“Knowledge comes, but wisdom lingers.”

- Lord Tennyson
CARDIOVASCULAR DISEASE (CVD) has emerged as a major health burden worldwide. CVD is responsible for almost one half of all deaths in the developed countries and for one fourth of deaths in the developing countries. By 2020, CVD will cause one of every three deaths worldwide. In U.S., each year 1.5 million are hospitalized for Acute Coronary Syndrome (ACS), and half of them usually die\textsuperscript{129}.

A rise in the prevalence of coronary heart disease (CHD) in early half of twentieth century and a subsequent decline in the latter half have been well documented in industrialized countries. However, the scenario is reversed in developing countries, especially India, with a steady escalation in prevalence of CVD\textsuperscript{130}.

The emergence of the CVD epidemic in the developing countries during the past two to three decades has attracted less comment and little public health response, even within these countries. It is not widely realized that at present, the developing countries contribute a greater share to the global burden of CVD than the developed countries. It has been estimated that 5/3 million deaths attributable to CVD occurred in the developed countries in 1990, where as the corresponding figure for the developing countries ranged between 8 to 9 million (i.e. a relative excess of 70%). Regional estimates of CVD mortality indicate that the difference would be even higher if the term 'developed countries' is restricted to established market economics only and exclude the former socialist economics.

188
This high, yet inadequately recognized, contribution of developing countries to the absolute burden of CVD is readily explained by the fact that 78% of the 49.9 million global deaths (from all the causes) in 1990 occurred in regions other than the established market economics of former socialist economics. Although the relative contribution of CVD deaths to total mortality was higher in the developed countries (~49%) than that in the developing countries (~23%), the excess total mortality in the latter is translated into excess absolute CVD mortality due to the large populations involved. Thus, in 1990 the developing countries contributed 68% of the total global deaths due to noncommunicable disease and 63% of world mortality due to CVD. Although the inadequacies and imperfections of cause-specific mortality ascertainment methods currently used in many developing countries call for cautious interpretation of these estimates, the conservative assumptions made by the analysts suggest that the absolute burden of CVD mortality is indeed likely to be high in developing countries.

Exact data regarding CHD epidemiology in our country are not available. Prevalence of CHD appears to be 10 times higher in our country as compared to that reported 40 years ago. Prevalence of CHD in migrant Indians is two to four times higher than in Caucasians. Prevalence of CHD in urban Indians is fast approaching the figures reported in migrant Indians. CHD in Indians is often premature, more extensive and more severe than among Caucasians. Mortality from CHD is slowly replacing infectious diseases as the leading cause of death in our country and CVD accounts for 24% of all deaths.
Thus, CHD remains a major cause of human mortality and morbidity worldwide. This is in spite of our increasing understanding of the pathophysiology and epidemiology of CHD and the continuing advances in prevention and management.

Established cardiovascular risk factors such as smoking, diabetes, hypertension and hyperlipidaemia do not fully explain the temporal and geographical variations in the prevalence of CHD over the past century. There is hence no surprise that intensive search is on for potential novel cardiovascular risk factors\textsuperscript{1,132}.

Recently, a number of newer cardiovascular risk factors like increased levels of homocysteine, fibrinogen, CRP, apolipoprotein (a) and infection with C. pneumoniae have been identified. These factors are of great interest in native Indians, where more than 60% of CHD remains unexplained by conventional risk factors\textsuperscript{1}.

Table 36 shows relative strength of established and newer cardiovascular disease risk factors for CHD, stroke and peripheral arterial disease.
Table 36 showing relative strength of established and newer cardiovascular disease risk factors for coronary heart disease, stroke, and peripheral arterial disease.

<table>
<thead>
<tr>
<th>Established</th>
<th>CHD</th>
<th>STROKE</th>
<th>PAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced age</td>
<td>+++</td>
<td>+++++</td>
<td>+++</td>
</tr>
<tr>
<td>Male gender</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Hypertension</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Low LDL cholesterol</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Newer</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Homocysteine</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>++++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>++++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>++</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

Table 36 has been adapted from the latest edition of Cecil's Textbook of Medicine. It shows comparative associations of established and newer risk factors in patients of CHD, stroke and peripheral arterial disease. Important differences include the strength of inflammatory factors in CHD, the dominance of hypertension for stroke and the importance of smoking and diabetes for peripheral arterial disease.

Studies on cardiovascular risk factors in native Indians are warranted as most risk factors for CHD have been derived from Western studies.

Comparative studies on newer risk factors illustrated that Asian Indians have higher CRP, plasminogen activator inhibitor and homocysteine levels.
Significance of C. pneumoniae infection as a novel factor for CHD has been stressed in the latest edition of Cecil's Textbook of Medicine and the number of published reports investigating role of this organism in atherosclerosis has more than tripled in last 5 years as compared to the previous decade. However, except for few studies, this topic has remained unattended in our country. At least, no work has been carried out from this part of the country.

An attempt has been made in the present study to correlate C. pneumoniae infection with ACS, and to evaluate the value of azithromycin, a specific antichlamydial macrolide antibiotic, on the outcome of adverse cardiovascular events during 6 months follow up in the seropositive patients.

ATHEROSCLEROSIS AND INFLAMMATION:
Atherosclerosis is the commonest cause of CHD. Though formerly considered as a bland lipid storage disease, atherosclerosis actually involves an ongoing inflammatory response.

Presently, there has been increasing interest in the relationship between infection, inflammation and the development of atherosclerosis.

At the turn of twentieth century, Sir William Osler (in 1908) and Ophulus (in 1921) proposed the concept that infection could be a causal factor in the pathogenesis of atherosclerosis. Frothingham in 1911 suggested that "the sclerosis of old age may simply be a summation of lesions arising from infections or metabolic toxins". On the basis of an examination of 4,00,000 sections from 40 autopsy cases in 1935, Leary...
used the term "abscess" to describe atheromatous plaques, because leukocyte infiltration suggested an inflammatory process\textsuperscript{18,53}.

Crucial advances in the understanding of the pathogenesis of atherosclerosis have been achieved during the last two decades. The two major historical hypotheses concerning pathogenesis, the "incrustation" and "lipid" hypotheses, have evolved into the new concepts that integrate several factors contributing to the initiation and the evolution of this disease.
Fig. 31 showing role of inflammation in atherosclerosis

**a Initiation of atherosclerosis**
- Endothelial dysfunction
- Inflammation
- Foam cells

**b Early lesion**
- Inflammation
- Foam cell (fatty streak)

**c Vulnerable plaque**
- Thin fibrous cap
- Lipid core: abundant foam cells
- Intense inflammation (shoulder region)

**d Advanced lesion**
- Fibroblast abundance
- Fibroblasts and matrix
- Extracellular calcification

Source: Nat Med © 2002 Nature Publishing Group
As seen in Fig. 31, dysfunctional endothelium and inflammation are now known to have pivotal roles in the initiation and progression of atherosclerotic disease, and are considered to be promoters of the disease. The atherosclerotic and thrombotic processes appear somewhat interdependent and may hence be integrated under the term atherothrombosis.

Pathologically, atherosclerosis involves injury, inflammation, infiltration, degeneration and thrombosis.

A substantial body of evidence now implicates inflammation and immune activation in the pathogenesis of atherosclerosis, thrombosis and neointimal thickening after arterial injury. A number of potential triggers capable of inducing proinflammatory responses have been identified. These include modified lipoproteins, cytokines, chemokines, angiotensin II, hypertension, hyperglycaemia, smoking, oxidative stress etc.

Atherosclerosis plaques are composed mainly of extra cellular matrix (including collagen, proteoglycans, fibronectin, and elastic fibers), lipids (crystalline cholesterol, cholesteryl esters, and phospholipids), inflammatory cells (monocyte – derived macrophages, T lymphocytes), smooth muscle cells, thrombi and calcium deposits. Varying proportions of these components occur in different plaques, giving rise to a wide variety of lesions.

To date, more than 20 inflammation associated cell adhesion molecules and almost 50 proinflammatory cytokines have been described. A significant number of these
reactants are also frequently present in advanced lesions, including fibrinogen, CRP, and serum amyloid-A\textsuperscript{131}.

Evidence indicates that both the composition of plaque and its propensity to rupture are major determinants of future ischaemic events. Disruption prone plaques in the coronary arteries, the so called high risk or vulnerable plaques, tend to have a thin, fibrous cap; a large lipid-core; and a high macrophage content, ACS often results from disruption of such modestly stenotic, lipid-rich, vulnerable plaques, which may not be detectable by X-ray angiography, leading to thrombotic complications\textsuperscript{134}

Fig. 32 showing macrophage accumulation in culprit plaque.

Fig. 32 represents a bar graph showing significantly more macrophages in culprit plaques responsible for unstable acute coronary syndromes (n = 18) than in those responsible for chronic stable angina (n = 8). Macrophages were identified by
responsible for chronic stable angina (n = 8). Macrophages were identified by immunohistochemical technique, using a specific monoclonal antibody against macrophages.

An accumulating body of recent evidence suggests that systemic factors may influence local plaque instability. Compared with stable angina, the temperature of culprit lesions is 0.6 °C higher in unstable angina and 1.0 °C higher in AMI. The plaque’s temperature correlates with the systemic level of circulating cell adhesion molecules, cytokines and CRP. These data suggest an interaction between systemic and local inflammatory processes.

Compared with controls, patients with unstable angina have more than twice the blood levels of interleukin-6, CRP and macrophage colony stimulating factor. These levels decrease after 6 weeks of aspirin treatment. Efficacy of aspirin in reducing early post hospitalization cardiac events is well known. Anti-inflammatory effect of aspirin may be additive to its antithrombotic effect in patients with plaque instability.

The exact stimulus for this inflammatory response is not known.

One of the most interesting developments in recent years has been the hypothesis that one or more infectious agents may play a role in atherothrombosis, either through a direct proinflammatory effect on the vessel wall (endothelial injury or dysfunction, smooth muscle cell proliferation and local inflammation) or, more commonly through a less specific long distance proinflammatory effect through chronic inflammation, cross
reactive antibodies, or changes in known or suspected cardiovascular risk factors such as lipids, coagulation proteins, oxidative metabolites or homocysteine\textsuperscript{3,17,53}.

Such potential mechanisms may act acutely to precipitate plaque rupture, or chronically to promote plaque growth.

Thus, a role for both the local inflammatory response in plaque (especially macrophages and T lymphocytes) and systemic inflammation, in patients at increased risk for coronary events, has been increasingly recognized and documented\textsuperscript{17,41,113}.

Our understanding of atherosclerosis has thus evolved beyond the view that lesions consist of lifeless collection of lipid debris.

Indeed, atherosclerosis is now accepted as an inflammatory disease.

**HOW INFECTION AGENTS CAUSE INFLAMMATION:**

The idea that microorganisms may cause inflammatory or immune—mediated, 'non-infectious' diseases is not unusual. H. pylori is established etiological factor in peptic ulcer disease. Other clinical scenarios include recognition of the role of oncogenic viruses eg. Epstein Barr virus in nasopharyngeal carcinoma, through to the more obscure roles of Tropheryma whippleii in Whipple's disease. Infection may therefore serve as an important and plausible biological link between inflammation and CHD.

Recently, it has been suggested that infectious agents may directly or indirectly trigger the cascade of biological and biochemical reactions, which ultimately lead to
inflammation, atherosclerosis and vascular thrombotic events\textsuperscript{2-4}. As far as ACS is concerned, cytomegalovirus and C. pneumoniae have received increasing attention. Whether these or other infectious agents are the cause, cofactor or an innocent commensal – bystander – relative to atherosclerosis is disputed\textsuperscript{2}. One thing is certain. With passage of time, many chronic inflammatory or degenerative diseases which were once upon a time considered noninfectious, are now being considered as infectious and that too, treatable with antibiotics. This is being dramatically exemplified by the response of peptic ulcer disease to antibiotic therapy directed at H. pylori, now known to play a key etiologic role.

More than a century ago, Robert Koch proposed three criteria that should be met to link an infectious agent causally with production of a disease:

1) The causative organism can be isolated from the affected host
2) The infectious agent can be identified by culture or directly by microscopy, and
3) On transfer to a susceptible host, the infectious agent must produce the disease.

At present, however, Koch's postulates in regard to an infectious cause of atherosclerosis remain unfulfilled in humans. The Marek's disease in chickens, however, fulfills all the Koch's postulates and provides strong experimental evidence in support of vital etiology of atherosclerosis. Observational evidence supporting involvement of infectious agents in atherosclerosis is summarized in Table-37 \textsuperscript{2,4}.
Two particular infectious agents, one bacterial (C. pneumoniae) and one viral (CMV), are currently linked with atherosclerosis and supported by emerging evidence. A large database of seroepidemiological studies has established grounds for a clinically relevant link between CP and CMV with atherosclerosis and in the case of CMV with restenosis after angioplasty. In the realm of seroepidemiological studies, increasing number of surveys of relatively small numbers of patients at a single centre is not likely to provide further illumination. Retrospective studies are subject to well known biases. It is difficult to compare results across various seroepidemiological studies because of differences in reagents and assay protocols. Perhaps a core testing laboratory would facilitate future seroepidemiological studies. Studies of geographically diverse populations with differing risks for atherosclerosis could provide new insight in testing of the relationship between infectious agents and atherosclerosis.
A recent review has concluded that the available evidence about chronic infections and coronary heart disease is still sparse and its interpretation is limited by potential biases\textsuperscript{17}. Evidence is weak for *Helicobacter pylori* infection. For CP the evidence of association is stronger, but the temporal sequence of infection and coronary heart disease is uncertain. For CMV, only a limited number of patients with classic atherosclerotic disease have been studied. Some of the uncertainties may be resolved by better and larger seroepidemiological or pathology-based studies\textsuperscript{17}.

The link between *C.pneumoniae* and atherosclerosis first came from seroepidemiological studies from Finland\textsuperscript{23}, and thereafter from the same and other groups\textsuperscript{25,77,113}.

How can infectious agents promote atherogenesis? Libby et al\textsuperscript{47} proposed that both direct and indirect effects on vascular wall cells may be involved.
As seen in Fig. 33, direct effects could be as follows:

(i) Infection may cause lethal lytic damage, or infected cells may survive but show altered function.

(ii) Endothelial dysfunction can manifest in the form of increased procoagulant activity, reduced intrinsic fibrinolysis, increased leukocyte adhesion and increased production of cytokines.

(iii) Smooth muscle cell dysfunction associated with increased proliferation, reduced apoptosis, increased cholesterol esterification and increased cytokine production.

(iv) Recruitment and activation of leukocytes.
Fig. 33 especially highlights the potential cross talk between intrinsic vascular wall cells (endothelium and smooth muscle), and among vascular cells and leukocytes.

Additionally, there may be indirect effects on vascular cells, or vessel associated leukocytes as seen in Fig. 34.

Fig. 34 showing **indirect effects** of infectious agents on intrinsic vascular wall cells.

(i) Mononuclear phagocytes may show increased procoagulant and decreased fibrinolytic activity, change in lipid metabolism, increased production of cytokines and release of toxic oxygen species.
T cells may show increased proliferation, increased cytokine production and increased lytic activity for vascular wall cells.

This Fig 34 also highlights potential cross talk between leukocytes and intrinsic endothelium and smooth muscle cells.

With respect to C.pneumoniae, little data is available regarding biology of vascular wall infection. C.pneumoniae commonly infects mononuclear phagocytes, and can infect epithelial cells persistently under certain conditions.

Chlamydial life cycle involves the elementary body (extra cellular, infectious form) and the reticulate body (intra cellular, replicative form). A metabolically inactive but viable form, the persistent body, has also been described during stressful conditions. All this reflects that C.pneumoniae are ideal candidates to produce chronic, persistent, nonlytic cellular infection.

Further, during infection, inflammatory cytokines like IL-1β, IL-6 and TNF-α increase. TNFα and IL-1 upregulate IL-8. Hepatocytes respond to IL-1 and IL-6 by producing CRP. In turn, CRP induces expression of monocyte tissue factor. All these interrelated inflammatory mediators enhance macrophage / monocyte activation.

**ATHEROSCLEROSIS: LIPID INFILTRATION OR CHLAMYDIA PNEUMONIAE INFECTION?**

Atherosclerosis manifests as lipid infiltrative type of lesions with primary attachment and infiltration of monocyte / macrophage foam cells. Pathological studies, however,
show additional features, such as the foam cell component consisting of a combination of macrophages and intimal smooth muscle cells, and containing not only lipid but C.pneumoniae organisms as well. Another feature is fragmentation and necrosis of C.pneumoniae-infected intimal smooth muscle cells, with a monocyte / macrophage response showing engulfment not only of lipid, but also of muscle fragments and C.pneumoniae organisms. When examined with an electron microscope, the central lipid rich core consists mainly of a colony of lipophilic C.pneumoniae organisms, not lipids as previously thought.

This type of arterial lesion seems to occur even in patients with cholesterol disorders. The association of the hypercholesterolemia and premature severe atherosclerosis in homozygous familial hypercholesterolemia (HFH) forms the strongest basis on which cholesterol is implicated in the atherosclerotic process. Patients with this rare, autosomal, co-dominantly inherited disorder are characterized by markedly elevated levels of plasma low-density lipoprotein cholesterol, tendon xanthomata, and severe, premature atherosclerosis, particularly coronary artery disease. If untreated, the majority of patients with this disorder die from accelerated atherosclerosis before the age of 30 years. Shor examined atherosclerotic lesions of a 16 year old female with HFH who died as a result of severe premature atherosclerosis. Somewhat surprisingly, the atherosclerotic lesions were not lipid infiltrative in nature, as one would expect, but showed the same pathological features as conventional atherosclerosis, with large numbers of C.pneumoniae organisms. Perhaps cholesterol is capable of enhancing the growth of C.pneumoniae organisms, as lipid is not only produced by Chlamydia nutrition, metabolism, and cell wall formation. Hypercholesterolemia is a symptom of HFH, the primary disorder that is a defect of...
the cholesterol receptors characterized by decreased cellular utilization of cholesterol. Relatively recently, it has been discovered that cholesterol plays a cellular role in mechanisms of transcription and post transcription events that affect meiosis, apoptosis, developmental patterning, protein cleavage, and protein degradation of cells.94 Perhaps defects in regulatory cholesterol-related events play a role. Whatever the association between lipid and this disease, pathological studies suggest human atherosclerosis is an infective Chlamydia lesion, rather than a lipid infiltrative type lesion.

**HOW C.PNEUMONIAE PRODUCES CHD:**

There has been a resurgence of interest in the infectious basis of coronary atherogenesis. Several recent reviews have suggested that coronary atherosclerosis may be an auto immune process triggered by an infectious agent, most likely C.pneumoniae.

Persistant elevation in IgA and IgG titers and the presence of immune complexes were clearly more common among case patients than among controls in several studies. This supports an association between chronic C.pneumoniae infection and the risk for CHD.

Experimental studies have demonstrated that C.pneumoniae can replicate and maintain infections in human macrophages, endothelial cells, and aortic smooth muscle cells.95 These cell types show particular susceptibility to infections with C.pneumoniae.110
Fig. 35 showing postulated mechanisms by which C. pneumoniae infection can produce increased risk of AMI.

**DIRECT EFFECTS**

Direct infection of arterial wall
- Smooth-muscle proliferation associated with p53 inactivation
- Local inflammation

Systemic infection
- Endothelial injury (or dysfunction due to circulating endotoxin)

Atherogenesis
- Plaque rupture
- Thrombosis

Autoimmunity
- Hsp60 cross-reactivity with bacterial antigens

Systemic inflammation
- C-reactive protein
- Leukocyte count
- Cytokines

Classic risk factors
- HDL cholesterol
- Fibrinogen
- Triglycerides

**INDIRECT**

As seen in Fig. 35, C. pneumoniae infection contributes to both atherogenesis and atherothrombosis through various locally and systemically operating mechanisms. These include direct infection of the arterial wall with smooth muscle proliferation and local inflammation. Besides, there is endothelial injury or dysfunction due to circulating...
endotoxin. Additionally, there is increased synthesis of inflammatory mediators like CRP, leukocytes, cytokines and fibrinogen; autoimmunity through cross-reactivity of heat shock proteins with bacterial antigens, lipoprotein disturbances, monocyte activation and enhanced activity of procoagulant mediators\textsuperscript{17}.

Several mechanisms could allow a chronic Chlamydial infection to affect the development of CHD including ACS. Chlamydia are gram negative bacteria with a notable tendency to produce chronic infections. C.pneumoniae commonly invades lungs and is responsible for producing 5-10\% of pneumonia in adults. C.pneumoniae multiplies in mononuclear phagocytes. A persistent chronic infection of alveolar macrophages may be of special importance because of their close association with circulatory system. This allows easy access to the blood stream for bacteria and bacterial components such as lipopolysaccharide. Lipopolysaccharide has powerful effects on vascular permeability and clotting mechanisms. Bacterial components can form immune complexes with currently present antibodies. This process can lead to tissue injury.

Chlamydial lipopolysaccharide binds with LDL, modifies the lipoprotein to make it immunogenic or toxic to endothelial cells, and foam cells are formed.

C.pneumoniae induces a chronic immune activation mediated by cytokines such as IL-1, IL-6 and TNF. C.pneumoniae bacterial endotoxin and TNF produce endothelial dysfunction.
Atherosclerosis is largely viewed as a chronic inflammatory disease. In this respect, the life cycle of Chlamydia, obligate intra cellular pathogens, appears particularly interesting. During the usual infective cycle generating new infectious progeny, Chlamydia express basal levels of two major antigens – the major outer membrane protein (MOMP) and the heat shock protein 60 (HSP 60; 60 stands for 60 kDa). Under some conditions, however, such as in the presence of interferon – α, a product of activated T cells within atheroma, certain Chlamydia can achieve a state of intra cellular chronic, persistent infections in which they remain viable but metabolically quiescent and do not replicate. During such chronic persistent infections HSP-60 production increases substantially, where as MOMP becomes almost undetectable.

Expression of HSPs, also called chaperonins, a ubiquitous family of highly conserved proteins, increases during a variety of conditions such as heat shock, nutrient deprivation, infections, and inflammatory reactions, functioning to stabilize cellular proteins. Atheromatous vessels contain endogenous human HSP-60. Human HP-60, when expressed by heat shocked endothelial cells, can provoke an autoimmune reaction mediating endothelial cytotoxicity. Microbial HSP-60, abundantly produced during a chronic chlamydial infection of the vessel wall, might augment atherosclerosis and/or stimulate humoral and cellular immunity in atheroma.

Although C.pneumoniae can infect most cells present in atheroma, it localizes mainly in plaque macrophages. Mediators elaborated by these phagocytic leukocytes probably contribute importantly to atherogenesis. Tumor necrosis factor α (TNF α) provides one example of cytokine produced by macrophages within atheroma. C.pneumoniae infection induces TNF α secretion by peripheral human monocytes.
This cytokine can induce a number of vascular cell function relevant to atherogenesis, including expression of endothelial leukocyte adhesion molecules and synthesis of interleukin – 1, mRNA by endothelial cells and smooth muscle cells. Lesional macrophages can also produce matrixs, metalloproteinases (MMPs), enzymes now accorded a major role in the degradation of connecting issue. Thus, macrophage – derived MMPs might promote plaque rupture and consequent thrombosis, the ultimate causes of acute coronary syndrome.

Arterial relaxation is largely regulated by endothelial nitric oxide (NO). Its diminished activity has been associated with incipient atherosclerosis. *C.pneumoniae* impairs arterial endothelial function and the NO pathway is principally involved. Cyclooxygenase-dependent vasoconstricting products may also account for the infection – induced impaired relaxation. Release of prostacyclin, a potent vasodilator and which also possesses strong antiplatelet activity is impaired. Consequently, thrombogenesis occurs. The infection – induced functional changes of the arterial endothelium precede the morphological changes (intimal thickening) in the aorta. These finding further support role of *C.pneumoniae* infection in atherosclerotic development.53.

Kol et al55 reported co localization of Chlamydia HSP 60 and human HSP 60 within carotid atherosclerotic plaque macrophages. They also showed that both these HSP 60 could activate macrophage TNF, which is a proinflammatory cytokine and matrix metalloproteinases which are enzymes that can degrade connective tissue, reduce the strength of atherosclerotic intimal cap and produce plaque rupture. Chlamydial HSP
may thus promote atherogenesis and atherothrombosis by direct antigenic stimulation and by acting as a signal transducer in macrophage activation\textsuperscript{2,38}.

As part of systemic effects, \textit{C.pneumoniae} infection may induce enhanced production of acute phase proteins like fibrinogen\textsuperscript{56} (a known risk factor for cardiovascular disease), CRP\textsuperscript{13,56} (strong independent predictor of subsequent cardiovascular events) and neopterin\textsuperscript{53} (a marker of monocyte activation). Chronic chlamydial infection produces hypercoagulable state through activation of monocyte-derived procoagulants eg. Tissue factor, upgradation of other inflammatory markers like monocyte integrins CD11b / CD11c, raised leukocyte count, and expression of adhesion molecules like VCAM-1 / ICAM-1\textsuperscript{2}.

Factor VII is not an acute phase protein but has been linked to CHD. Factor VII antigen in responsible for most of factor VII coagulant activity. Chronic chlamydial infection can activate factor VII antigen via a tissue factor dependent process, inducing a procoagulant state and thereby influencing the risk of CHD\textsuperscript{56}.

Raised malondialdehyde concentrations due to release of oxidative free radicals suggests another possible mechanism whereby \textit{C.pneumoniae} may influence CHD\textsuperscript{53}.

All these mechanisms which may link \textit{C.pneumoniae} infection and CHD are shown in Table 38.

\textbf{Table 38 showing specific factors that may link \textit{Chlamydia pneumoniae} Infection and Coronary Artery Disease.}

<table>
<thead>
<tr>
<th>Systemic inflammation</th>
<th>Increased C-reactive protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increased leukocyte count</td>
</tr>
</tbody>
</table>

211
<table>
<thead>
<tr>
<th>Systemic infection</th>
<th>Increased cytokines (interleukin 6, interleukin 8, tumor necrosis factor α)</th>
<th>Activated monocytes and monocyte integrins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelium</td>
<td>Endotoxin (lipopolysaccharide) secreted by C. pneumoniae</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Endothelial dysfunction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enhanced expression of endothelial adhesion molecules</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increase in tissue factor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlamydia heat shock protein-60</td>
<td></td>
</tr>
<tr>
<td>Vascular smooth muscle cells</td>
<td>Activate the nuclear factor for the expression of immunoglobulin α light chain in the B-lymphocytes pathway (nuclear factor α-B).</td>
<td></td>
</tr>
<tr>
<td>Activation of macrophages (macrophages ingest C.pneumoniae)</td>
<td>Activation of monocyte-derived macrophages</td>
<td>Alteration of cholesterol metabolism and lipid oxidation</td>
</tr>
<tr>
<td>Autoimmunity in genetically susceptible individuals</td>
<td>Cross reactivity of bacterial antigens with vasculature antigenic mimicry</td>
<td>Chlamydia heat shock protein 60</td>
</tr>
<tr>
<td></td>
<td>Mycobacterial heat shock protein 60</td>
<td>Increased antibodies to specific C.pneumoniae antigens</td>
</tr>
<tr>
<td>Alteration of classic risk factors</td>
<td>Increased triglycerides</td>
<td>Increased total cholesterol levels</td>
</tr>
<tr>
<td></td>
<td>Decreased high-density lipoprotein cholesterol levels</td>
<td>Increased lipoprotein levels</td>
</tr>
<tr>
<td></td>
<td>Glucose intolerance</td>
<td></td>
</tr>
<tr>
<td>Hypercoagulability</td>
<td>Increased fibrinogen</td>
<td>Increased thrombin</td>
</tr>
<tr>
<td></td>
<td>Increased expression of tissue factor (factor VIIa antigen)</td>
<td>Increased plasminogen activator inhibitor</td>
</tr>
<tr>
<td></td>
<td>Increased platelet accumulation / adhesion</td>
<td>Increased platelet accumulation / adhesion</td>
</tr>
<tr>
<td></td>
<td>Enhanced activity of homeostatic and procoagulant</td>
<td></td>
</tr>
</tbody>
</table>

212
The same mechanisms are separately shown in the form of a schematic representation in Fig. 35.

Therefore, several mechanisms which have been suggested whereby *C. pneumoniae* infection contributes to atherosclerosis and thereby development of CHD including ACS include:

1. Cytopathologic effects of infection on endothelial and smooth muscle cells\(^{25,47}\).
2. Formation of circulating toxins or immune complexes that could deposit on vessel walls, eliciting an inflammatory response\(^{23}\).
3. Induction of alterations in serum lipid metabolism\(^{2,17}\).
4. Enhanced activity of procoagulant mediators\(^{66}\).

Blassi et al\(^ {112}\) proposed that *C. pneumoniae* reinfection could lead to instability within atherosclerotic plaques via reactivation of a chronic or latent infection and / or immune mediated endothelial damage. Thus, an acute infection, superimposed on a chronic or latent infection (reinfection) may trigger the onset of AMI.

**C. PNEUMONIAE - WHETHER CAUSE OR BYSTANDER?**

At present, the evidence for an association of Chlamydia pneumoniae and atherosclerosis does not constitute causation. Data regarding whether infection...
That the presence of the organism is related to the pathogenesis of disease is circumstantial.

Three possibilities can be examined. 
(i) The organism persists in vascular cells but does not contribute to pathologic abnormality.
(ii) Causes the initial injury and induces the atherosclerosis process or
(iii) Accelerates the severity or progression of the disease.
The "innocent bystander" hypothesis argues that C. pneumoniae could merely be a secondary passenger transported via circulating monocytes from the respiratory tract and thereafter to remain dormant in atherosclerotic vascular tissue, and that it has no role in atherogenesis or atherothrombosis\textsuperscript{31}. This is perhaps supported by the fact that C. pneumoniae has been also detected in sites other than cardiovascular system eg. in lungs, liver, spleen, bone marrow, lymph nodes and granulomatous specimens\textsuperscript{31}. However, this argument loses ground when one considers that c-pneumoniae is after all a ubiquitous organism. Further, though C. pneumoniae was found in noncardiovascular tissue, it was found much more frequently in the cardiovascular tissue\textsuperscript{21}.

It has been also argued that infection with C. pneumoniae and the development of antibody may have followed the development of CHD. However, no relationship has been noted between CHD and antibodies to other organisms like Mycoplasma pneumoniae, which also happens to be a common respiratory pathogen.

If the organism is involved its role must fit within the context of events in atherogenesis. Chronic infection of macrophages may contribute to inflammatory processes\textsuperscript{56} or trigger a procoagulant state through tissue factor expression\textsuperscript{53,56}. The bacterial lipopolysaccharide cell wall itself may contribute to direct endothelial cell damage. Kuo et al\textsuperscript{86} showed that chances of identifying C. pneumoniae within atherosclerotic lesions was inversely related to serum antibody. They suggested that hypersensitivity to C. pneumoniae may play a role in atherogenesis. This observation can be compared to
involvement of C.trachomatis in the pathogenesis of trachoma\textsuperscript{56}. However, Kuo et al\textsuperscript{86} studied small number of autopsy coronary atheroma specimens and thereafter reported such findings. In that case, it is easy to understand that antibody titer from haemolysed specimen is difficult to evaluate. Therefore, their proposition does not carry much weightage. When similar diagnostic techniques were followed in a later study, results demonstrated C.pneumoniae organisms within coronary atherectomy specimens of patients with angina, & they did not notice any inverse relationship between the likelihood of detecting the organisms and the height of the anti Chlamydia pneumoniae antibody titer\textsuperscript{86}.

Davidson et al\textsuperscript{111} proposed that replication of C.pneumoniae within human macrophages, endothelial cells and vascular smooth muscle cells gives biological plausibility to the concept of a chronic intravascular infection that produces rather than follows an immune response.

In an attempt to clarify the issue whether C.pneumoniae plays a pathogenetic or commensal role, Anderson et al\textsuperscript{113} repeatedly infected 30 rabbits intranasally with C.pneumoniae or saline, then randomly assigned them to 2 months of azithromycin or no treatment, and assessed aortic maximal intimal thickness (MIT) and plaque area index (PAI), at 3 months. MIT and PAI were increased 3 fold in infected, untreated animals compared to uninfected controls. In contrast, infected animals treated with antibiotics showed MIT and PAIs similar to uninfected animals. These results suggest that C.pneumoniae infection may accelerate atherogenesis, and anti chlamydial therapy may prevent it.
Animal models are important in demonstrating the pathological role of and temporal
relationship between C.pneumoniae infection and the development of atherosclerotic
lesions. The synergistic effect and interaction between C.pneumoniae infection and
cardiovascular risk factors eg. Hypercholesterolaemia and genetic predisposition, can
very well be judged when animal models are employed. Whether animal models
(where atherosclerosis is acutely induced in laboratory), can translate into human
atherosclerosis (which develops over several years) remains to be established53.

Davidson et al111 reported that serological evidence for C.pneumoniae infection
frequently precedes both the earliest and the more advanced lesions of coronary
atherosclerosis that harbor this intracellular pathogen, suggesting a chronic infection
and causal developmental role in CHD.

Helsinki Heart Study77 provided serological data suggesting that infection does not
represent a proclivity for C.pneumoniae to land in injured cardiac tissues or for
myocardial damage to reactivate a latent infection.

Several studies20,29,43,113,118 have reported increased association between positive
antichlamydial antibody titer and the risk of future adverse cardiovascular events.
Some studies20,118-120 have also observed beneficial effects of azithromycin, a specific
antichlamydial antibiotic, in reducing adverse cardiovascular events. Azithromycin, by
eradicating or suppressing infection, may have helped to "stabilize" active plaque
lesions, in part by dampening inflammation and hypercoagulation.
Blassi et al\textsuperscript{112} reported that C. pneumoniae infection may act as a trigger for AMI. C. pneumoniae reinfection could lead to instability within the atherosclerotic plaques via a reactivation of a chronic or latent infection and/or immune-mediated endothelial damage. They summarized that an acute infection superimposed on a chronic or latent infection (reinfection) may trigger the onset of AMI.

These findings suggest a direct causal role of C. pneumoniae in atherogenesis.

**BASIC SCIENCES VERSUS CLINICAL STUDIES:**

The concept that C. pneumoniae contributes to atherothrombosis in humans needs to be documented. This is in spite of initial encouraging positive results. Data from laboratory studies and clinical trials seem to diverge. There is a greater evidence in favour of a role of C. pneumoniae emerging from basic laboratory studies. As against that, clinical trials of patients with stable and unstable CHD failed to demonstrate a significant benefit of antibiotic therapy\textsuperscript{22,57}. Several prospective, seroepidemiological studies have failed to confirm the results of retrospective studies which had reported a positive association between serological evidence of C. pneumoniae infection and CHD. While negative serology does not necessarily rule out infection, substantial inconsistencies should be properly settled to validate the hypothesis.

Danesh et al\textsuperscript{45} reported that their metaanalysis of prospective seroepidemiological studies failed to demonstrate existence of any independent association between persistent C. pneumoniae infection and CHD. The combined odds ratio was only 1.15 (0.97 to 1.36), which is much weaker than the combined weighted odds ratio for
atherosclerosis of 20 (15 to 32) obtained from pathology based studies that assessed human arterial specimens for endovascular markers of C.pneumoniae (DNA, antigens, elementary bodies or viable organisms)\textsuperscript{75}.

What might account for this 20 fold discrepancy? Pathology based studies are essentially retrospective. Therefore, they pose uncertainty about whether local C.pneumoniae infection is a cause or consequence of atheroma. Prospective studies examined evidence of infection several years before CHD was diagnosed.

Secondly, most pathology based studies can be blamed for selection biases, and lack of adjustment for possible confounders such as age, sex and smoking\textsuperscript{75}. However, these explanations are much too big to explain the 20 fold difference.

Lastly, the role played by different definitions of vascular disease (atheroma versus major CHD events) and the different markers of infection (endovascular markers such as DNA and antigens versus circulating antibody titers) employed in different sets of studies needs to be certified, and may be responsible for part of discrepancy.

Epidemiological variations like these need to consider also the number of patients to be studied to arrive at a proper statistical significance. If the 20 fold odds ratio reported in pathology based studies suggested a causal effect that was largely reversible, then antichlamydial treatments might be expected to achieve a significant reduction of adverse cardiovascular events. To confirm or refute claims of such positive results, future studies should involve similar number of patients, having similar risk and identical follow up.
In case prospective studies tend to show any positive association between C. pneumoniae infection and CHD, trials larger than those previously conducted or currently in progress should be carried out to achieve a proper statistical significance. Even if there is a 10% excess risk due to C. pneumoniae that is fully reversible by antibiotics, none of the trials shown in Table 39 would be large enough to confirm or refute its existence. The largest current trials as shown in Table 39 are not sufficiently powered to detect reductions in coronary events that are less than 25%.

Table 39 showing large sized randomized trials of antichlamydial strategies for prevention of CHD, either in progress or recently completed.

<table>
<thead>
<tr>
<th>Study</th>
<th>Location</th>
<th>Planned size</th>
<th>Entry criteria</th>
<th>Drug Duration (months)</th>
<th>Follow up (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACES</td>
<td>United States</td>
<td>4000</td>
<td>Previous myocardial infarction or coronary revascularization</td>
<td>Azithromycin/1 2</td>
<td>4</td>
</tr>
<tr>
<td>PROVEIT</td>
<td>United States</td>
<td>4000</td>
<td>Acute coronary syndrome</td>
<td>Gatifloxacin/1 8</td>
<td>1.5</td>
</tr>
<tr>
<td>WIZARD</td>
<td>United States</td>
<td>3800</td>
<td>Previous myocardial infarction or coronary revascularization</td>
<td>Azithromycin/3</td>
<td>3</td>
</tr>
<tr>
<td>MARBLE</td>
<td>United Kingdom</td>
<td>1300</td>
<td>Waiting for coronary artery bypass graft surgery</td>
<td>Azithromycin/3</td>
<td>1</td>
</tr>
<tr>
<td>STAMINA</td>
<td>United Kingdom</td>
<td>600</td>
<td>Previous myocardial infarction</td>
<td>Azithromycin plus drugs against Helicobacter pylori/0.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Patients randomized irrespective of C pneumoniae serostatus.

**ISSUE OF “TOTAL PATHOGEN BURDEN”:**
Evidence showing relationship between specific viral and bacterial infection and atherosclerosis, and therefore with CHD, continues to accumulate. However, any causal link remains tantalizing, awaiting to be proven, and thus remains to be demonstrated.

Previous studies have produced inconsistent results with respect to exact identity of the relevant pathogens (viruses, bacteria or both)\textsuperscript{18}.

In order to evaluate the role of previous infection with atherosclerosis, several investigators carried out prospective controlled studies. These studies were directed to ascertain the exposure status of the organism before the onset of thrombosis.

Some of the studies of C.pneumoniae\textsuperscript{75,77,82}, H.pylori\textsuperscript{79,136}, herpes simplex\textsuperscript{53,76}, and cytomegalovirus\textsuperscript{76} have not provided a strong evidence of association. However, none of these studies evaluated exposure due to simultaneous multiple infections.

This is a potentially important issue, because it has been hypothesized that multiple pathogens may be involved, and that this local pathogen burden may be a more relevant marker of risk infections alone\textsuperscript{18}. The total burden of pathogen may be a critical factor in determining atherogenesis. Experimental evidence suggests that infections with several agents can lead to accelerated atherosclerosis without thrombosis\textsuperscript{47}. 

221
The use of an antibiotic to prevent the development of clinical CHD is based on the hypothesis that an underlying infection could predispose the individual to progression of an atherosclerotic plaque. Espinola – Klein et al.\textsuperscript{136} demonstrated that patients with seropositivity to multiple pathogens have an increased prevalence of advanced atherosclerosis. They have increased risk of death. When seropositive findings were 0 to 3, there was limited atherosclerosis and the mortality rate was only 1.4%. When seropositive findings ranged from 6 to 8, there was more advanced atherosclerosis and the mortality rate was 20%.

Although numerous pathogens have been identified to serve as infectious stimuli, it is for C.pneumoniae that the most supportive data exists. The prospective data evaluating relation between baseline antibody titers against various plausible organisms producing simultaneous infection and the risk for cardiovascular disease are not only sparse but often contradictory.

Ridker et al.\textsuperscript{76} found little evidence of an association between risk for cardiovascular events and baseline IgG seropositivity for antibodies against C.pneumoniae (rate ratio, 1.1 [95% CI, 0.7 to 1.8]), H.pylori (rate ratio, 0.9 [95% CI, 0.6 to 1.4]), herpes simplex virus (rate ratio, 1.2 [95% CI, 0.6 to 2.1]), and cytomegalovirus (rate ratio, 0.9 [95% CI, 0.6 to 1.5]). Further any association between a participant's total number of infections and subsequent cardiovascular risk was weak and not significant (P>0.2).

In contrast, Roivainen et al.\textsuperscript{16} emphasized that combination of at least 2 different infections are potential triggers of the inflammatory reaction, one of the key events in atherosclerosis. They measured baseline levels of CRP and antibodies to adenovirus,
enterovirus, cytomegalovirus, and herpes simplex virus as well as C.pneumoniae and H.pylori in 241 subjects, who suffered either myocardial infarction or coronary death during the 8.5 year trial in the Helsinki Heart Study, a coronary prevention trial.

When the joint effects between high antibody levels and high CRP were correlated in their study, vast differences emerged between herpes simplex virus infection and C.pneumoniae infection. High herpes simplex virus level increased risk of CHD even in subjects with low CRP, but, to increase the risk, high C.pneumoniae antibody levels required the presence of high CRP. The independent contribution of both high herpes simplex virus and high C.pneumoniae antibody levels in the joint effect analysis indicates that chronic infection with either of these two agents alone increases the risk, and coexistence of the other is close to additive with regard to CHD risk.

Thus, risk of CHD associated with high antibody levels alone was moderate, but when the CRP level was simultaneously high, the risk was increased substantially.

Ongoing inflammation increases CRP. Simultaneous infection due to multiple organisms will profoundly increase CRP, and in turn, atherosclerotic CHD risk.

This clarifies that aggregate exposure to many candidate infectious agents ("pathogen burden") appears to be a stronger, more consistent risk marker.

**SEROLOGIC DIAGNOSIS OF C.pneumoniae INFECTION**

The increasing interest in chronic C.pneumoniae infection has led to many new investigators and laboratories getting involved in research on C.pneumoniae. The
focus of most of the investigators has been on C.pneumoniae and atherosclerosis. Several seroepidemiological studies have yielded conflicting results about the association of C.pneumoniae and AMI, possibly due to lack of uniformity in methods used (i.e. different classes of antibodies tested, different methods, different cutoff points) and by the high prevalence of C.pneumoniae exposure in the population. Because of these reasons, the role of C.pneumoniae infection in AMI has remained controversial and unresolved.

Most of the studies have relied on results of serological tests. Unfortunately and very importantly, we do not have any reliable serological marker for chronic or persistent C.pneumoniae infection.

However, the presence or absence of C.pneumoniae antibody is not a perfect measure of past infection. Following an acute infection with C.pneumoniae, antibody levels usually drop over a period of months to years and may become undetectable in some people. Therefore, the presence of antibody probably does not simply distinguish subjects ever infected from those never infected, but rather may distinguish subjects with frequent, recent, severe, or chronic infections from those with, less frequent, less recent, or milder infections.

Primary chlamydial infections are characterized by a predominant IgM response, delayed IgG response, and a weak or absent IgA response in the MIF test, whereas secondary infections are characterized by an absence of IgM response, and prompt IgG and IgA responses.
Roivainen et al. pointed out the data of Personen that a process that eventually leads to CHD is initiated in early life after infections acquired in childhood. However, assessment of the chronicity of an infection is a complicated issue, and it is not clear whether increased levels of IgG antibodies reflect the duration of infection, reactivation of a latent infection, reinfection, or some unknown immunological features of the host.

While presence of elevated antibody titer is generally considered indicative of past chronic infection, no clear information is available about the time span of persistence of chlamydial antibodies in individuals after a single episode of infection.

Persistently raised IgG and IgA titers, determined by MIF or a variety of ELISAs, have been considered as one of the criteria for chronic infection. However, there are no uniform cutoff titers to define seropositivity. It is not clear whether seropositivity reflects chronic, or merely, past infection. Though the MIF test is technically challenging, prone to subjective variations compared to the more commonly used ELISAs, it is considered as the gold standard for detection of Chlamydia antibody.

No method of detecting antibody to C.pneumoniae is entirely specific.

Since the majority of tests used are in house developed, and the results from many of the studies are contradictory and confusing, standardization of the methods has been called for. Therefore, guidelines have recently been formulated to ensure that results are as reproducible and comparable as possible.
CURRENT RECOMMENDATIONS FOR C.pneumoniae SEROLOGICAL TESTING:

As part of a workshop on standardization of C.pneumoniae diagnostic methods, the Centers for Disease Control and Prevention\textsuperscript{83} proposed some modifications of the serologic criteria for diagnosis. MIF test was considered to be the only currently acceptable serologic test. The criteria were made extremely stringent.

Acute infection, using the MIF test, was defined by a four fold rise in the IgG titer or an IgM titer of $\geq 16$; use of a single elevated IgG titer was discouraged.

An IgG titer of $\geq 16$ was felt to indicate past exposure, but neither elevated IgA titers nor any other serologic markers were valid indicators of persistent or chronic infection.

Available data support these recommendations, especially the poor predictive value of a single high IgG titer\textsuperscript{20,25}.

Many factors influence selection of appropriate diagnostic test for serological detection of chlamydial antibodies. These include

(i) Number of samples to be processed.

(ii) Cost of the test and

(iii) Expertise available.

Moreover, before using a new test, sensitivity and specificity of each assay needs to be considered. Keeping this in mind, in the present study, ELISA test was utilized for
the entire population of 205 ACS patients, and its efficacy was compared with IIFT in 100 of these 205 patients.

IgG positivity against C.pneumoniae accounts for > 50% in healthy population\textsuperscript{19}. Further, cross reactivity with other chlamydial species may lead to unacceptably high ratio of false positives in patient groups with other prevalent chlamydial infections.

As per multivariate analysis from USA, out of 246 patients of CHD, 65% had IgG seropositivity to C.pneumoniae as compared to 55% of healthy controls\textsuperscript{74}.

42.77% of blood donors in Delhi were seropositive to C.pneumoniae as judged by MIF test\textsuperscript{137}.

Serological evidence of past C.pneumoniae infection was reported in up to 20% in UK population\textsuperscript{36}.

Out of 266 patients of CHD, Sharma et al\textsuperscript{114} showed 41.3% seropositivity using MIF, compared to 30% age and sex matched controls.

Abhijit Chaudhury et al\textsuperscript{35} reported high infection prevalence of C.pneumoniae in India to the tune of 81% of healthy adult population and 70.1% among the CHD patient, giving a mean seroprevalence of this agent in Andhra Pradesh, South India at around 75% as detected by ELISA test.
Studies in Chinese population has revealed a similar high seroprevalence of 61.5% in normal population\textsuperscript{35}.

Rajasekhar et al\textsuperscript{34} reported seropositivity in 75% of their patients of unstable angina and chronic stable angina as compared to 62% of healthy controls.

In the present study, 52.7% of the 205 patients of ACS and 29% of healthy controls were seropositive to \textit{C.pneumoniae} by ELISA test.

Therefore, need for a species specific test is often felt in larger community based studies to assess association between infection with \textit{C.pneumoniae} and CHD.

**METHODOLOGICAL CONSIDERATIONS**

Comparison of different assays for large scale clinical trials are certainly valuable. But then, the results should be interpreted with caution. It is possible to make any assay appear sensitive by comparing it with suboptimal test, or by comparing the results of specimens of newer, more specific assays\textsuperscript{67}. Several tests that detect chlamydial antibody by ELISA are commercially available. But, MIF is the only serological test that detects species and serovar – specific responses against genus \textit{Chlamydia}\textsuperscript{36}. MIF and ELISA tests also detect reactivity to genus - specific antigen of chlamydial elementary or reticulate bodies\textsuperscript{36}. 

228
I. **Different serological methods are being used, and the different results may be related to the choice of method:***

Some investigators have used methods detecting species-specific MOMP antibodies. Others have used methods detecting genus-specific LPS antibodies. MIF is established as 'gold standard' for detecting chlamydial antibodies in seroepidemiological studies. But, MIF methods differ from commercial methods to in-house MIF techniques. Antigen composition varies between tests. Some workers have relied on IgA positivity, some have relied on IgG, and some have used a combination of the two.

Use of several different methods would be no problem, provided there is high grade agreement between the tests, eg. In one of the studies, there was great discrepancy noted between Medac rELISA and Labsystems MIF, both tests being species specific. 41% of the individuals were IgA seropositive by one method and seronegative by the other, versus 39.7% for IgG. This observation could have been due to technical methodological differences, but it may also reflect differences in the immunological response among individuals.

The difference in specificity between tests could also be one reason why some subjects are seronegative by MIF and seropositive by Medac rELISA.

In the same study, some samples were seropositive by Lab systems MIF and seronegative by Medac rELISA. This could have been because of higher sensitivity of the MIF method.
However, besides differences in sensitivity and specificity, other explanations can also exist.

It is difficult to understand why some individuals have a dominance of persisting Chlamydia LPS antibodies and others have a dominance of species-specific C.pneumoniae antibodies detected by MIF. Whether this is caused by differences in the properties of the infectious agent, the clinical infection they induce, or the human immunological response remains to be settled\textsuperscript{67}.

Some workers have shown that children with respiratory C.pneumoniae infection may develop antibodies that are not detected by MIF, and it has been suggested that the MOMP is not the dominant protein for immune responses. Further research is required to explain this phenomenon. More work should be done to determine if these responses are of pathogenetic importance\textsuperscript{67}.

Schumacher et al\textsuperscript{67} observed that the difference in prevalence of antibodies between CHD patients and healthy controls was significant when Chlamydia LPS antibodies were measured, while no significant differences between the study groups were observed by the other two methods which detected C.pneumoniae MOMP antibodies. They concluded that LPS antibodies were related to atherosclerotic disease, while antibodies detected by MIF were not.

In the present study, ELISA and IIFT were compared in 100 patients of ACS. ELISA test revealed seropositivity in 66\% of cases as compared to 48\% by IIFT. The sensitivity and specificity of ELISA were 70.8\% and 38.4\% respectively as compared
to IIFT. The PPV was 51.5%, the NPV was 58.8% and the diagnostic accuracy of ELISA for C.pneumoniae was 54.46%.

These results compare favourably with the findings published by Satpathy et al.\textsuperscript{36} Using Immunocomb assay, 73.7% serum samples were positive for C.pneumoniae, while MIF assay detected 50.8% serum samples as positive. The sensitivity and specificity of the Immunocomb assay were 79.3% and 32.14% respectively in detecting C.pneumoniae antibodies. PPV of Immunocomb for C.pneumoniae was 54.8% and NPV was 60%. The diagnostic accuracy of Immunocomb test for C.pneumoniae was 56.1%.

The results of the two studies are compared in Table 40

Table 40 showing comparison of ELISA and MIF tests in the two studies.

<table>
<thead>
<tr>
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<th>Satpathy</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seropositive %</td>
<td>ELISA</td>
<td>73.70%</td>
</tr>
<tr>
<td></td>
<td>MIF</td>
<td>50.80%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td>79.30%</td>
</tr>
<tr>
<td>Specificity</td>
<td></td>
<td>32.14%</td>
</tr>
<tr>
<td>PPV</td>
<td></td>
<td>54.80%</td>
</tr>
<tr>
<td>NPV</td>
<td></td>
<td>60.00%</td>
</tr>
<tr>
<td>Diagnostic accuracy of ELISA</td>
<td></td>
<td>56.10%</td>
</tr>
</tbody>
</table>
The results of the present study indicates that ELISA was inferior to IIFT in detecting C.pneumoniae antibodies in serum samples. Therefore, ELISA can only be used as a presumptive test. Confirmative diagnosis should include combination of tests. One should not derive inference on the basis of results of a single test.

II. Different titer limits are used in different studies:
Most studies classify individuals as seropositive or seronegative based on low titer limits of IgG or IgA: However, low titers will not distinguish between passed and persistent infection.

In a study carried out by Schumacher et al results with MIF serology did not support the hypothesis of higher titers in CHD patients than in healthy individuals, but there was a higher proportion of CHD patients than healthy individuals with high titers of LPS IgA and IgG.

III. The time interval from an acute event to sampling time differs in different reports, published in the literature.

Immunomodulation in relation to an acute cardiac event may alter the antibody titer.

It is generally agreed upon that serology tested on an average 2 weeks or more after the acute event is representative of the patient's antibody status towards C.pneumoniae.
IV. Different study populations with different stages of atherothrombotic
disease may account for the diverging results:

However, some studies have also reported that there was no difference noted
between patients with a sustained AMI compared to those who never had an AMI.
Relatively small numbers in the subgroups and methodological limitations of serology
may explain such discrepancy.

Such disagreement between different serological methods can throw enough light on
the diverging results published in different studies.

Despite this, it is also clear that both positive and negative correlations have
been observed with MIF as well as by Chlamydia LPS serology. This indicates that a
methodological aspect is not the only explanation of the noticeable discrepancies.

SEROLOGICAL EVIDENCE OF ASSOCIATION OF C. pneumoniae WITH CHD:

The prevalence of antibodies to C. pneumoniae increases with age, and is about 50-70
% in normal middle aged individuals as reported in Western literature. In few
studies carried out from our country, the prevalence of seropositivity ranged from 0-
81%. In the present study, 26 (29%) out of 90 subjects serving as controls were
detected seropositive to C. pneumoniae.

The possible association between C. pneumoniae and atherosclerosis was first
reported in 1988 by Saikku et al, who noted that antichlamydial antibodies were
present more often among patients with AMI than among matched controls. Since then, several other serological studies have yielded conflicting results about this association.

In the present study also, an increased prevalence of seropositivity in patients of ACS as compared to healthy controls was observed. Seropositivity to C.pneumoniae (IgG titer > 20 RU by ELISA) was noted in 108 (52.7%) out of 205 patients of ACS as compared to 26 (29%) of 90 age and sex matched healthy controls. The difference was statistically significant ($X^2 = 13.34, P < 0.001; OR 2.95 [0.91<012<10.57]$). The relation persisted virtually unchanged after adjustment for a wide range of possible confounding factors. These observations argue strongly against a spurious relation arising from chance or from confounding factors.

Among 108 seropositive ACS patients, seropositivity was significantly more in patients with ST elevation myocardial infarction group as compared to unstable angina and non-ST elevation myocardial infarction group ($X^2 = 4.87, P= 0.0273$).

Patients in study group showed higher antibody concentrations titer by ELISA than subjects in the control group. Amongst 108 seropositive cases, IgG antibody concentrations were 40 RU/ml in 8 patients (7.4%), 60 RU/ml in 10 patients (9.2%), 80 RU/ml in 26 patients (24%), 100 RU/ml in 20 patients (18.5%), 120 RU/ml in 20 patients (18.5%), 140 RU/ml in 18 patients (16.6%), 160 RU/ml in 6 patients (5.5%).
As against that, in 26 seropositive controls, IgG antibody concentrations were 40 RU/ml in 6 patients (23%), 60 RU/ml in 8 patients (31%), 80 RU/ml in 6 patients (23%), and 100 RU/ml in 6 patients (23%).

While none of the controls had antibody concentration >100 RU/ml, 44 out of 108 seropositive study cases (40.7%) had antibody concentration >100 RU/ml.

As has been observed in the present study, a number of published studies of different designs in Europe and the US generally support associations of elevated IgG and/or IgA C.pneumoniae antibodies with AMI\textsuperscript{17,23,32,33,62,77,111,112,114,138}, angiographic evidence of CHD\textsuperscript{25,27}, and carotid wall thickening\textsuperscript{138}, although there are inconsistencies between reports.

The findings in the present study are at variance with the reports of other research workers\textsuperscript{75,76,78,81,82}.

In the initial small Finnish population-based study of men aged ≤ 50 years, Saikku et al\textsuperscript{23} reported that 27(68%) out of 40 patients of AMI and 15 (50%) out of 30 patients of chronic angina had raised IgG (≥128) and / or IgA (≥32) titers in the MIF test. Both frequencies were significantly higher than in the controls (7% and 17% respectively).

The OR estimates for seropositive and for high IgG and IgA titers were between 3.6 and 7.6 for AMI patients and 4.2 to 5.4 for chronic angina patients. The titers remained elevated without seroconversion in cases over a 4-week period and no IgM antibodies were detected in the MIF test. These findings pointed to chronic C.pneumoniae infection in both groups of patients. Among AMI patients, 68% seroconverted to Re-LPS antigen versus none in the chronic angina patients and 2% in the controls, raising
the possibility that AMI may also be associated with acute exacerbation of chronic chlamydial infection.

In 1992, the same workers demonstrated chronic C.pneumoniae infection as a significant and an independent risk factor for CHD in the Helsinki Heart Study. The risk was increased by 40% when IgG titers were ≥ 1: 128. Elevated IgG titers were present at baseline and 6 months before a coronary event in 33% and 31% of case patients and in 22% and 21% of controls respectively. The corresponding values for elevated IgA titers were 23% and 22% in case patients and 14% and 10% in controls. The presence of LPS immune complexes was more variable. In the beginning of the study, 33% of case patients and 30% of controls had immune complexes, where as 6 months before the coronary event, the corresponding values were 52% and 33% respectively. Using a conditional logistic regression model, odds ratios for the development of CHD were 2.7 (95% CI, 1.1 to 6.5) for elevated IgA titers, 2.1 (95%CI 1.1 to 3.9) for the presence of immune complexes, and 2.9 (95%CI 1.5 to 5.4) for the presence of both factors. When adjusted for other CHD risk factors eg. age, hypertension and smoking, the corresponding values were 2.3 (95%CI, 1.9 to 6.2), 1.8 (95%CI, 0.9 to 3.6) and 2.6 (95%CI, 1.3 to 5.2), respectively. Persistent elevations of IgA and IgG titers and the presence of immune complexes were clearly more common among case patients than among controls, thus supporting an association between chronic C.pneumoniae infection and the risk for CHD.

These associations are taken as evidence against myocardial infarction merely reactivating a latent C.pneumoniae infection or boosting C.pneumoniae titers through cross-reactivity with cardiac antigens such as heat shock proteins.
A German case control study did not show a statistically significant association of AMI with IgG or IgA C.pneumoniae antibodies. However, a strong association with C.pneumoniae specific immune complexes (OR = 3.9, 95% CI : 2.2 – 7.2) was interpreted as evidence for an effort of chronic infection. The population had high prevalence of IgG antibodies indicating a probable C.pneumoniae epidemic in recent years. The authors suggested that recent wide spread infection may have concealed an association with IgG and IgA antibodies, where as that with immune complexes remained intact.\(^\text{138}\)

A large hospital-based case control study in the UK\(^\text{139}\) reported associations of both acute and chronic C.pneumoniae infections as judged by serological criteria (IgG, IgA and IgM) with acute admissions for unstable angina, AMI, stroke and transient cerebral ischaemia – compared with other hospitalized controls. Odds ratios calculated from the data were similar for acute and chronic infection and ranged between 2 and 3.

Blassi et al\(^\text{112}\) observed a significantly higher prevalence of IgG titers in patients of AMI (35/61) compared to blood donors (18/61) (P=0.003). They confirmed the possible role of C.pneumoniae infection in CHD and suggested that reinfection may trigger the onset of AMI.

Roivainen et al\(^\text{16}\) measured higher baseline levels of antichlamydial antibodies in 241 subjects who suffered either AMI or coronary death, as compared to controls, during the 8.5 years treatment in the Helsinki Heart Study. This study emphasized that high
levels of antibodies and/or circulating immune complexes against C.pneumoniae were risk factors for future coronary events in a prospective cohort of middle aged dyslipidaemic men.

In Atherosclerotic Risk in Communities Study\textsuperscript{74}, the prevalence of high titer (both 1:512 and 1:1024) was greater among patients with CHD than among controls.

In a retrospective investigation, Davidson et al\textsuperscript{111} showed presence of intracellular C.pneumoniae organism in coronary arteries obtained at autopsy and a serological diagnosis of infection in the same individuals 5 to 14 years earlier. They concluded that serological evidence for C.pneumoniae infection preceded the earliest as well as more advanced lesions of coronary atherosclerosis.

Siscovick et al\textsuperscript{78} noted negligible association with low to moderate C.pneumoniae antibody titers of ≤1:512 (OR 1.1, 95% CI 0.7 to 1.8). However, there was definitely an evidence that high titer (1:1024) was associated with increased risk (OR = 2.2, 95% CI 1.1 to 4.4) of AMI and coronary death. This risk was particularly large for events that occurred within the first 2.1 years after the blood draw.

A number of crosssectional case control studies of patients with angiographically confirmed CHD support an association\textsuperscript{25,27}. In Seattle, the OR for a comparison of cases and angiographically negative controls was 1.6 (95% CI, 1.0 – 2.7) for positive IgG\textsuperscript{27}.

238
A second study using population-based controls confirmed the association (OR = 2.6 [95% CI, 1.4 – 4.8]), but this was confined to smokers only. No additional effect was evident at high IgG titers. No interaction with smoking was detected in the present study.

In the UK, a comparison with the controls selected from a general practice setting showed a stronger association at high IgG titers (OR = 7.4 [95% CI, 1.7 – 33.1]) than at lower titers.

A Finnish study with age-matched population based controls showed an association with C.pneumoniae LPS containing immune-complexes, interpreted as evidence for chronic infection.

A case control investigation in US ARIC study of carotid wall thickness determined by ultrasonography showed risk factor adjusted association with positive IgG (OR = 2.0, 95% CI, 1.19 – 3.35) which was significantly stronger for participants aged 45-54 than for those aged 55-64. The present study did not confirm an effect modification by age.

In general, the Finnish studies suggested a stronger relation with IgA than IgG, whereas in the US studies, only IgG was assessed.

In 1997, Danesh et al reviewed 18 published studies on association of C.pneumoniae and the risk of CHD (Fig. 36).
Fig. 36 showing odds ratios in epidemiological studies of C. pneumoniae seropositivity and vascular diseases.

<table>
<thead>
<tr>
<th>Study design</th>
<th>Cases/ Controls</th>
<th>Disease of case</th>
<th>Degree of adjustment</th>
<th>Odds ratio (seropositivity in cases: controls) &amp; 95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prospect studies</td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Meittinen,</td>
<td>202/1791</td>
<td>MI or CHD death</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Saikku,</td>
<td>102/102</td>
<td>MI or CHD death</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Ossewarde,</td>
<td>54/108</td>
<td>Myocardial infarct</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Population controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patel</td>
<td>83/305</td>
<td>Angina or ECG</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Melnick,</td>
<td>326/326</td>
<td>Carotid stenosis</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Thom</td>
<td>171/120</td>
<td>coronary stenosis</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Dahlen,</td>
<td>60/60</td>
<td>coronary stenosis</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Saikku,</td>
<td>70/41</td>
<td>MI or angina</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Leinonen,</td>
<td>44/44</td>
<td>Myocardial infarct</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Linnanmaki,</td>
<td>46/46</td>
<td>coronary stenosis</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Haidi</td>
<td>38/68</td>
<td>CHD by ECG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandali</td>
<td>103/67</td>
<td>MI or coronary stenosis</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Weiss</td>
<td>65/28</td>
<td>coronary stenosis</td>
<td>++</td>
<td>←</td>
</tr>
<tr>
<td>Wimmer</td>
<td>58/52</td>
<td>Ischaemic stroke or TIA</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Thom</td>
<td>461/95</td>
<td>coronary stenosis</td>
<td>+</td>
<td>←</td>
</tr>
<tr>
<td>Aceti</td>
<td>37/60</td>
<td>Myocardial infarct</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cock</td>
<td>408/1297</td>
<td>MI or unstable angina</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>Geitters</td>
<td>400/400</td>
<td>Clinical stages of CHD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Most of these studies found at least two fold or larger odds ratios and some reported increasing odds ratios with increasing antibody titers. The general consistency of their findings in a total of 2700 cases supports the existence of some real association between C. pneumoniae and CHD.

Comparatively, very little work has been carried out from our country on this subject.

In 1995, Sharma et al\textsuperscript{114} reported that seropositivity to C. pneumoniae was present in 41.3% of 266 CHD patients as compared to 30% of age and sex matched controls.

Later, Sonia Miglani et al\textsuperscript{32} concluded that C. pneumoniae infection is significantly related to CHD. IgG antibodies as measured by ELISA were raised in 32 (64%) of 50 patients of AMI as compared to 10 (29.4%) out of 34 controls (P=0.0038).

Choudhary et al\textsuperscript{33} detected IgG positivity in 37.5% of their patients of AMI and in none of the controls (P<0.02), suggesting a strong association between C. pneumoniae infection and CHD.

Abhijit Chaudhury et al\textsuperscript{35} found no significant association between atherosclerosis and seroprevalence of C. pneumoniae. They studied 117 patients of unstable angina, 16 patients of chronic stable angina and 90 healthy controls. Their study exposed the high infection prevalence of C. pneumoniae in India to the tune of 81% of health adult population and 70.1% among the CHD patients (66% of unstable angina and 94% of chronic stable angina patients), giving a mean seroprevalence of this agent in Andhra Pradesh, South India at around 75%.
Rajasekhar et al\textsuperscript{34} observed seropositivity to C. pneumoniae in 66\% of patients of unstable angina, 94\% of chronic stable angina and 62\% of healthy controls. Significant difference between seropositivity to C. pneumoniae in unstable angina and control group was observed (P<0.001).

Negative seroepidemiological studies have also emerged over the last few years. As a part of Women's Health Study in 1999, Ridker et al\textsuperscript{76} did not find any association between risk for cardiovascular events and baseline IgG seropositivity (rate ratio 1.1 [95\% CI, 0.9 to 1.8]).

Later, in a large scale study of socioeconomically homogenous men that controlled for age, smoking and other cardiovascular risk factors, the same workers\textsuperscript{75} did not find any evidence between C. pneumoniae IgG seropositivity and risks of future AMI.

Siscovick et al\textsuperscript{78} observed almost identical prevalence of IgG seropositivity amongst controls (82.7\%) and cases (84.0\%) that experienced an incident AMI and coronary death.

Wald et al\textsuperscript{82} demonstrated that the distributions of IgG and IgA antibody concentrations were similar in 647 men who subsequently died of IHD and 1294 age matched controls that did not. No important relation was found between C. pneumoniae infection and CHD.
Danesh et al. reported that 200 (40%) of the 496 men with CHD were in the top third of C. pneumoniae titers compared with 329 (33%) of the 989 controls. The corresponding odds ratio for CHD was 1.66 (95% CI 1.25 to 2.21), which fell to 1.22 (0.82 to 1.82) after adjustment for smoking and indicators of socioeconomic status. They denied any strong association between C. pneumoniae infection and risk of CHD.

Danesh et al. had earlier carried out a metaanalysis of 18 studies, but majority of these studies were retrospective.

The same workers later on identified 15 prospective studies of C. pneumoniae IgG titers and CHD. The studies included a total of 3169 cases of nonfatal myocardial infarction or coronary death. The weighted mean follow up was of 10 years. All adjusted for smoking and other classic risk factors.

Ten of these 16 studies (which includes study by Danesh et al.) used MIF assays (seven studies used ≥ 1:64 as cutoff titer for seropositivity, one study used ≥ 1:32, one used ≥ 1:32 and one did not specify the cutoff), and five used other methods (two used ELISAs), two used time resolved fluorimetry and one did not specify the exact method used. Despite these differences, there was no significant heterogeneity among the 15 studies ($X^2 = 10.5$, df =14; P>0.1) and the combined analysis yielded an odds ratio of 1.15 (95% CI, 0.97 to 1.36) for CHD.

Subsidiary analysis yielded similar results in the 10 studies, (1521 cases) that used MIF assays (combined odds ratio 1.11 (0.87 to 1.42)), and in the 9
studies \cite{16,17,62,63,73,74,79,80,82} (1816 cases) that reported risk in relation to C. pneumoniae IgA titers (combined odds ratio of 1.13 (0.90 to 1.41)). There was no significant heterogeneity among studies in any of these subsidiary analyses.

Danesh et al.\textsuperscript{61} reliably excluded possibility of any strong association between C. pneumoniae IgG titers (or IgA titers) and CHD.

It is strange that the same group of workers who demonstrated positive association of seropositivity\textsuperscript{17} with CHD, later on backed out and did not claim any strong association between seropositivity to C. pneumoniae and CHD\textsuperscript{61}. However, the concerned workers themselves have outlined the reasons to justify this discrepancy.

Initial metaanalysis included many retrospective and fewer prospective studies. The studies were done in different populations, used different criterias for cases, adjusted for potential confounders to differing degrees, and were therefore prone to different biases. Prospective studies, which should be less liable to selection bias, had been small.

Most of seroepidemiological studies in the earlier metaanalysis\textsuperscript{17} detected C. pneumoniae antibodies by MIF. The results of this test need to be interpreted by expert microscopists. Even then, poor reproducibility is well known. Random measurement errors would be plentiful. Owing to regression dilution, such errors would weaken chances of real association. In contrast, systematic measurement errors could produce biases that may either weaken or exaggerate strength of any
association. In very minority of studies, the disease status was concealed from microscopists. Studies that used chlamydial immune complexes or LPS for diagnosis of chlamydial infection might have produced spurious association with CHD due to cross-reaction with some antigen, such as cardiolipin, which is associated with CHD.

Further, in the initial review\(^1\), studies employed various combinations of antibody fractions or various cut off titers to define C.pneumoniae seropositivity that were not chosen until an exploration of the data had shown which seemed to be most strongly related to the disease. Some groups of investigators used different definitions of seropositivity in different studies (Table 41).

Table 41 showing diagnostic criteria for C.pneumoniae infection adopted in prospective seroepidemiological studies
positive IgG > 64 considered high positive

<table>
<thead>
<tr>
<th></th>
<th>2000</th>
<th>78</th>
<th>IC EIA (Medac rELISA)</th>
<th>IgG ≥ 32 IgA (0.7 OD)</th>
<th>IgG antibodies were detected in IC by MIF (Kajaani 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glader et al</td>
<td>2000</td>
<td>78</td>
<td>IC EIA (Medac rELISA)</td>
<td>IgG ≥ 32 IgA (0.7 OD)</td>
<td>IgG antibodies were detected in IC by MIF (Kajaani 6)</td>
</tr>
<tr>
<td>Danesh et al</td>
<td>2000</td>
<td>496</td>
<td>TRF</td>
<td>IgG – NS</td>
<td></td>
</tr>
<tr>
<td>Wald et al</td>
<td>2000</td>
<td>647</td>
<td>TRF</td>
<td>IgG – NS</td>
<td></td>
</tr>
<tr>
<td>Choussat et al</td>
<td>2000</td>
<td>71</td>
<td>MIF (NS)</td>
<td>IgG – NS</td>
<td></td>
</tr>
<tr>
<td>Siscovick et al</td>
<td>2000</td>
<td>213</td>
<td>MIF (TW 183)</td>
<td>IgG ≥ 8</td>
<td>IgG ≥ 8 considered past infection</td>
</tr>
</tbody>
</table>

Where IC – Immune complex
NS – Not specific
OD – Optical density

Such posthoc analyses could produce misleadingly strong associations. Such apparently positive results might then have been published.

Extreme findings in selected subgroups (eg. Diabetic, smoker, etc.) may likewise be statistically biased. This is especially true when we consider that most subgroup analyses were based on sparse data.

In the final metaanalysis, Danesh et al. reported that for arriving at any reliable association between C.pneumoniae infection and CHD, odds ratio should not be weaker than at least 1.5. The present study showed a significant correlation of seropositivity and ACS, as the difference between seropositivity in patients and controls was highly significant ($X^2 = 13.34$, $P<0.001$, OR 2.95 ($0.91<0.12<10.57$)).
Results of studies demonstrating negative association of chlamydial infection with CHD may raise the possibility that specific patterns of infection with Chlamydia pneumoniae that result in a high titer serologic response (which may be due to reinfection, reactivation, or infection with possibly different serotypes with different immunogenecities or altered host immune responsiveness) may be more relevant to its potential pathophysiological role in atherothrombosis.

It should also be noted that lack of a positive serology might not definitely rule out the presence of Chlamydia pneumoniae infection in the vessel wall.

While positive seroepidemiological studies at their best may imply a genuine association rather than underlying causative mechanism, negative seroepidemiological data, due to limitations of serological methods, cannot conclusively disprove a causative role of C.pneumoniae in CHD.

Some studies have included only elderly subjects; most previous studies have included younger and middle aged subjects; Ridker et al have studied only women. Thus, the data may or may not apply to all age groups and both genders.

Interestingly, no positive association between the presence of C.pneumoniae in atheromas and positive IgG serology has been found. In fact, an inverse relation was apparent. These latter data raise the question as to the informativeness of serological studies in assessing the association between C.pneumoniae and CHD, and could explain inconsistencies between serological studied.
Hahn has proposed that regional variation in MI incidence may be influenced by seroprevalence of C. pneumoniae\textsuperscript{138}. Therefore, association of antibodies to C. pneumoniae with manifestations of CHD may not be ubiquitous, and C. pneumoniae infection may be a risk factor in some populations but not in others\textsuperscript{138}.

Pathological studies support serological evidence of a positive association.

The presence of C. pneumoniae, detected by PCR, ICC & EM has been repeatedly documented in a number of different populations in atheromas of coronary arteries\textsuperscript{86,94,143}, carotid artery\textsuperscript{64}, iliac and femoral arteries\textsuperscript{97} and in restored coronary vessels\textsuperscript{108}, suggesting a role in atherogenesis (although one study from US did not confirm these observations\textsuperscript{60}). Moreover, C. pneumoniae has been identified only in atheromatous lesions but not in normal vessels\textsuperscript{21,26,64,143}. 

248
RELATION OF SEROPOSITIVITY WITH INDIVIDUAL RISK FACTORS

Fig. 37 showing proposed relationships among risk factors, endothelial dysfunction, atherogenesis, and progression to cardiovascular events.

- Aging
- Smoking
- Diabetes
- Hypertension
- Dyslipidemia

- Estrogen withdrawal

- Sedentary lifestyle

- Infection / inflammation

- Homocysteine

Endothelial dysfunction

- Decreased NO
  - Vasoconstriction
  - Platelet adhesion
  - VSMC proliferation
  - Leukocyte adhesion

- Other Vasocoactive agents
  - Prostaglycerin
  - Endothelin
  - EDHF
  - Carbon monoxide

- Decreased t-PA, PAI-1
  - Impaired fibrinolysis
  - Other procoagulants

- Adhesion molecules expression
  - Monocyte adhesion
  - Foam cell formation
  - Plaque inflammation
  - Other inflammation cytokines

Atherosclerotic lesion formation and progression
- Plaque activation and rupture
- Decreased blood flow due to thrombosis and vasospasm

Coronary events and stroke
Fig. 37 shows proposed relationships among risk factors, endothelial dysfunction, atherogenesis, and progression to cardiovascular events.

One has to accept that CHD in a multifactorial disease. C. pneumoniae infection may interact with smoking*, hypertension*, hyperlipidaemia* and in certain genetically susceptible subjects* and then predispose to atherosclerosis. It has been suggested that smoking, low HDL and adiposity may be associated with enhanced persistence of the infection, or increased susceptibility of reinfection. Alternatively, lower HDL concentration may result from C. pneumoniae infections. C. pneumoniae is also associated with age* and male sex*. Hence, it will be important to account for these potential confounding risk factors while trying to establish an independent role of C. pneumoniae in CHD.

An attempt was made to correlate the serological status with classic individual coronary risk factors in the present study.

Smoking has been emphatically proposed as both a confounder and an effect modifier of the association between C. pneumoniae and CHD.

Out of 205 study cases, 131 patients (64%) were males and 77 of them (58% of males) were smokers. None of the females was a smoker. In these 77 males who were smokers, seropositivity was observed in 47 patients (61%) and seronegativity in the remaining 30 patients (39%). The difference in serological status was statistically not significant ($X^2 = 2.94, P=0.0865$). In 90 controls, there were 60 males (66.6%) and
40 (66.6%) of them were smokers. Amongst controls, 21 (52.5%) of 40 smoker males were seropositive, while 19 (47.5%) were seronegative. Thus, seropositivity was more prevalent in smokers as compared to nonsmokers in both study groups and controls. Though larger number of smokers in the study group (61%) were seropositive as compared to 52.5% in control group, the difference was statistically not significant ($X^2 = 0.3014, P>0.05$).

This finding compares favourably with report of Ridker et al and Strachen et al, who also did not find association between smoking and seropositivity. However, it is at variance, with reports of Thom et al, Roivainen et al, and Choudhary et al. In a study carried out by Thom et al, association between C.pneumoniae antibody and CHD was limited to subjects who ever smoked. In smokers, OR was 3.5 (95% CI, 1.7 to 7.0). Among nonsmokers, OR was 0.8 (95% CI, 0.3 to 1.9).

Roivainen et al also reported that the risk associated with high C.pneumoniae antibody titer was mainly confined to smokers. The relative risk was 1.44 (0.82 – 2.33) in nonsmokers and 4.88 (2.42 – 9.81) in smokers.

A significant correlation was found between C.pneumoniae infection and smoking in the AMI group ($p<0.05$) in the study reported by Chowdhary et al.

When other classic coronary risk factors were considered, it was observed that seropositivity to C.pneumoniae was not related to presence or absence of positive family history of CHD, or associated hypertension, diabetes, hyperlipidaemia,
hypertension plus diabetes, hypertension plus hyperlipidaemia, diabetes plus hyperlipidaemia or hypertension plus diabetes plus hyperlipidaemia. There was no evidence of confounding by commonly recognized risk factors for CHD. Thus, none of the risk factors alone or in combination demonstrated any statistically significant association with seropositivity, indicating an independent causal relationship of seropositivity to C.pneumoniae as a risk factor in patients of ACS. The relationship persisted virtually unchanged after adjustment for a wide range of possible confounding factors. These observations argue strongly against a spurious relation arising from chance or from confounding factors.

Saikku et al\textsuperscript{77} also concluded that effect of C.pneumoniae infection on the risk for CHD related events appears to be independent of the classic risk factors for CHD. This observation is shared by several other workers\textsuperscript{20}. In a study from Finland, no association between elevated C.pneumoniae titers and coronary risk was overall reported. Although a nonsignificant increase in risk was observed in the subgroup of nondiabetic men (RR = 1.8, 95\% CI 0.9 to 3.7)\textsuperscript{116}.

Strangely enough Rajasekhar et al\textsuperscript{34} observed that patients seropositive for C.pneumoniae in unstable angina group showed lower levels of total cholesterol, triglycerides, LDL-C but higher percentage of smoking (57\%) and alcoholism (38\%) than patients seronegative to C.pneumoniae; however, no significant difference was observed between the two groups. Similarly, subjects in control group seropositive to C.pneumoniae showed higher levels of total cholesterol, LDL-C and higher percentage of diabetes,(20\%) and alcoholism (40\%) as compared to seronegative controlled patients.
Rajasekhar et al\textsuperscript{34} have quoted the study by Laurila et al, who have reported increased triglyceride, total cholesterol and decreased HDL in subjects who were seronegative to \textit{C.pneumoniae}.

**BASELINE IgG ANTIBODY TITRE**

Examination of the association between antibody concentrations to \textit{C.pneumoniae} and mortality from CHD is complicated by the fact that there is no agreed or validated cut off point for concentrations of IgG or IgA that identifies individuals who have, and who have not been infected with \textit{C.pneumoniae}. Consequently, there is no accepted understanding as to what proportion of adult population has been infected.

All chlamydial species have a tendency to cause chronic infections, and recurrence of the disease is frequent despite treatment with antibiotics. Primary chlamydial infections are characterized by a predominant IgM response, delayed IgG response and a weak or absent IgA response, where as secondary infections are characterized by an absence of IgM response and prompt IgG and IgA responses\textsuperscript{23}.

With a primary \textit{C.pneumoniae} infection, IgM antibodies are usually first detected after 3 weeks. It is a further 3 to 5 weeks before a significant increase in IgG occurs. This means that antibody detection is possible later than with many other infections. With a reinfection, the increase in IgG comes after 1 to 2 weeks, although sometimes it is missing entirely. A weak increase in IgM can occur with a reactivated infection.
Seropositivity is an imperfect marker of persistent chlamydial arteritis and is associated with uncertain and possibly, substantial rates of false positivity and negativity.

Some studies\textsuperscript{60,108} have suggested that C.pneumoniae titers are not positivity associated with the presence of Chlamydia in atheroma.

Presence of serum antibodies does not necessarily indicate the persistence of active infection at any site, or persistent exposure of the coronary arteries to any type of insult. C.pneumoniae antibody titers may fall substantially in a variable period of seroconversion, and may increase substantially if reinfection occurs. Such temporal variation means that any associations between CHD and antibody titers for C.pneumoniae measured at just one time will, owing to regression dilution, be substantially weaker than associations of CHD with long term average antibody concentrations, or with direct evidence of persistent infection at the relevant anatomic site\textsuperscript{17}.

All the 108 patients in the study group in the present study had stable elevated IgG titers when rechecked at 3 months, consistent with persistent antigenic stimulation resulting from chronic active infection. This was in contrast to only 5 (20\%) of control group who had persistently elevated IgG titers after 3 months.

High IgG antibody level was considered as a marker of previous infection in the present study. Patients in study group showed higher antibody concentrations titer by ELISA than subjects in the control group. Amongst 108 seropositive cases, IgG
antibody concentrations were 40 RU/ml in 8 patients (7.4%), 60 RU/ml in 10 patients (9.2%), 80 RU/ml in 28 patients (25.9%), 100 RU/ml in 20 patients (18.5%), 120 RU/ml in 21 patients (19.4%), 140 RU/ml in 17 patients (15.7%) and 160 RU/ml in 4 patients (3.7%).

As against that, in 26 seropositive controls, IgG antibody concentrations were 40 RU/ml in 6 patients (23%), 60 RU/ml in 8 patients (31%), 80 RU/ml in 6 patients (23%) and 100 RU/ml in 6 patients (23%).

While none of the controls had antibody concentrations > 100 RU/ml, 42 (38.8%) out of 108 seropositive study cases had antibody concentration > 100 RU/ml.

There is disagreement as to whether sequentially higher titers imply greater burdens of infection. However, some of the studies reported by Danesh et al showed increasing odds ratios with increasing titers.

Thom et al reported 2.6 fold increase in the risk of CHD associated with the presence of antibody (95% CI, 1.4 to 4.8), but no evidence for an increasing association with increasing antibody titer.

Gupta et al observed that the frequency of adverse events increased with rising antichlamydial antibody titers, which persisted after correction of confounding variable.

Siscovick et al reported that there was little evidence of an association between IgG antibody titers of ≤ 1:512, but the prevalence of an IgG antibody titer of 1:1024 was
higher among cases (15.1%) than among control subjects (8.7%). High titer (1:1024) C.pneumoniae antibody was associated with an increased risk (OR = 2.2, 95% CI, 1.4 to 4.4) and association of high titer with risk was particularly large for events that occurred within the first 2.1 years (for early events, OR 4.2, 95% CI, 1.7 to 10.7; for later events, OR 1.3, 95% CI 0.7 to 2.7; P for interaction = 0.045).

In addition to efforts to prevent adverse cardiovascular events developing in patients of AMI with antichlamydial antibiotic, a trial of roxithromycin to prevent restenosis following coronary artery stenting has also been reported. This double blind trial randomized 1010 patients immediately following stenting to roxithromycin 300 mg daily for 28 days or placebo. Restenosis of ≥ 50% was noticed at the 6 months follow up angiograms in 31% of antibiotic treated patients and in 29% of the controls (P=0.43). At 1 year, the rates of death and AMI were slightly but insignificantly higher in patients randomized to antibiotic. There was, however, a significant benefit of antibiotic therapy in patients with high antibody titers in both reduction in restenosis and the need for revascularization in the first year of follow up. In patients with negative or low antibody titers, however, the placebo patients had less restenosis and required fewer revascularization procedures than those receiving antibiotic therapy. (Fig. 38) There was, however, no detectable interaction between treatment and C.pneumoniae titer with respect to combined rate of death and AMI (adjusted model P=0.95).
ASSOCIATION OF ELEVATED IgG ANTIBODIES WITH OTHER MARKERS:

The present study investigated several biological mechanisms proposed as links between C.pneumoniae infection and CHD.

In the present study, in seropositive group, mean serum fibrinogen level was 348.52 ± 25.83 mg% (range 200-390 mg %). In seronegative group, mean serum fibrinogen level was 260.72 ± 25.24 mg% (range 210 to 340 mg %). Thus, serum fibrinogen levels were higher in 108 seropositive cases as compared to 97 seronegative patients. The difference was statistically significant (P<0.05). The significant association of seropositivity to mean fibrinogen levels was maintained even after adjustment of all
confounding variations like age, smoking, diabetes, hypertension, hyperlipidaemia and previous coronary revascularization.

This observation compares favourably with that reported by Patel et al but is at variance with the reports of Siscovick et al and Strachen et al. Patel et al noted that fibrinogen concentrations were significantly raised and directly related to seropositivity. They emphasized that effect of C.pneumoniae infection on mean fibrinogen concentration was equivalent to currently smoking 10–15 cigarettes a day. They suggested that persisting immune responses to C.pneumoniae may raise the fibrinogen concentration as part of an inflammatory response through release of the cytokine tissue necrosis factor α and IL-6.

On the other hand, Strachen et al and Siscovick et al did not confirm any association of IgG antibody titer with increased fibrinogen concentration.

With regard to cardiovascular risk prediction, CRP has been the most extensively studied of all markers of inflammation. Elevated CRP levels are a marker of increased production of IL-1 and IL-6, the two proinflammatory cytokines that also have prothrombotic properties, and CRP itself can activate monocytes to produce tissue factor and induce monocyte and endothelial cell release of IL-1 and IL-6. However, the mechanisms that can lead to initiation of such inflammatory reaction may be multiple and to date are largely unexplained. The mechanisms that can lead to the persistence of this "acute-phase" reaction also are unexplained.
There is now robust evidence from numerous large scale prospective studies that CRP predicts a variety of cardiovascular outcomes in numerous clinical settings\textsuperscript{45}. In the preliminary prevention setting, CRP has emerged as a strong independent determinant of future MI, stroke, cardiovascular death, need for coronary revascularization, development of peripheral vascular disease, and sudden cardiac death (Fig. 39).
Fig. 39 showing prospective studies of CRP and future cardiovascular events.

In these studies, those with a lower quartile have a 2-4 increased risk compared with the lowest quartile.

Kuller CHD death
Ridker MI
PHS 1997 Stroke
Ridker CHD
PHS 1997
Tracy CHS / RHPP
1997
Ridker PAD
PHS 1998, 2001
Ridker CVD
WHS 1998, 2000, 2002
Koenig CHD
MONICA 1999
Roivainen CHD
HELSINKI 2000
Mendell CHD
CAERPHILLY 2000
Danesh CHD
BRHS 2000
Gussekloo Fatal stroke
LEIDEN 2001
Lowe CHD
SPEEDWELL 2001
Packard CV events
WOSCOPS 2001
Ridker CV
AFCAPS 2001 events
Rost Stroke
FHS 2001
Pradhan MI, CVD
WHI 2002 death
Albert MI, CVD
PHS 2002 death
Saikknen MI
HHS 2002

Increased risk compared with the lowest quartile.
CRP predicts risk among patients with stable and unstable angina, in the chronic phase after AMI, and among patients undergoing revascularization procedures. Importantly, among those patients presently with ACS, the predictive value of CRP is independent of, and additive to, that of established markers of myocyte necrosis, such as troponin^{2,13}.

The precise trigger to increased CRP among patients presenting with ACS remains speculative. Data suggest that focal plaque rupture may not be the cause, but rather that increased CRP levels may be a marker of hyperresponsiveness of the inflammatory system to even minor stimuli^{41}, eg. CRP levels do not change after balloon angioplasty in patients with stable or unstable CHD, who have normal preprocedural levels, but they do increase after angioplasty in unstable patients with increased preprocedural CRP levels. Moreover, even diagnostic angiography without intervention caused an increase in CRP levels among patients with increased levels at baseline.

In the present study, CRP was increased in 83 (76.8%) out of 108 seropositive patients as compared to 50 (51.5%) out of 97 seronegative patients. The difference was statistically significant (P< 0.05). The mean CRP values were significantly higher in seropositive group as compared to seronegative group (P< 0.05). In seropositive group, mean CRP value was 16.19 ± 6.35 mg/L (range 6 to 23 mg/L). In seronegative group, CRP value was 9.87 ± 5.40 mg/L (range 2 to 25 mg/L).
The significant association of seropositivity to mean CRP values was maintained even after adjustment of all confounding variations like age, smoking, diabetes, hypertension, hyperlipidaemia and previous coronary revascularization.

Individuals seropositive to C.pneumoniae had higher plasma concentration of CRP\textsuperscript{17}.

Raised concentration of CRP (> 4 mg/L) were present in 54\% of patients who were seropositive for C.pneumoniae as compared to 37\% of seronegative patients (adjusted OR 1.55; 1.07 to 3.51) in the study reported by Patel et al\textsuperscript{56}.

Rojvainen et al\textsuperscript{16} noted that high C.pneumoniae IgG antibody levels increased the risk of AMI in presence of high CRP levels (OR 5.4, 95\% CI, 2.4 to 12.4).

Sonia Miglani\textsuperscript{32} also observed that 15 out of 32 cases of AMI and 4 out of 10 healthy controls had both C.pneumoniae infection and raised CRP levels (OR = 2.86).

Rajsekahr et al\textsuperscript{34} noted higher levels of CRP in unstable angina patients seropositive to C.pneumoniae as compared to healthy controls and therefore concluded that infection played a strong role in causing coronary syndrome.

Vahdat et al\textsuperscript{46} observed that elevated CRP levels did not show significant association with ECG-defined CHD independent of seropositivity to C.pneumoniae. But, concurrent elevated CRP levels (> 10 mg/L) and anti- C.pneumoniae IgG antibodies
[OR = 1.68 (CI, 1.24-2.59; P=0.04)] were associated with ECG defined CHD in the general population.

Biasucci et al reported that seropositivity for C.pneumoniae very well correlated with CRP levels and was associated with recurrence of instability during follow up.

However, Ridker et al, Siscovick et al, Danesh et al and Danesh et al could not correlate C.pneumoniae seropositivity with raised CRP levels. As seen in Table 42, Ridker et al found no evidence of association between CRP and IgG seropositivity regardless of the titer evaluated.

Table 42 showing median levels of CRP for study participants according to baseline IgG antibody titer directed against *Chlamydia pneumoniae*

<table>
<thead>
<tr>
<th>IgG Titer</th>
<th>CRP mg/L</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 1:16</td>
<td>1.18</td>
<td>0.4</td>
</tr>
<tr>
<td>&lt; 1:16</td>
<td>1.49</td>
<td></td>
</tr>
<tr>
<td>≥ 1:32</td>
<td>1.16</td>
<td>0.3</td>
</tr>
<tr>
<td>&lt; 1:32</td>
<td>1.41</td>
<td></td>
</tr>
<tr>
<td>≥ 1:64</td>
<td>1.18</td>
<td>0.7</td>
</tr>
<tr>
<td>&lt; 1:64</td>
<td>1.24</td>
<td></td>
</tr>
<tr>
<td>≥ 1:128</td>
<td>1.09</td>
<td>0.8</td>
</tr>
<tr>
<td>&lt; 1:128</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>≥ 1:256</td>
<td>0.80</td>
<td>0.8</td>
</tr>
<tr>
<td>&gt; 1:256</td>
<td>1.24</td>
<td></td>
</tr>
</tbody>
</table>

C.pneumoniae infection could be a stimulus responsible for the persistent inflammatory state or for the recurrence of instability. This possibility is supported by
recent reports of a reduction in cardiovascular events (recurrence of angina, AMI and death) in unstable angina and survivors of AMI after antibiotic treatment for C.pneumoniae\textsuperscript{20,118}.

Vahdat et al\textsuperscript{46} concluded that elevation of CRP in C.pneumoniae seropositive subjects indicates an active "smoldering" infectious / inflammatory process (arteritis?) that accelerates athrothrombotic progression where as low CRP in C.pneumoniae seropositive subjects suggests a resolved or inactive infection.

Previous studies have been inconsistent regarding effect of C.pneumoniae infection on circulating lipid concentration. Strachen et al\textsuperscript{79} suggested that this is an unlikely mechanism linking this infection with CHD. In the present study also, the levels of serum cholesterol, HDL, LDL, and triglycerides did not differ significantly in seropositive group when compared to levels in seronegative group. C.pneumoniae infection did not influence serum lipids. This observation tallies with reports of other studies\textsuperscript{25,33,56,78,81}.

However, Sonia Miglani\textsuperscript{32} from our country noted that 26/32 cases of AMI and 0/10 healthy controls having C.pneumoniae infection had raised LDL levels (P=0.000039).

Similarly the mean values of fasting blood sugar and total leukocytes were not significantly different in two groups in the present study, and therefore seropositivity was not associated with these cardiovascular risk factors in the present study.
Besides, Patel et al\textsuperscript{56} estimated higher levels of sialic acid, factor VII antigen and malondialdehyde in seropositive patients as compared to patients seronegative to C. pneumoniae infection. Higher concentrations of sialic acid are predictors of CHD\textsuperscript{56}. Infected macrophages may express tissue factor, activate factor VII antigen and thus increase the risk of local or distant thrombosis\textsuperscript{56}. Raised malondialdehyde concentrations due to release of oxidative free radicals suggests another possible mechanism where by C. pneumoniae may influence CHD\textsuperscript{56}.

Besides CRP, Danesh et al\textsuperscript{45} studied baseline levels of serum amyloid A protein, leukocyte count and serum albumin in 506 patients who died from AMI. After including additional 551 cases reported in three previous studies, they carried out a meta analysis of 1057 patients. Though no strong association of these factors was noted with IgG antichlamydial antibodies, they agreed low grade inflammatory process may be relevant to CHD.

Harnessing the basic and clinical biology of inflammation in atherothrombosis should refine our predictive ability, aid rational and cost- effective development of preventive strategies, and also promote the development and evaluation of novel therapies to reduce the overall impact of atherothrombosis and its complications like ACS.
EFFECT OF C. PNEUMONIAE INFECTION ON DEVELOPMENT OF FUTURE ADVERSE CARDIOVASCULAR EVENTS:

All the 205 patients of ACS were followed up for 6 months to record occurrence of any adverse cardiovascular events.

Among 97 seronegative patients, adverse events occurred in 11 patients (11.3%). These adverse events comprised of cardiovascular death in 3, nonfatal myocardial infarction in 3, and hospitalization for unstable angina, recurrent angina, need for PTCA, CABG and nonfatal stroke in 1 patient each (Table 43).

Table 43 showing adverse events in seronegative and seropositive patients during 6 months follow up.

<table>
<thead>
<tr>
<th>Content</th>
<th>Seronegative (n=97)</th>
<th>Seropositive (n=108)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular death</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Nonfatal Myo. Infarction</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Hospitalization for unstable Angina</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Recurrent Angina</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PTCA</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>CABG</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Nonfatal stroke</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total events</strong></td>
<td><strong>11</strong></td>
<td><strong>23</strong></td>
</tr>
<tr>
<td><strong>(11.30%)</strong></td>
<td><strong>(21.30%)</strong></td>
<td></td>
</tr>
</tbody>
</table>
Among 108 seropositive patients, adverse events occurred in 23 (21.3%) cases. These 23 adverse events included cardiovascular death in 5, nonfatal myocardial infarction in 6, hospitalization for unstable angina and recurrent angina in 1 case each, need for PTCA and CABG in 4 patients each, and nonfatal stroke in 2 patients (Table 43).

Adverse events occurred more frequently among seropositive patients (21.3%) as compared to seronegative patients (11.3%), but the difference was spastically not significant ($X^2 = 3.51, P>0.05$). Failure to reach significant level can be explained by less number of adverse events, occurring in small number of cases, during a short observation period.

There is disagreement as to whether sequentially higher titers imply greater burdens of infection. Some of the studies reported by Danesh et al showed increasing odds ratios with increasing titers. This was noted in the present study also. The frequency of adverse events certainly increased with higher C.pneumoniae IgG titer. This relationship persisted even after correction of confounding variables.

The 23 seropositive patients who experienced adverse events had high initial IgG titers ($\geq 120$ RU). In 5 patients in whom cardiovascular death occurred, initial IgG titers were 160 RU in 3 patients and 140 RU in 2 patients. In 6 patients who had nonfatal myocardial infarction, initial IgG titers were 140 RU in 2 and 120 RU in 4 patients. One patient who had to be hospitalized for recurrent angina had initial IgG titer of 120 RU. In 4 patients who were subjected to PTCA, initial IgG titer was 140 RU in 2 and 120 RU in other 2 patients. Similarly, in 4 patients who underwent CABG, their initial IgG
titers were 140 RU in 2 and 120 RU in the remaining 2 patients. Both the patients who experienced nonfatal stroke had initial IgG titer of 120 RU. Thus, adverse events were mainly confined to higher IgG titers.

This is in contrast to observation of Thom et al\textsuperscript{25}. They reported 2.6 fold increase in the risk of CHD and adverse cardiovascular events associated with the presence of antibody (95% CI, 1.4 to 4.8), but no evidence for an increasing association with increasing antibody titer, as seen in Table 44.

Table 44 showing association between C. pneumoniae antibody and CHD.

<table>
<thead>
<tr>
<th>Antibody Level</th>
<th>Cases</th>
<th>Controls</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent (&lt;1:8)</td>
<td>No. 56</td>
<td>% 33</td>
<td>No. 62</td>
<td>% 52</td>
</tr>
<tr>
<td>Present (&lt;1:8)</td>
<td>115 67</td>
<td>58 48</td>
<td>2.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Level &lt;1:8</td>
<td>56 33</td>
<td>62 52</td>
<td>1.0‡</td>
<td>1.0‡</td>
</tr>
<tr>
<td>1:8</td>
<td>16 09</td>
<td>07 06</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>1:16</td>
<td>29 17</td>
<td>15 13</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>1:32</td>
<td>31 18</td>
<td>15 13</td>
<td>2.3</td>
<td>3.4</td>
</tr>
<tr>
<td>1:64</td>
<td>26 15</td>
<td>14 12</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>≥1:128</td>
<td>13 08</td>
<td>07 06</td>
<td>2.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* Adjusted for age, gender, and quarter of blood drawing (factored).  
† CI indicates confidence interval  
‡ Reference group.

Gupta et al\textsuperscript{20} also noticed increase in incidence of adverse cardiovascular events with increasing antichlamydial titer. Seropositive patients had a four fold increased risk for adverse cardiovascular events compared with the seronegative group (OR 4.2; 95% CI, 1.2 to 15.5; \( P = 0.03 \)).
In another study, Ridker et al.\textsuperscript{75} found little evidence, if any, of an association between risk of adverse cardiovascular events and baseline IgG seropositivity against C. pneumoniae (rate ratio 1.1 [95\% CI, 0.7 to 1.8]).

**ANTIBIOTIC INTERVENTION:**

a) **Importance of antibiotic treatment:**

Data have shown that immunological and microbiological factors may play a part in the pathogenesis of atherosclerosis and cell proliferation of the injured vessel wall\textsuperscript{117}.

A substantial number of patients with unstable angina have an adverse clinical outcome despite the use of aspirin and heparin during the acute and chronic phases of angina\textsuperscript{117}. This loss of an initial benefit could be explained in part by the rebound activation of the coagulation system that follows the discontinuation of heparin infusion and the continuing inflammatory process that persists for up to 6 months from the onset of a coronary event.

Several antibiotic trials have been published or are ongoing on the basis of infection and inflammation as the root cause of atherosclerosis. This is being continued even when some of the recent seroepidemiological studies or evaluations of the performance of MIF and PCR are raising doubts against the causal relationship of C. pneumoniae in development of CHD.
b) **Choice of antibiotic:**

Four classes of antibiotics have activity against C.pneumoniae: quinolones, macrolides, tetracyclins and antituberculars (rifapentine, rifampine). Some classes appear to have anti-inflammatory activity. All the antibiotic trials reported thus far used macrolides.

Azithromycin is rapidly absorbed, is widely distributed, achieves high and persistent tissue concentrations (tissue half life ≥ 72 hours), is well tolerated and has been effective in animal models. Therefore, azithromycin and roxithromycin have been selected for human pilot studies and ongoing major clinical coronary prevention trials.

Antibiotics are known to have pharmacological effects other than antimicrobial.

Table 45 showing anti-inflammatory effect of macrolides.

<table>
<thead>
<tr>
<th>Endothelial function</th>
<th>Improved endothelial function (azithromycin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines (from monocytes or macrophages)</td>
<td>Decreased interleukin 1 (azithromycin, clarithromycin, roxithromycin)</td>
</tr>
<tr>
<td></td>
<td>Decreased interleukin 6 (azithromycin clarithromycin erythromycin, roxithromycin)</td>
</tr>
<tr>
<td></td>
<td>Decreased tumor necrosis factor α (azithromycin clarithromycin erythromycin, roxithromycin)</td>
</tr>
<tr>
<td></td>
<td>Decreased granulocyte / monocyte colony – stimulating factor (clarithromycin)</td>
</tr>
<tr>
<td></td>
<td>Decreased monocyte chemotactic protein1 (azithromycin)</td>
</tr>
<tr>
<td>Systemic inflammation</td>
<td>Decreased E-selectin (azithromycin)</td>
</tr>
<tr>
<td></td>
<td>Decreased C-reactive protein (roxithromycin)</td>
</tr>
<tr>
<td>Coagulation</td>
<td>Decreased von Willebrand factor levels (azithromycin)</td>
</tr>
</tbody>
</table>
Table 45 shows anti-inflammatory effect of macrolides. Macrolides suppress production of proinflammatory cytokines, interleukin -1β, interleukin 6 and TNF. They also significantly inhibit lipopolysaccharide (LPS) induced generation of nitric oxide and other free radicals.

Macrolides, through their antichlamydial activity may eradicate organism and thereby suppress reactivation of chronic infection within the atherosclerotic plaque.

By their protective anti-inflammatory action, they may attenuate persistent infection in the plaque, dampen hypercoagulation phase and lead to stabilize active plaque lesions\textsuperscript{20}.

Macrolides may also exert antioxidant, antithrombotic or a direct effect or a combination of all these effects.

Moreover, tetracyclines are known to inhibit macrophage matrix metalloproteinases and hence may also stabilize the vulnerable atherosclerotic plaque.

By virtue of these properties, antichlamydial antibiotics may lower the risk of further adverse cardiac events. This is the rationale of using such antibiotic in patients of ACS\textsuperscript{11,20}.

**EFFECT OF AZITHROMYCIN IN SEROPOSITIVE CASES:**

Several small trials of antibiotic therapy directed towards C.pneumoniae in patients of CHD have now been reported, and several larger trials are ongoing. The rationale for these studies is that treatment of a putative, active C.pneumoniae infection will reduce the risk of further cardiac morbidity and mortality in patients of AMI. Azithromycin at various doses and durations of therapy has been the drug of choice in most of the
studies\textsuperscript{20,113,116,124,125} and roxithromycin\textsuperscript{57,117,122} or clarithromycin\textsuperscript{120} in the remaining studies. Practically all studies have used serology alone for diagnosis, essentially limiting themselves to a clinical end point.

Three main populations with positive C. pneumoniae serology that were randomized to antibiotics have been studied.

Table 46 shows randomized controlled trials of antibiotics in Post – AMI patients.

Table 46 showing randomized controlled trials of antibiotics in Post – AMI patients.

<table>
<thead>
<tr>
<th>TRIAL</th>
<th>STUDY POPULATION</th>
<th>TREATMENT</th>
<th>END POINT</th>
<th>RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>St George’s Hospital London</td>
<td>220 consecutive male outpatients tested for IgG to Chlamydia pneumoniae; C. pneumoniae negative, anti C. pneumoniae IgG 1:8 – 1:32 and anti C. pneumoniae IgG ≥ 1:64</td>
<td>Randomized patients with IgG &gt; 1:64 to 3-6 days of azithromycin, 500 mg/d or placebo; follow up, 18 months</td>
<td>Primary (composite); nonfatal MI, unstable angina, cardiovascular death</td>
<td>IgG anti C. pneumoniae negative; OR, 1.0 IgG 1:8 – 1:32; OR, 2.0 (P=.10) IgG≥1:64 plus azithromycin; OR, 0.9 (P=NS) IgG ≥1:64 plus placebo; OR, 4.2 (95% CI, 1.2-15.5; P=0.03 vs group C. pneumoniae negative) 43% of azithromycin treated patients had a decrease in anti-C. pneumoniae titers compared with 10% of controls (P=0.02)</td>
</tr>
<tr>
<td>WIZARD International Multicenter</td>
<td>7724 patients (83% male) ≥ 18 yr with</td>
<td>Azithromycin (Zithromax) for 3 months</td>
<td>Primary (composite); recurrent MI, all</td>
<td>No significant reduction n the primary</td>
</tr>
<tr>
<td>Trial</td>
<td>Study population</td>
<td>Treatment</td>
<td>End point</td>
<td>Results</td>
</tr>
<tr>
<td>-------</td>
<td>------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>---------</td>
</tr>
<tr>
<td>ACADEMIC</td>
<td>302 pts (69% men) with chronic CAD (previous MI, bypass surgery, or &gt; 50% angiographic stenosis of ≥ 1 major coronary artery) &gt; 18 y, IgG anti Chlamydia pneumoniae titer ≥ 2</td>
<td>3 mo of azithromycin treatment (500 mg/day for 3 days then 500 mg/wk) vs placebo; follow up, 6 mo</td>
<td>Primary: cardiovascular events (cardiovascular death, nonfatal MI or stroke, hospitalization for unstable angina, resuscitated cardiac arrest)</td>
<td>Clinical cardiovascular events at 6 mo did not differ between groups; azithromycin reduced a global rank sum score of 4 inflammatory markers (C-reactive protein, interleukin 1, fibrinogen)</td>
</tr>
</tbody>
</table>

Table 47 shows randomized controlled trials of antibiotics in patients of CHD.

Table 47 showing randomized controlled trials of antibiotics in CHD patients.
### Table 48

<table>
<thead>
<tr>
<th>Trial</th>
<th>Study population</th>
<th>Treatment</th>
<th>End point</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AZACS</strong></td>
<td>1439 patients (74% men) enrolled shortly after admission for acute coronary syndrome, 826 of whom</td>
<td>Azithromycin (single dose of 500 mg followed by 4 days of 250 mg initiated 3-4 days after admission) vs placebo; follow</td>
<td>Primary (composite): death, cardiac arrest, nonfatal MI, and revascularization. Secondary: unstable angina, congestive heart</td>
<td>No significant difference in composite primary end point (12.6% in placebo vs 12.3% in azithromycin group; P = NS)</td>
</tr>
<tr>
<td>Study</td>
<td>Patients</td>
<td>Comparator</td>
<td>Primary End Point</td>
<td>Secondary End Point</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>------------</td>
<td>-------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>CLARIFY</td>
<td>148 patients (70% men) 105 with non-Q-wave MI and 43 with unstable angina</td>
<td>Clarithromycin (500 mg/d) for 85 days vs placebo</td>
<td>Primary (composite): death, MI or unstable angina  Secondary: death, MI unstable angina, ischemic stroke, and critical limb ischemia</td>
<td>No significant difference in primary end point within 3 mo (19 events in placebo vs 11 events in clarithromycin group; ( P = 0.10 )) significant difference in secondary end point throughout mean follow up (median, 555 days): 27 events in placebo vs 16 events in clarithromycin group, risk ratio, 0.49; ( P = 0.03 )</td>
</tr>
<tr>
<td>ROXIS</td>
<td>202 patients (76% men) with unstable angina from 8 coronary care units; age &gt; 21 yr</td>
<td>Roxithromycin (150 mg twice daily) for 30 days vs placebo; follow up. 6 mo</td>
<td>Primary (composite): cardiac ischemic death, MI severe recurrent ischemia  Secondary: anti Chlamydia pneumoniae IgG titers, C-reactive protein</td>
<td>Day 30: the primary triple end point rates were 9% in placebo vs 2% in roxithromycin group (( P = 0.03 )) Day 90: 12.5% vs 4.37%, respectively (( P = 0.06 )) Day 180: 14.6% vs 8.69%, respectively (( P = 0.26 ))</td>
</tr>
<tr>
<td>PROVE IT</td>
<td>4000 patients with acute coronary syndrome for &lt; 10 days and a total cholesterol level of 150-240 mg/dL</td>
<td>2 by 2 factorial trial of pravastatin vs atorvastatin and gatifloxacin (400 mg/d for 10 days, then 10 days per month for trial duration) vs</td>
<td>Primary: death, MI stroke, hospitalization for angina, revascularization  Secondary: lipid levels, high sensitivity C-reactive protein</td>
<td>Expected</td>
</tr>
<tr>
<td>Study</td>
<td>Description</td>
<td>C. pneumoniae IgG titers</td>
<td>Primary</td>
<td>Secondary</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>--------------------------</td>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>Single-center trial</td>
<td>84 patients with acute coronary syndrome admitted to a single hospital in Bangkok, Thailand</td>
<td>Roxithromycin (150 mg twice daily) for 30 days vs placebo; follow-up 90 days</td>
<td>Primary: cardiac death, unplanned revascularization, recurrent angina / MI</td>
<td>Secondary: anti-C. pneumoniae titers (IgG and IgA)</td>
</tr>
<tr>
<td>STAMINA</td>
<td>324 patients from 4 hospitals in south London admitted with an acute coronary syndrome (MI or unstable angina)</td>
<td>Antibiotics given during hospitalization: azithromycin, metronidazole, and omeprazole; amoxicillin, metronidaole, and omeprazole; or placebo; follow-up 1y</td>
<td>Primary: unstable angina or MI</td>
<td>Secondary: antibiotics to C. pneumoniae and <em>Helicobacter pylori</em></td>
</tr>
</tbody>
</table>

AZAS = Azithromycin in Acute Coronary Syndrome; CLARIFY = Clarithromycin in Acute Coronary Syndrome patients in Finland, MI = myocardial infarction, PROVE IT = Pravastatin or Atorvastatin Evaluation and Infection Therapy, ROXIS = Randomized Trial of Roxithromycin in non-Q-wave Coronary Syndrome; STAMINA = South Thames Trial of Antibiotic in Myocardial Infarction and Unstable Angina

The effects of azithromycin were assessed in 108 seropositive patients in the present study. 8 patients were excluded because of their serious medical condition. Of the remaining 100 patients, 60 patients received azithromycin (500 mg / day for 3 days) and 40 patients received placebo.
Table 49 lists the various adverse events which occurred in either azithromycin or placebo group.

<table>
<thead>
<tr>
<th>Adverse events</th>
<th>Azithromycin (n=60)</th>
<th>Placebo (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular death</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Nonfatal Myo. Infarction</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Hospitalization for unstable Angina</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Recurrent Angina</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>PTCA</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>CABG</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Nonfatal stroke</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>6 (10%)</strong> *</td>
<td><strong>12 (30%)</strong> *</td>
</tr>
</tbody>
</table>

* $X^2 = 6.50, P = 0.0108; RR = 0.33 (0.014 < RR < 0.82)$.

As noted in Table 49, in azithromycin group, 6 patients (10%) developed adverse cardiovascular events (cardiovascular death 1, nonfatal myocardial infarction 1, hospitalization for unstable angina 1, need for CABG in 2 and PTCA in 1).

As against that, in 40 patients receiving placebo, 12 patients (30%) suffered adverse events (cardiovascular death 4, nonfatal myocardial infarction 5 recurrent angina 1 and need for CABG in 2) (Table 48).

Thus, adverse events occurred more frequently in the group which received placebo as compared to patients who received azithromycin. The difference in statistically significant ($X^2 = 6.50, P = 0.0108$). The relative risk was 0.33 ($0.14 < RR < 0.82$).

The benefit of azithromycin treatment are obvious, as occurrence of adverse events (10%) in seropositive patients receiving azithromycin was nearly same as 11.3% prevalence of adverse events in seronegative patients, and three times less than 30% prevalence in seropositive patients receiving placebo.
The results of the present study are in conformity with those reported by several smaller trials and at variance with some larger studies.

Gupta et al stimulated interest in antibiotic trials in humans with a pilot study in the United Kingdom: 60 survivors of AMI with persistently elevated antichlamydial antibody titers (IgG≥1:64) were randomized to receive placebo, a single 3 day course of azithromycin (500 mg/day) or 2 courses 3 months apart. Compared with patients in the placebo group plus a nonrandomized group with high antibody titers, azithromycin treated patients showed an apparent reduction in cardiovascular events within 6 to 18 months i.e. from 28% to 8% (Odds ratio 4.2 [95% CI, 1.2 to 15.5; P =0.03]). Thus, seropositive patients not receiving azithromycin had four times increased risk of experiencing adverse cardiovascular events as compared to seronegative patients. Further, seropositive patients who had received azithromycin had 8% incidence of adverse cardiovascular events, which was almost same as 7% incidence in seronegative patients (OR 0.9 [95% CI, 0.2 to 4.6; P = NS]) (Table 46).

Gurfinkel et al reported on a pilot antibiotic trial (ROXIS study) (Table 48). From Argentina: 202 patients presenting with ACS were randomized on hospital admission to roxithromycin 150 mg twice daily or placebo for 30 days. The patients were followed up for 6 months. Rates of recurrent angina (2 versus 5), AMI (0 versus 2), cardiac ischaemic death (0 versus 2), or any event (2 versus 9) tended to be reduced at 1 month. The primary triple end point rates were 2% in roxithromycin versus 9% in placebo group (P=0.03) at 30 days, 4.37% in roxithromycin versus 12.5% in placebo group (P=0.06) at 90 days, and 8.69% in roxithromycin versus 14.6% in placebo group (P=0.26) at 180 days. The authors concluded that roxithromycins anti-inflammatory
effects played the beneficial role. Unfortunately, ROXIS study suffers from the small number of events, poor characterization of patients and short follow up. However, like present study, it certainly raises the question of the role of inflammation in unstable coronary syndromes and the possibility that antibiotic therapy might provide benefit in the acute or sub acute setting.

The Clarithromycin in Acute Coronary Syndrome patients in Finland (CLARIFY study)\textsuperscript{119} (Table 47) treated 148 patients with non-Q wave AMI or unstable angina patients with Clarithromycin 500 mg/ day or placebo for 85 days. They found a trend towards reduced primary end point events (death, AMI or unstable angina) at 3 months. There were 19 events in placebo versus 11 events in Clarithromycin group (P=0.10). However, when all cardiovascular events were included (secondary end point) there was a significant reduction of events, beginning at the 3 months period and continuing until about 10 months, then remaining approximately the same thereafter (Table 47). During the mean 555 days follow up period, 27 events occurred in placebo versus 16 events in clarithromycin group (risk ratio 0.49; P=0.03).

Compared to ROXIS study\textsuperscript{117}, CLARIFY study\textsuperscript{119} had more patients with first time angina and fewer patients with previous myocardial infarction or revascularization. However, the patients condition was more unstable, with the number of events during the first 3 months in the placebo groups being higher (26% in CLARIFY vs. 13% in ROXIS). CLARIFY suggests that the beneficial effect starts during the treatment phase (3 months) and continues for at least another 7 months (continued separation of event curves).
The results of the South Thames Trial of Antibiotics in Myocardial Infarction and Unstable Angina (STAMINA)\textsuperscript{120} also showed a superiority of a combination antibiotic therapy after 1 year in 324 patients of ACS. The antibiotics selected (triple therapy analogous to that used to eradicate H. pylori in gastric ulcers) were aimed at controlling infection by both C. pneumoniae and H. pylori (Table 47). The surprising finding in this small study was that the antibiotics produced beneficial effects whether or not the patient was a carrier of one or both infections. Patients who received antibiotic therapy with either azithromycin or amoxicillin had a 40% reduction in unstable angina or AMI. This suggests that the antibiotics may be working either against a different infection or in an alternative manner.

The results of these earlier, smaller studies appear overly enthusiastic.

In contrast to these already mentioned small studies, in Antibiotic Therapy After an Acute Myocardial Infarction (ANTIBIO study)\textsuperscript{122}, no beneficial effect of a 6 week treatment with roxithromycin 300 mg daily in 872 patients of AMI was observed during a 12 months follow up period. There was no difference in total mortality (6.5% in the roxithromycin group compared with 6% in the placebo group; (OR, 1.1, 95% CI, 0.6 to 1.9, \(P=0.739\)), and no differences at 12 months for the combined end point of death, AMI, stroke or angina leading to hospitalization (25.1% versus 20.8%, \(P=0.138\)).

Similarly, Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with Chlamydia (ACADEMIC) study\textsuperscript{113} also showed discouraging results with antibiotic therapy. In 302 patients, no beneficial effect of a 3 months treatment with azithromycin was found after 6 and 24 months follow up. During the trial, 47 primary events
occurred, 22 events in azithromycin group, and 25 in placebo group. There was no significant difference in the primary end point between the two groups (hazard ratio for azithromycin 0.89; 95% CI, 0.51 to 1.61; P= 0.74). Events included 9 versus 7 occurring within 6 months, and 13 versus 18 between 6 and 24 months in the azithromycin and placebo group respectively (Table 47).

The AZACS (Azithromycin in Acute Coronary Syndrome) trial\textsuperscript{67} enrolled 1439 patients shortly after admission for ACS. After a brief course of azithromycin versus placebo, no significant difference was noted in composite primary end points (death, cardiac arrest, nonfatal AMI and revascularization) (12.6% in placebo group vs 12.3% in azithromycin group; P=NS) or in any of its components. Also, there was no difference in rates of end points in patients enrolled with AMI or with antibodies to C.pneumoniae. (Table 47).

The WIZARD (Weekly Intervention with Zithromax for Atherosclerosis and its Related Disorders) trial\textsuperscript{116} was the logical extension of the study carried out by Gupta et al at St.George's Hospital in London. This is by far the largest trial of antibiotic therapy for CHD, with 7724 patients enrolled at 271 centers in 9 different countries. (Table 45). All patients had documented myocardial infarction in stable phase. This myocardial infarction had occurred more than 6 weeks before screening. All patients were seropositive to C.pneumoniae. Patients were randomized to treatment with azithromycin (600 mg per day for 3 days and then weekly for the following 11 weeks) or placebo. After 2.5 years follow up, there was no significant effect on cardiovascular events (hazard ratio 0.93; P = 0.23). There appeared to be an early benefit after treatment with azithromycin in the risk of MI, but it was not sustained. The maximal
benefit, although not significant, was obtained in smokers (hazard ratio, 0.76; 95% CI, 0.58 – 1.00; P=0.05) and in diabetic patients (hazard ratio, 0.8; 95% CI, 0.64 – 1.01; P = 0.06) (Table 46).

The WIZARD trial\textsuperscript{116} had two phases: 3300 patients were enrolled in the original trial; subsequently, the trial reopened enrollment and recruited to 7724 patients. It would be instructive to know whether patients in the original cohort who were followed up longer (3 years vs 1.5 years) had a significant event reduction compared with those enrolled later.

The small single-centre study at Siriraj Hospital in Bangkok\textsuperscript{56} used the same protocol as ROXIS and found no significant differences in events at 90 days. There were 17 cardiac events in roxithromycin group versus 16 in placebo group (Table 48).

Some other studies are still in progress.

The small ongoing CROAATS (Croatian Azithromycin in Atherosclerosis Study\textsuperscript{56}) is analyzing the effect on cardiovascular events of approximately 3 weeks of azithromycin therapy given on 3 days to post – MI patients with 2 positive anti-C. pneumoniae IgG titers obtained 2 months apart (Table 46).

The ACES (Azithromycin and Coronary Events Study\textsuperscript{124}) is unique in that it will study 4000 patients with stable CHD. It will treat patients with azithromycin 600 mg / week vs placebo for 1 year. Seropositivity to C.pneumoniae is not an inclusion criterion but the investigators expect about 80% of patients to be seropositive with an IgG titer.
In addition, ACES will study C. pneumoniae DNA (detected by PCR) located in PBMCS. The patients will be followed up for 4 years (Table 47).

The ongoing MARBLE (Might Azithromycin Reduce Bypass List Events) trial is treating patients who are awaiting bypass surgery with long term antibiotic therapy in an attempt to decrease cardiovascular events (Table 47).

PROVE – IT (Pravastatin or Atorvastatin Evaluation and Infection Therapy) is a well powered study (N=4000) examining long term use of gatifloxacin in patients presenting with an ACS and elevated cholesterol levels. Results are awaited (Table 48).

All these data suggest as follows.

1) Short term antibiotic treatment in patients with a history of AMI and positive C. pneumoniae titers do not significantly reduce cardiovascular events (Table 46).

2) No large randomized controlled trial has shown a significant reduction in cardiovascular events with short term antibiotic treatment in patients with a history of chronic CHD and positive C. pneumoniae titers; a possible benefit on inflammatory markers was suggested in one small trial (Table 47).

3) In patients with ACS, no large well powered randomized well controlled trial has been completed. Results of available trials showed benefit on total cardiovascular events (ROXIS, CLARIFY and Present study): One small pilot trial showed a decrease in events with several triple therapeutic antibiotic regimens (STAMINA) (Table 48).
This data shows conflicting results for the value of antibiotic therapy in patients of CHD.

EFFECT OF ANTIBIOTIC THERAPY ON IgG TITER:

Possible explanations for the association between elevated antichlamydial antibody titers and the risk of adverse cardiovascular events include direct involvement of C.pneumoniae in atherogenesis. If antichlamydial titers decrease after treatment with azithromycin, which is a specific antichlamydial antibiotic, then that will provide additional proof of involvement due to C.pneumoniae. This may also account for the reduction in adverse cardiovascular events in the treated group.

In the present study, IgG titers decreased in 14 (26%) out of 54 patients who received azithromycin as compared to 6 (21.4%) out of 28 patients who received placebo, at the end of 6 months (P>0.05). Thus, antibody titers were not significantly reduced by antibiotic therapy in the present study. Patients having baseline IgG titer of ≥ 120 RU mainly manifested this decrease in titer. None of the patients showed increase in IgG level. None of the seropositive patients became converted to seronegative at end of 6 months.

Whatever the relationship between antichlamydial antibody titer and cardiovascular outcome, the reduction in event rate after treatment with azithromycin is intriguing, particularly because the benefit was not restricted to subjects who had a decrease in titers.
Gupta et al. noticed that at the end of 6 months, antichlamydial antibody titers fell to <1:16 in 43% of patients in the azithromycin group versus 10% of subjects in the placebo group (P=0.02). They also showed surprise that favourable treatment outcome in terms of reduction of adverse cardiovascular events was not restricted to individuals who showed a decrease in titer.

Torgano et al. randomly assigned 97 patients with CHD to receive either clarithromycin 500 mg B.D. for 30 days, if they were seropositive to H. pylori irrespective of C.pneumoniae status, or 500 mg B.D. for 14 days if they were seropositive (IgG titers ≥ 64) for C.pneumoniae, or placebo. They demonstrated a significant reduction in the geometric mean IgG titer in the treated patients.

However, this opinion is not shared by other workers. In ACADEMIC study, quantitative measures of antichlamydial IgG and IgA titers at baseline and 3 & 6 months in the two treatment groups were as shown in Table 50.
Table 50 showing effect of therapy on antichlamydial antibodies in ACADEMIC study.

<table>
<thead>
<tr>
<th>Measure (n)</th>
<th>Azithromycin</th>
<th>Placebo</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change score (ruv.) 3 mo vs baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ IgG (146)</td>
<td>1.11 ± 1.87 (0.89)</td>
<td>1.25 ± 2.08 (0.79)</td>
<td>0.90</td>
</tr>
<tr>
<td>Δ IgA (87)</td>
<td>0.76 ± 1.09 (0.85)</td>
<td>1.14 ± 0.92 (1.15)</td>
<td>0.093</td>
</tr>
</tbody>
</table>

| Change score (ruv.) 6 mo vs baseline | | | |
| Δ IgG (216) | 0.41 ± 2.12 (0.0) | 0.30 ± 1.84 (-0.05) | 0.39 |
| Δ IgA (271) | 0.27 ± 0.86 (0.16) | 0.27 ± 0.84 (0.11) | 0.68 |

Titers were stable or tended to increase slightly over time. No difference in changes in titers of either immunoglobulin by treatment group was observed at 3 or 6 months. Thus, in ACADEMIC study, antibody titers were unaffected by therapy.

In Wizard study¹¹⁶, no significant association between C.pneumoniae titers and treatment effect was observed (Fig 40).
No association between titers to C.pneumoniae and the likelihood of developing a primary event was seen within the group of patients randomized to either azithromycin or placebo. There was no difference between treatment groups at baseline or end of follow up in the distribution of C.pneumoniae titers. The risk reduction for the primary event was unchanged when the log of the baseline C.pneumoniae titer was included in the Cox proportional hazards model.

Jackson et al treated 88 patients who had undergone PTCA with placebo or azithromycin for 28 days. They found that although azithromycin was well tolerated, there was no effect on either IgG or IgA titers.
Similar findings were reported by Sinisalo et al. They reported that treatment with doxycyclin 100 mg twice daily for 4 months had no effect on antibody titers in men with angiographically confirmed CHD and prior bypass surgery.

**EFFECT OF ANTIBIOTIC THERAPY ON INFLAMMATORY AND OTHER BIOLOGICAL MARKERS**

Several studies have searched for the effects of antichlamydial antibiotic on inflammatory markers. Although the association of antichlamydial antibodies and inflammatory markers was evaluated in the present study, the effect of azithromycin on any change in inflammatory markers was not specifically looked for.

Gupta et al. observed reductions in some of several markers of inflammation, specifically, monocyte/macrophage tissue factor and the surface adhesion molecule CD 11b.

In a double blind, randomized, moderately large secondary prevention study in CHD patients with serological evidence of prior C.pneumoniae exposure, Anderson et al. showed no difference in global inflammatory marker scores for CRP, IL-1, IL-6 and TNFα at the end of 3 months period. However, at 6 months, the global difference score was lower in the active-therapy group, both the change scores (P=0.03) and actual value scores (P=0.01). All measures trended in a favourable direction, with significance reached for CRP, IL-1 and IL-6 on one or the other test.
Gurfinkel et al\textsuperscript{118} reported that roxithromycin treatment decreased CRP levels compared to placebo in the ROXIS study.

Muhlestein et al\textsuperscript{121} reported that inflammatory markers decreased gradually, first becoming evident at 6 months, and therefore, to confirm / refute effect of antibiotic, only long term studies should be encouraged.

Torgano et al studied 84 patients with chronic IHD, H.pylori and/or C.pneumoniae antibodies, and normal acute – phase reactants. They were randomly assigned to treatment or no treatment. Treatment consisted of omeprazole, clarithromycin, and tinidazole in H.pylori – piositive patients and claritromycin alone in C.pneumoniae – positives. The effet of treatment and other baseline variables on fibrinogen levels, determined at 6 months, was evaluated by multivariate analysis. Treatment significantly reduced fibrinogen level at 6 months in the overall study population and in the groups of patients divided according to H.plori or C.pneumonie positivity. In the 43 treated patients, mean (± SD) base fibrinogen was 365 ± 50 mg% and mean final fibrinogen was 309 ± 52 mg% (P < 0.001), whereas in the 41 untreated patients, mean basal and final fibrinogen levels were 345 ± 70 and 361 ± 71 mg%, respectively. The largest decrease was observed in patients with both infections. Fibrinogen changes were also significantly and negatively correlated with age. \textit{Their data suggest that a short, safe, and effective course of antibiotic therapy might be suggested as a means of interacting with an “emerging” risk factor.}

However, Semann et al\textsuperscript{128} treated 40 patients of documented CHD having IgG titer >16 and 20 controls, who had normal coronary arteries, with azithromycin 500 mg/day for 3 days, then twice weekly for 3 months, or placebo. They observed that
azithromycin treatment had no effect on the levels of soluble markers of endothelial activation, intercellular cell adhesion molecule – 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM -1) and E-selectin in plasma.

Sinisalo et al\textsuperscript{119} also did not find any significant difference in CRP, total cholesterol, HDL and LDL between seropositive patients who received doxycyclin or placebo\textsuperscript{11}.

**CONFLICTING RESULTS OF STUDIES USING ANTIBIOTIC THERAPY**

Some investigators\textsuperscript{20,117,119,120,126} have reported encouraging results when patients of CHD underwent antibiotic intervention. On the other hand, several studies\textsuperscript{113,116,118,122,124,125} failed to demonstrate beneficial response to antibiotic treatment. The data, therefore, is full of conflicting information for the value of antibiotic therapy in patients with CHD.

It must be remembered that the chief and the large evidence in favour of a role of C.pneumoniae emerged from basic laboratory studies. However, clinical trials of patients with stable and unstable CHD failed to demonstrate significant benefits of antibiotic treatment\textsuperscript{66}. These data from laboratory studies and clinical trials seem to divulge.

But then, does a true contradiction really exist between laboratory data and clinical trials? Exact answer is not available. However, some explanations can be put forward.
Most laboratory data comes from in vitro experiments, and their true validity is difficult to confirm in vivo.

Most in vivo experimental data are derived from small-animal models. This has inherent limitations. Data from large-animal models of C. pneumoniae infection are not available.

Lastly, animal models are just what they are, i.e. nothing beyond experimental models. Scientific literature is full of studies of interventions that are extremely successful in animals, but they fail to have identical effects in patients.

As far as clinical trials are concerned, several reasons can be put forward to explain discrepancy in the results, noted during antibiotic intervention.

1) The choice of antibiotic (macrolide or otherwise)
2) Type of macrolide (roxithromycin, azithromycin, clarithromycin)
3) Underlying condition of patients (stable CHD, unstable angina, AMI, old myocardial infarction, ACS)
4) Dosing of the drug
5) Duration of therapy (3 days to 12 weeks)
6) Duration of follow up (31 days to 18 months)
7) Number of patients studied
8) Selection of patients being seropositive for C. pneumoniae.

All these may have a major impact on the outcome of antibiotic therapy.
In terms of dosage and duration of treatment, none of the antibiotic regimens used previously or being used currently has been evaluated or demonstrated to be effective in eradication of C.pneumoniae from the respiratory tract or any other site.

Further, the weekly dosing regimens of azithromycin used in the ACADEMIC study and other intervention studies may result in long periods of subinhibitory levels of drug, which may lead to development of resistance.

Higgins\textsuperscript{66} reported that some clinical studies reveal early benefit that is lost when antibiotic is discontinued.

Antibiotics might have been more effective in acute or subacute setting. Since late antibiotic treatment of C.pneumoniae infection does not prevent atherogenesis in animal models\textsuperscript{68}, it is possible that inclusion of stable patients at a median time of 31 months after the acute event as in WIZARD trial may have minimized the chances of obtaining beneficial effects with azithromycin therapy.

Favourable response with antibiotic will be available only if patients are followed up for sufficiently long period. Response of antibiotic producing anti-inflammatory effects will be delayed. Prolonged persistence of LPS and HSP 60 in atherosclerosis plaque even after the organism has been killed by antibiotic, and very gradual reduction in inflammatory markers index (IL-1, IL-6, CRP, and TNF) may explain improvement in reduction of adverse clinical events only after a prolonged period of observation.
Several studies suffered from small number of events, poor characterization of patients, and short follow up, and hence beneficial effects of antibiotic might not have been observed.

In general, the larger trials which included more patients, showed negative results compared with the smaller trials.

Are the negative results of antibiotic therapy studies due to the reason that C. pneumoniae does not play any role in atherogenesis – or because a proper antibiotic regimen has not been used in the proper clinical setting?

Before completely rejecting “infection hypothesis” of atherosclerosis, we should not forget lessons from the past. Thrombolytic therapy with streptokinase in AMI was considered marginally effective in 1950s. Next two decades exposed improper patient selection and inadequate dosage in initial studies as responsible for poor efficacy. Strangely enough, at present, thrombolytic therapy with streptokinase forms cornerstone in early management of AMI.

Will antibiotic therapy for C. pneumoniae follow up the same pattern?

C. pneumoniae INFECTION IN INDIAN CONTEXT:

Cardiovascular diseases especially atherosclerotic CHD are responsible for major disability in the developed as well as in developing countries. WHO has predicted that
by 2020 AD up to three quarters of deaths in developing countries would be resulting
from noncommunicable disease and that CHD will top the list of killers\textsuperscript{140}.

Data suggests that epidemiological transition as seen by aging and changing lifestyle
and culminates in epidemic of hypertension and CHD is rapidly occurring in India and
other developing countries\textsuperscript{141}.

Prevalence of CHD is very low in rural populations of India, and it is significantly more
in urban Indians\textsuperscript{141}.

A metaanalysis showed that prevalence of CHD in urban population increased from
3.42\% in 1960'\textquotesingle s to 3.62\% in 1970'\textquotesingle s and to 9.45\% in the 1990'\textquotesingle s ($\chi^2$ for trend = 5.63,
P=0.018). In rural area, the prevalence increased from 2.06\% in 1970'\textquotesingle s to 4.15\% in
the 1990'\textquotesingle s. ($\chi^2 = 2.94$, P=0.086)\textsuperscript{140,141}. Established risk factors like hypertension,
diabetes, cholesterol levels, truncal obesity, and sedentary life style help explaining
only part of the urban rural difference. A substantial proportion of this difference
remains unexplained\textsuperscript{140}.

It has been postulated that in countries like India with low socio-economic conditions,
there may be a link between CHD and some undefined environmental exposures,
including infections.

Infections in genesis of atherosclerosis is an attractive hypothesis to explain the
epidemic of CHD in India. Epidemiological transition in demographic distribution of
CHD, i.e. from initial higher prevalence in upper socioeconomic strata to lower socioeconomic status subjects presently can also be explained by chronic infections in pathogenesis of atherosclerosis. This demographic shift is similar to epidemics of other infections (Tuberculosis, HIV) in India\textsuperscript{1,140-142}.

Elevated CRP levels have been considered independent risk factors for an acute coronary event\textsuperscript{11-13}. Only a few Indian studies have evaluated markers of inflammation in CHD\textsuperscript{10}.

Autopsy studies in 1950's and 1960's in India have shown that among asymptomatic subjects and accident victims, prevalence of coronary atherosclerosis is very high\textsuperscript{140}. The prevalence and nature of lesions is similar in rural and urban individuals\textsuperscript{140}. However, none of the earlier studies have highlighted the nature of atherosclerosis in detail; Evidence of inflammation in early or late atheroma has not been evaluated. Further studies in this direction are needed. Again, in these autopsy studies, no information regarding microscopic appearance of lesions is available. In Indians, microscopic study of atherosclerotic lesions is needed to identify the nature of atherosclerotic lesions.

Kavishwar et al\textsuperscript{143} recently reported findings of their autopsy study to detect C.pneumoniae inclusion bodies in 80 atherosclerotic and 20 apparently normal coronary arteries by direct immunofluorescence. They observed that C.pneumoniae positively was seen in 30 out of 80 atherosclerotic lesions (46.25%) and in only one out of 20 control cases (5%). C.pneumoniae was more prevalent in men and after fifth decade. The fluorescent inclusion bodies were observed for size, shape, location,
arrangement and majority were present in intimal and subintimal tissue (81%), but also seen in tunica media. Out of 38 deaths due to acute coronary insufficiency, 26 cases (68.18%) were positive for C.pneumoniae. Statistical analysis by Chi square test showed C.pneumoniae positivity to be significantly associated with coronary atherosclerosis (p value – 0.005) and cardiac mortality (p value – 0.0089).

The authors concluded that C.pneumoniae is significantly associated with coronary atherosclerosis. It may be a risk factor for cardiac mortality by initiating and/or exacerbating atherosclerosis.

A few recent studies in India have shown the presence of C.pneumoniae antibodies in CHD patients. Emigrant South Indians also have higher titers of C.pneumoniae antibodies than other groups. Larger and intensive studies are needed to confirm these preliminary observations, especially as infections have multiple mechanisms of damage in atherosclerosis.

It is possible that cardiovascular risk increases with cumulative or earlier exposure to more pathogens or specific potentially atherogenic microbes. Besides C.pneumoniae high sero prevalence of H.pylori has been documented in several Indian studies.

In countries like India, a person gets exposed to multiple pathogens right from infancy, which often goes untreated or incompletely treated. Some of these may be detected while others may pass undetected. This may result in a disease burden. Some of these infectious agents may work in tandem to produce a cumulative atherogenic effect.
A plea is made that atherosclerosis among Indians could partly be related to chronic infections. The atherosclerotic lesion should certainly be studied for presence of inflammation and infections. C.pneumoniae infection is a prime candidate but other infections should also be evaluated. Seroepidemiological studies should be performed for identifying initial direction. Pathological examination of the atheroma specimens should be routinely carried out.

For additional support, a variety of techniques like ICC, PCR, EM and finally culture support for confirmation of the organism are required.

**WHAT NEEDS TO BE DONE IN FUTURE?**

1. Epidemiological studies of C.pneumoniae infection and CHD are needed from our country. These studies should be large enough for medium-sized effects to be assessed or refuted reliably. They should involve repeated antibody measurements in at least a subsample to allow correction for regression dilution. Importantly, in such studies, the effects of residual confounders need to be kept to a minimum.

2. The association of vascular risk factors with CHD usually tend to be stronger in younger than in older subjects. Therefore, future studies in young adults might be especially informative.

   i. Results of recent studies suggest that laboratories using the same methods with the same specimens may obtain different results.
In addition to lack of consistent serological criteria, there are inherent problems with performance of most widely used serologic methods.

ii. Reliable serological marker for chronic persistent C.pneumoniae infection must be unanimously agreed upon.

iii. The methods of detection need to be uniform, and universally standardized.

iv. Diagnosis based on a single test could be considered as presumptive. Confirmatory diagnosis then deriving inference on the basis of single test results.

3. Local quantification of different cytokines in correlation to the endovascular presence of C.pneumoniae and possible consecutive increased smooth cell proliferation may help to clarify the underlying pathological mechanism. Further studies quantifying inflammatory markers and the level of cytokines (e.g. TNF-α, IL-1, IL-4 and IL-6), and comparing these levels with the proliferation rate of smooth muscle cells are warranted to further elucidate the underlying mechanism of vessel damage by C.pneumoniae.

4. The causal relation between inflammatory responses and neointima formation after percutaneous coronary interventions is well established by mechanistic studies in animals and clinical studies.

More than 90% of angiographically detectable late lumen loss after stenting is caused by neointima formation.
Acute inflammatory responses at the stented segment start within hours and peak around end of first week after stenting. Most inflammatory and proliferative responses are completed within first month. Further late lumen loss does not extend beyond 6 months. A 4 week course of antibiotic treatment suffices to cover period of principal inflammatory responses that lead to neointima formation. Effect of such treatment on restenosis can be assessed within first year after intervention.

Hence, neointima formation might be especially suited to investigate new treatment strategies based on the putative link between C.pneumoniae and vascular inflammation.

5. Selecting patients on the actual presence of the organism, perhaps by PCR analysis of circulating white blood cells, may prove to be a better way to test for the role of antibiotic therapy directed against C.pneumoniae.

6. Even if chronic C.pneumoniae infection is causally linked with CHD, effects of infection on CHD risk might not be rapidly and fully reversible. Hence, intervention trial should randomize large number of individuals and observe them for some years to assess reliably the moderate effects on CHD that are possible.

7. Future studies should randomize subjects irrespective of their antibody status. Baseline blood samples should be stored for future testing with any improved
8. Considering the potential impact of total pathogenic burden, while evaluating a drug like clarithromycin, which acts not only on C.pneumoniae, but also on H.pylori, the interpretation of results should remain guarded.

9. WIZARD study\textsuperscript{116} is the largest antibiotic treatment trial to date. It has essentially ruled out the possibility of a large and durable benefit in secondary prevention with a 3 month course of azithromycin therapy. However, post hoc analysis showed trends towards a favourable effect of antibiotic therapy in men who smoke or who have diabetes or hyperlipidaemia; a significant 55\% reduction in events was noted in individuals who have diabetes and who smoke\textsuperscript{22}.

These observations should point investigations towards consideration of alternative durations of therapy or essentially, focus on analyses of different subsets of patients with cardiovascular disease.

10. In trials in which observation period extends over number of years, duration of therapy need to be longer. Since, reduction of events is known to wane with time, longer suppressive therapy may be advantageous in this patient population\textsuperscript{124}.
11. Although azithromycin has significant activity against C. pneumoniae, certain subpopulation of organisms may be less susceptible to eradication. Further efforts to understand the organism, in this state may prove fruitful, both for selection of antibiotics or combinations of antibiotics and for manipulating the microenvironment to move organisms out of this state.

12. Treatment of patients, even seronegative to antichlamydial antibiotics, with azithromycin might help to answer the query of specific versus nonspecific effects of azithromycin.

All the available data are based on work done in Western countries. There is tremendous paucity of research work done on this subject from our country.

The present study has demonstrated a positive association of the prevalence of ACS with potentially treatable infection, which is common in general population. The results of the study imply that between one third and one half of the current CHD in this population from West India was statistically attributable to C. pneumoniae infection.

What are required now from several centers of our country, are well conducted prospective studies and eradication trials to evaluate the causal relationship of this infection to haemostatic function.
progression of atherosclerosis, and the suitable timing, dose & duration of antibiotic therapy to reduce cardiovascular morbidity and mortality.

LIMITATIONS OF PRESENT STUDY

The potential limitations of this study merit discussion.

1. In the present study, estimation of antichlamydial antibodies was relied upon for the diagnosis of chronic C.pneumoniae infection. Serum samples were analyzed with ELISA and IIFT. With kits provided by EUROIMMUN Medizinische Labordiagnostika AG.

For ELISA, the antigen preparations were HEp-2 cells infected with the “CDC/CWL-029” strain of C.pneumoniae. Elementary bodies purified from cell lysates were treated with sodium dodecylsulphate. The used solution contains all relevant antigens localized in the outer membrane of the elementary bodies. The outer membrane is composed of lipopolysaccharide (LPS) and numerous proteins (outer membrane proteins, OMPs). The main portion is provided by the MOMP antigen.

For IIFT, cells (EU 38) infected with C.pneumoniae are the standard substrate used for the determination of antibodies to C.pneumoniae.

As per the claims of the manufacture, the sensitivity and specificity of IIFT were 98% and 100% respectively. Further, sensitivity and specificity of ELISA with reference to IIFT were 84.2% and 81.5% respectively.
No cross reactivity with other bodies has been noticed with either method according to the manufacturer.

The three chlamydial species, - C.trachomatis, C.psittaci and C.pneumoniae are very similar. For this reason, antibodies against C.trachomatis and C.psittaci almost always show cross reactions with the LPS as well as the MOMP antigen of C.pneumoniae. Thus, an exclusive determination of species – specific antibodies against C.pneumoniae is not possible with the currently available tests systems.

Because ELISA, in contrast to the earlier complement fixation tests for chlamydial LPSs, is based on a Chlamydia – specific small fragment of the LPS content, the probability of cross – reactions to other gram negative bacteria is far lower than with earlier tests.

The manufacturer has investigated cross reactions with sera positive for the following parameters (the % of the samples that showed a positive reaction in the IIFT is indicated in the parenthesis)

IgG: EBV-CA (79%), CMV (70%), HSV-1 (52%), Legionella (83%), Mycoplasma pneumoniae (62%), Influenza – A (75%) and Chlamydia trachomatis (96%). Since the prevalence for C.pneumoniae lies at 50-80%, only cross – reactivity with C.trachomatis is to be expected.
Cross reactivities with C.trachomatis and C.psittaci cannot be ruled out either in the IIFT with infected cells as the substitute or in MIF with elementary bodies as the substrate.

Specific methods for C.psittaci were not included in the present study, because no such ELISA is available. Further, the prevalence of C.psittaci antibodies is too low to have any significant influence on the result.

Some cross-reactivity between MOMP of the different chlamydial species might have occurred in the current estimation in this study.

Cross-reactivity with C.trachomatis can't be ruled out. However, the prevalence of C.trachomatis seropositivity is very low in our country. In one of the studies, Immunocomb assay detected 74% of specimens as positive for C.pneumoniae as compared to only 18% for C.trachomatis antibodies. Unlike C.pneumoniae, role of C.trachomatis in CHD is not clear. Since, Immunocomb assay employs LPS extracted from C.trachomatis L2, and LPS extracted from C.pneumoniae elementary bodies on two separate antigenic spots, interpretation of results remains crucial, and the remote possibility of cross reactivity between C.pneumoniae and C.trachomatis cannot be ruled out even in the present study.

I agree that this point is of vital importance, because, if assay used to detect C.pneumoniae titers is insensitive or nonspecific, then systematic error will have led to a spurious end result.
2. The second limitation of the study was that only IgG was estimated, and IgA and IgE were not evaluated. However, I do not believe this to be a major limitation due to the following reasons.

i. The great majority of cross-sectional and retrospective studies which suggest a positive association for C. pneumoniae have relied primarily or exclusively on IgG serology to determine exposure status.\textsuperscript{17}

ii. Completed as well as ongoing clinical trials of antibiotic therapy to reduce cardiovascular risk have used IgG titers as a critical determinant of eligibility.\textsuperscript{53,118}

iii. It has been demonstrated that IgG titers but not IgA or IgM titers correlate with the ability to directly detect Chlamydia within human coronary arteries obtained at autopsy.\textsuperscript{111}

iv. The Current Recommendations for C. pneumoniae Serologic Testing by Centers for Disease Control and Prevention\textsuperscript{83} suggest that IgG titer of \( \geq 16 \) indicates past exposure, but neither elevated IgA titers nor any other serological markers are valid indicators of persistent or chronic infection.

3. 60 Seropositive patients received 500 mg / day azithromycin for 3 days in the present study. The optimal dose and duration of azithromycin therapy are unknown in the setting of chronic CHD. A persistent, low grade indolent infection might require a longer duration of therapy than acute infections. Chlamydia in a persistent, metabolically inactive state might not be immediately susceptible to antibiotic therapy. Longer term and / or intermittent (eg. Monthly)
treatment may be required to ensure lasting benefit. Reinfection with C.pneumoniae is known to occur, even in adulthood, and appears to occur to the extent of 2% to 3% annually.

In light of all this, 3 days treatment might have been inadequate, and longer duration of treatment could have benefited many more.

4. The present study does not clarify whether the effects of azithromycin are due to a specific antichlamydial action and not a mere general anti-infectious or anti-inflammatory or antiatheromatous effect.

Additional work needs to be done in this direction. In the mean time, the beneficial effect of azithromycin on atherosclerosis, whether by specific or nonspecific mechanisms, is of great interest.

5. The effects of azithromycin were studied in all the seropositive patients in the present study. Response in specific subpopulations eg. Smokers, diabetics, hyperlipidaemics or hypertensives was not studied. This would have thrown brighter light on the subject. This was perhaps not possible due to small number of adverse cardiovascular events occurring in a limited number of patients belonging to a small group.

6. In the present study, only seropositive patients were administered azithromycin for secondary prevention of cardiovascular events. Treatment of seronegative patients might have helped to answer the question of specific versus nonspecific antichlamydial effects of azithromycin.
7. The limited utility antibody titers to C.pneumoniae has been well described\textsuperscript{11}. The findings in this study regarding antibody titers are not inconsistent with these views, because the benefit of azithromycin was not restricted to only those patients who demonstrated fall in titers with therapy. Selecting patients based on the actual presence of the organism, perhaps by PCR, may prove to be a better way to test for the role of antibiotic therapy directed against C.pneumoniae.

Finally, a plea is made that studies like the present one obviously have natural short comings and restrictions. Results of this study should be considered more as hypothesis – generating.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Several reports have linked CHD with various infections, such as with C.pneumoniae.
- Plasma concentrations of CRP and other sensitive markers of systemic inflammation may be correlated with future risk of CHD in the general population.
- It is not known whether these associations are causal or merely due to confounding by classic risk factors, chronic infective processes, or early disease.
- Persistent infection with C.pneumoniae has been suggested to be an avoidable cause of CHD.
- Most previous studies on this topic have been small and prone to biases.
• Data from laboratory studies and clinical trials seem to divulge. More evidence in favour of a role of C.pneumoniae emerged from basic laboratory studies, whereas clinical trials of patients with stable / unstable CHD failed to demonstrate significant benefit of antibiotic therapy.

WHAT THIS STUDY ADDS

• Concentrations of IgG antibody of C.pneumoniae was higher in patients of CHD than among controls.
• Baseline antichlamydial IgG antibody concentrations were strongly associated with
  a) Prevalence of CHD
  b) Major coronary events
  c) Risk factors like inflammatory markers.
• Azithromycin, a specific antichlamydial antibiotic, is beneficial in secondary prevention of adverse cardiovascular events in patients of ACS.
• Important association is found between chronic Chlamydia pneumonia infection and CHD.
• These findings suggest that low grade inflammatory processes may be relevant to CHD.