inflammatory mechanisms contribute to decline in forced

expiratory volumes and progression of atherosclerosis, studying COPD patients could yield

useful data on the interaction of inflammation, infection and smoking in these processes.

These studies would corresponded to the proposed "Phase 2" risk marker studies, of

investigating the effect of therapies on surrogate markers. If intermittent winter dosing of

azithromycin decreases *C. pneumoniae* DNA and decreases inflammatory marker levels, this

would justify exploration of this therapeutic strategy in larger controlled trials powered to

detect clinically important outcome measures.

Conclusions

In conclusion, *C. pneumoniae*, as measured by serology, was associated neither with

cardiovascular outcome, nor with inflammatory markers. *C. pneumoniae* DNA detection in

blood was associated with smoking, but not with cardiovascular disease per se. Thus, on

balance, I found no epidemiologic evidence to support an association of *C. pneumoniae*

infection with human atherosclerotic heart disease.

The association of *C. pneumoniae* with smoking suggests that previous associations

may have been confounded by smoking status. However, the association of *C. pneumonaie*

DNA detection with smoking and season suggest we need to design studies specifically to

investigate whether *C. pneumoniae* contributes to smoking and season-associated

cardiovascular events. Such studies will require DNA markers rather than serology, but

much work remains to develop and validate such tools.

Unlike the relationship between *Helicobacter pylori* and peptic ulcers, *C. pneumoniae* does

not appear to be *the* cause of cardiovascular disease, although these studies do not rule out a

potential *contributing cause*. In concert with ongoing attempts to develop an effective vaccine,

molecular epidemiologic studies of candidate infections may eventually improve our understanding and therapy of human atherosclerotic heart disease.

Final remarks

In this thesis, as with the mythical voyage to Ithaca, the journey was likely more important than the destination. To all my readers, I thank you for sharing that journey.

This is not the end.
This is not even the beginning of the end.
It is, perhaps, the end of the beginning...

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