ATHEROSCLEROSIS AND
CHLAMYDOPHILA (CHLAMYDIA) PNEUMONIAE

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SUMMARY

The concept that atherosclerosis was induced by environmental factors such as smoking and high fat intake was perturbated when inflammation was found to be prominent in atherosclerotic lesions. *Chlamydophila pneumoniae* but also other microbes inducing chronic endothelial infections have been implicated, particularly cytomegalovirus (CMV) and the gastric pathogen *Helicobacter pylori*. Large sero-epidemiological studies in different parts of the world have shown seroconversion to *C. pneumoniae* in patients with myocardial infarction, stroke and other forms of cardiovascular disease. The seroprevalence rates vary between 50 and 75%. Older individuals show higher seropositivity rates, suggesting that re-infection may be common. Using polymerase chain reaction (PCR) and immunohistochemistry, several studies have shown *C. pneumoniae* DNA in atherosclerotic lesions but not in normal vessels, although other studies have failed. Animal models with *C. pneumoniae* inducing atherosclerotic lesions have been established. The chlamydia LPS induces foam cell formation of monocytes, and the heat shock protein (Hsp) 60 oxidises low-density lipoproteins. Hsp 60 causes transcription of NF-κB and initiates deleterious immune response. Hsp 60, cross-reacting with human Hsp60, may also be involved in molecular mimicry which is part of the chronicity of *C. pneumoniae* infection. A peptide produced by *C. pneumoniae* mimics human heart muscle protein, which causes immune sentries. A co-infection of *C. pneumoniae* with *H. pylori* increased expression of vascular cell adhesion molecules (VCAM-1) in ApoE knockout mice, which may enhance atherogenesis.

INTRODUCTION

Atherosclerotic heart disease is the leading cause of morbidity in the Western hemisphere with manifestations of coronary artery disease, cerebrovascular disease and renal vascular disease. Contribution of inflammation to the pathogenesis of atherosclerosis was first hypothesised by Virchow in 1859 (*Verkooyen* et al., 1992). The finding of *Chlamydia* particles in damaged heart valves of a fairly great number of bird owners left the cardiologists and infectious disease specialists untouched (*Ward* and *Ward*, 1974). Experimental infection with avian herpesvirus in germ-free chicken produced arterial disease, resembling atherosclerosis (*Fabricant* et al., 1978). Elevated levels of C-reactive protein and fibrinogen, which is typical for infections, were associated with
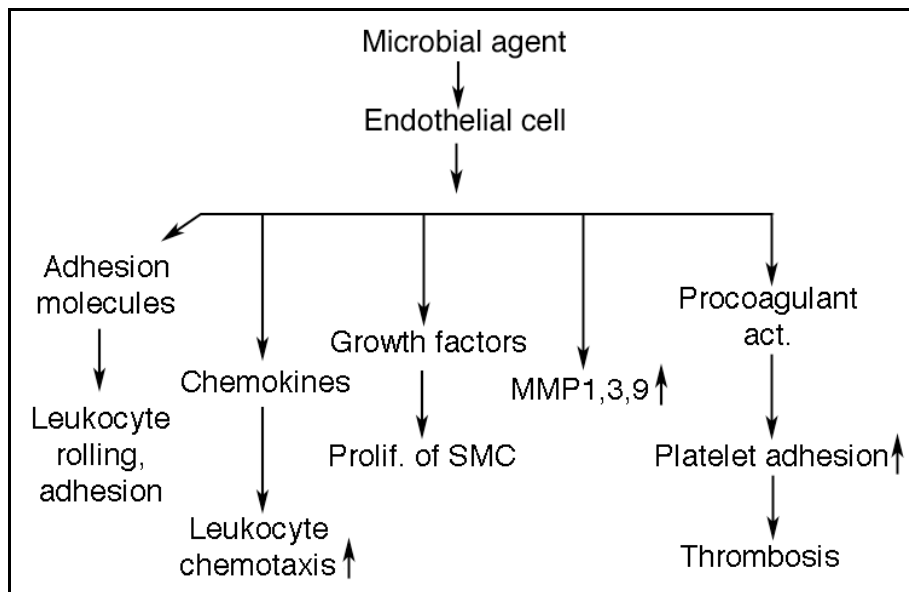
Figure 1: Pathogenesis of atherosclerosis.

coronary artery disease as well as with unstable angina (*Danesh* et al., 2000). Since then, multiple microbes have been investigated as possible aetiological agents of atherosclerosis, such as *C. pneumoniae, H. pylori,* cytomegalovirus, human herpesvirus and periodontogenic bacteria (*Kalayoglu* et al., 2002; *Kusters* and *Kuipers,* 1999; *Nieto,* 1999; *Beck* et al., 2001). No association was found between coronary artery disease and seropositivity to *Bartonella* sp. but a modest association with *Coxiella burnetti* (*Ender* et al., 2001). Before that, the general concept was that atherosclerosis was induced by elevated blood pressure, high caloric (cholesterol) intake, low physical activity and smoking. Gradually, the central role of inflammation as main part of atherosclerosis was accepted.

PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis starts with fatty streaks in the endothelium which, with time, develop into fibrous plaque, i.e. a lipid core with a fibrous cap. Monocytes and T-cells are recruited to the vessel wall across an intact epithelium. This requires expression of leukocyte adhesion molecules (e-selectin, ICAM-1 and VCAM-1) which are transcriptionally regulated by NF-κB. Modified smooth muscle cells (SMC), macrophages, monocytes, T-lymphocytes and several inflammatory cytokines are abundant in the plaques (*Noll,* 1998). This is a result of endothelial dysfunction with accumulation of monocytes, macrophages and lymphocytes in the intima. Macrophages ingest lipid and become foam cells. SMCs proliferate and secrete extracellular matrix (ECM). When sufficient lipid has accumulated the core of the lesion becomes necrotic, and in the
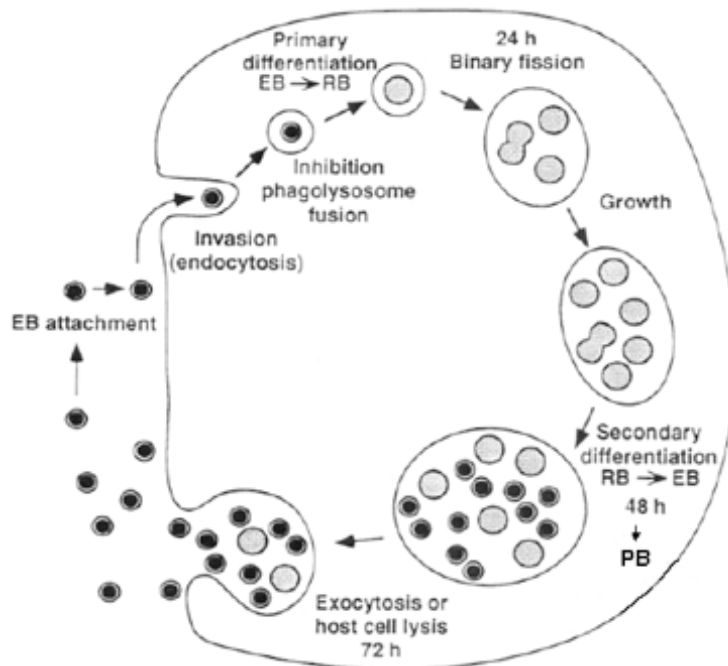
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Figure 2: The life cycle of Chlamydia organisms (EB: elementary bodies , RB: reticulate bodies and PB: persistent bodies .

latter stages they become calcified. During atherogenesis, cytokines, growth factors, lipids, nitric oxide (NO) and other small molecules induce and regulate migration and proliferation of cells as well as interfere with lipid and ECM protein synthesis. Of these, TNF-α, often found in atheromatous plaques, enhances production of platelet-derived growth factor, which promotes proliferation of SMCs. These secrete a proteoglycan matrix important for the uptake of low-density lipoproteins (LDL). TNF-α further induces increased expression of cell adhesion molecules and leukocytosis, and inhibit lipoprotein lipase which leads to (i) aggregation of lymphocytes on the endothelium, and (ii) altered lipid metabolism and accumulation of triglycerides in the blood (*Coles* et al., 1998). Matrix metalloprotease (MMP) expression in plaques is also induced by TNF-α whereas NO synthesis can be suppressed. Decreased

NO availability is common in early stages of atherosclerosis.

Homocysteine has been shown to have toxic effects on endothelial cells, promote proliferation of vascular SMCs, and enhance monocyte chemotaxis, all factors which can be involved in the pathogenesis of atherosclerosis (*Poddar* et al., 1997). Hyper-homocysteinaemia was further shown to activate NF-κB in endothelial cells as a result of oxidative stress (*Au-Yeung* et al., 2004).

Microbial agents may promote atherogenesis by evoking local inflammation in the arterial wall or by inducing endothelial injury during systemic infection (Figure 1). The human heat shock protein 60 (HSP60) can cross-react with bacterial antigens. Microbes may also promote atherogenesis indirectly by the evoked inflammatory reaction or by inducing changes in lipids, coagulation factors, homocysteine, MMPs or oxidative metabolites.

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CHLAMYDOPHILA PNEUMONIAE –THE BACTERIUM

C. pneumoniae was first isolated from the conjunctiva of a child in 1965 in Taiwan, and therefore labelled TW-183. In 1983, it was isolated from the respiratory tract and designated AR-39. During some years it was hence designated "TWAR" but DNA homology studies and ultrastructural analyses defined it as its own species, *C. pneumoniae,* in 1989, beside *C. trachomatis, C. psittaci* and *C. pecorum,* a cattle pathogen (*Grayston* et al., 1989). In 1999, based on DNA homology studies, *C. trachomatis* remained within the genus *Chlamydia* whereas *C. pneumoniae* and *C. psittaci* were transferred to the genus *Chlamydophila* (*Everett,* 1999).

The life cycle of chlamydial organisms has three distinct forms (Figure 2):
*   the infectious form, elementary bodies (EB), specialised to survive extracellularly,
*   an intracellular form, reticulate bodies (RB), which are metabolically active and capable of reproduction, and
*   persistent bodies (PB).

EBs are phagocytosed by endothelial cells and monocytes in the respiratory tract, and differentiate into RBs which localise in inclusion bodies. RBs can revert to EBs which are released by cell lysis or turn into metabolically inactive PBs which may remain dormant for many years (*Ngeh* and *Gupta,* 2000): PBs are unsusceptible to antibiotics as well as to the immune system.

CLINICAL MANIFESTATIONS

C. pneumoniae was first established as a cause of a variety of infections in the upper respiratory tract. Middle-aged adults have a 50-70% prevalence of seropositivity. Industrialised countries encounter epidemics every four to seven years. Re-infection appears to be common. The organism can also cause conjunctivitis and keratoconjunctivitis, acute myocardial infarction and endocarditis. More recently, it has been linked to chronic, inflammatory diseases, viz. atherosclerosis, Alzheimer's disease, multiple sclerosis, arthritis, myocarditis and reactive arthritis (*Gilbert* and *Grayston,* 2000; *Balin* et al., 1998; *Sriram* et al. 1998; *Gdoura* et al., 2002), and lung cancer (Koyi et al., 2001; *Anttila* et al., 2003).

VIRULENCE TRAITS IN *C. PNEUMONIAE*

Binding of heparan sulphate-like glycosaminoglycan was shown to mediate adhesion of the organisms to eukaryotic cells (*Wuppermann* et al., 2001). EBs are known to invade cells, and the role of glycosaminoglycans on the EB as well as on the host cell surface has been debated. *C. pneumoniae* as well as *C. trachomatis* were shown to use glycosaminoglycans on both EBs and host cells for invasion of bronchial epithelial but not of human umbilical vein endothelial cells (*Beswick* et al., 2003). LPS induces foam cell formation by mononuclear phagocytes. *C. pneumoniae* infected monocytes exhibited enhanced heat-resistant adhesion to endothelial cells, suggesting that it was LPS-mediated (*Kalayoglu* et al., 2001). *Chlamydophila* HSP (cHsp) 60 oxidises low-density lipoproteins. Both cHSP60 and human HSP60, which are co-localised in human atheroma, were shown to induce expression of the adhesion molecules e-se-

lectin, VCAM-1 and ICAM-1 which facilitate adhesion of leukocytes to the endothelial wall (*Kol* et al., 1999). They further induced production of IL-6 in endothelial and smooth muscle cells and macrophages similar to that induced by Gram-negative bacterial LPS, effected by transcription of NF-κB to the cell nucleus. *C. pneumoniae* and cHSP were recently shown to stimulate proliferation of human vascular SMCs via TLR4 and protein kinase activation (*Sasu* et al., 2004). Some features of the *C. pneumoniae* atherosclerosis pathogenesis are listed in Table 1. Another outer membrane protein, shown to be immunogenic and to be a pro-inflammatory activator (IL-1, IL-6 and TNF-α), is Outer Membrane 2, OMP2 (*Ciervo* et al., 2002). This protein (of about 60 kDa) is expressed late in the growth cycle and prevalent in EBs. Genus- and species-specific B- and T-cell epitopes have been identified in OMP2 (*Watson* et al., 1994). SMCs infected with *C. pneumoniae* secreted MMP-1 and MMP-3 but not gelatinases (MMP-2 and MMP-9) (*Rödel* et al., 2003). MMP may degrade ECM proteins of the fibrous cap and cause rupture of plaques. Non-immune cells (endothelial and epithelial cells) were reported to respond to *C. pneumoniae* infection by producing pro-inflammatory chemokines, cytokines and growth factors (*Stephens,* 2003). Dendritic cells are the key cells in the initiation and regulation of immune responses and are present in atherosclerotic lesions. The detection of *C. pneumoniae* in dendritic cells obtained from atherosclerotic plaques of 17/60 patients therefore links *C. pneumoniae* stronger to a subset of atherosclerotic patients (*Bobryshev* et al., 2004). Unfortunately, the study did not include diagnostics for other bacterial species.

Hyperhomocysteinaemia and elevated titres to *C. pneumoniae* IgG were correlated in patients with coronary artery disease but a role of *C. pneumoniae* in hyperhomocysteinaemia has not been found (*Stanger* et al., 2002).

In the Apo-E mouse, repeated infection with *C. pneumoniae* resulted in endothelial dysfunction, principally mediated by the NO pathway (*Liuba* et al., 2000).

LABORATORY DIAGNOSTICS

C. pneumoniae is an obligate intracellular bacterium and must be cultured within eukaryotic cells. This is the golden standard of diagnostics. There are some, but few reports on isolation of *C. pneumoniae* from atherosclerotic lesions (*Ramirez*, 1996).

Micro-immunofluorescence was first developed and has become the standard for serology (*Wang* and *Grayston,* 1986). A suspension of EBs with or without LPS are used as antigens. Microscopic reading of the test and lack of standardisation of antigen makes automatisation impossible and evaluation of results between different laboratories difficult. Later, commercial EIAs were introduced with shifting quality. Different antigens are used – some use complete LPS-containing *C. pneumoniae* antigens, some use LPS-free *C. pneumoniae*-specific antigens (*Hermann* et al., 2002). In one study, N-lauroylsarcosine extract of EBs was used, showing 2-5 times higher absorbance values than with native EB as antigen (*Quevedo Diaz* et al., 2002). Recombinant OMP2 was used as antigen in EIA for *C. trachomatis* and *C. pneumoniae* (*Portig* et al., 2003). The sensitivity of the assay was high. The antibody levels of patients infected with *C. pneumoniae* declined

faster in the EIA than with MIF. Chlamydial HSP60 was not suitable for serodiagnosis (*Peeling* et al., 1997). Western blot analyses have identified antigens of 40 and 60 kDa for *C. pneumoniae* (*Ijima* et al., 1994; *Wagels* et al., 1994). *C. pneumoniae* has only one serovar or immunotype while there are numerous serologically distinct strains among other *Chlamydia* species which should simplify establishment of serological diagnostics for this organism. The role of IgA antibodies in serodiagnosis of *C. pneumoniae* is not established but some studies have emphasised IgA antibodies as a good marker of chronic infection (*Paldanius* et al., 2003).

Immunohistochemistry has been employed to demonstrate the presence of *C. pneumoniae* in atherosclerotic tissue. In several studies, problems with high background staining, false-positive results in damaged lesions, and false-negative results because of the small area examined have been encountered (see *Boman* and *Hammerschlag*, 2002).

Nucleic acid amplification techniques caused a break-through in diagnostics of *C. pneumoniae* on tissue samples. Methodological aspects on the different steps of polymerase chain reaction (PCR), including sampling, sample preparation, choice of primers, purification of DNA and RNA to get rid of inhibitors were reviewed by *Boman* and colleagues (1999). A low concentration and patchy distribution of *C. pneumoniae* DNA was shown in carotid artery specimens. This emphasises the need to investigate multiple samples (*Cochrane* et al., 2003). Amplification of *C. pneumoniae* mRNA from atheromas is proof of viable organisms in the lesion (*Gieffers* et al., 2001). Quantitative (real-time) PCR has also been developed which may prove useful to monitor intracellular replication of *C. pneumoniae* (*Bonanomi* et al., 2003).

ANIMAL MODELS

Intranasal infection of New Zealand White rabbits resulted in pneumonia, fatty streaks and atherosclerotic lesions in aortas (*Fong* et al., 1997). When rabbits were re-infected 3 weeks later they developed intimal thickening or fibroid atherosclerotic-like plaques within 4 weeks. Immunohistochemistry was positive for *C. pneumoniae* (*Saikku* et al., 1998). The apo-lipoprotein (apoE)-deficient mouse develops atherosclerosis spontaneously and the C57BL/6J mouse does so when fed an atherosclerotic diet. Following single or repeated intranasal inoculation of *C. pneumoniae* in the apoE mouse *C. pneumoniae* was detected in internal organs and in atherosclerotic lesions after 20 weeks (*Moazed* et al, 1999). In different monkeys, intranasal inoculation of *C. pneumoniae* resulted in systemic spread and persistence but producing mild clinical symptoms (*Holland* et al, 1990).

EPIDEMIOLOGICAL STUDIES

In a case-control study of 250+250 patients and controls, elevated anti cHSP60 antibodies, but no anti-human or anti-*E. coli* homologues were independently associated with coronary artery disease (*Mahdi* et al., 2002).

Helicobacter pylori

Several studies have provided evidence for a causal relation between *H.*
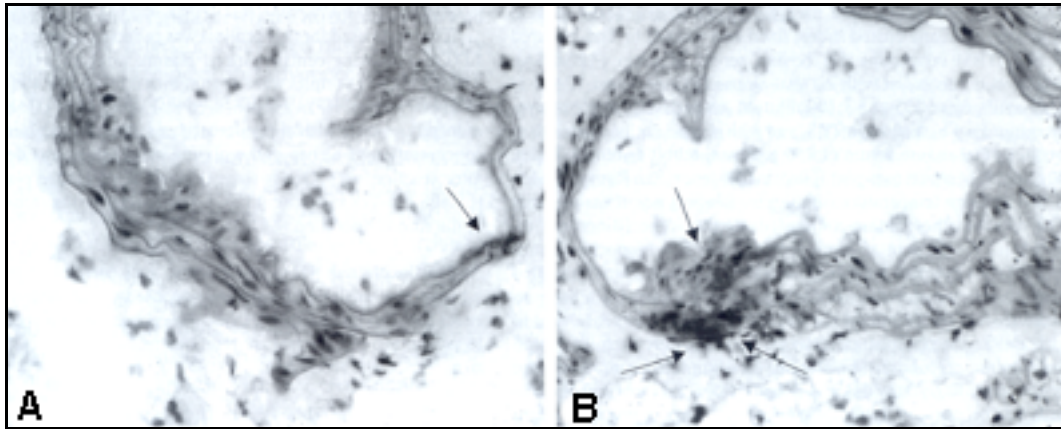
Figure 3: ApoE-knockout mice infected with *C. pneumoniae* (A) and with *C. pneumoniae* and *H. pylori* (B). VCAM-1 staining of branching site of aorta.

pylori and chronic heart disease, and interestingly, the peak in the incidence of coronary artery disease in the U.S. coincided with a peak in duodenal ulcer disease (*Blaser,* 1998). However, meta-analysis of multiple studies failed to show any relation between blood pressure, plasma fibrinogen concentration, blood lipid concentrations, C-reactive protein and other known cardiovascular risk factors and *H. pylori* (*Danesh* and *Peto,* 1998). *Blasi* and colleagues (2000) detected *C. pneumoniae* DNA but not *H. pylori* DNA in atherosclerotic plaques. *H. pylori* transcribes NF-κB, induces formation of pro-inflammatory cytokines and expression of cell adhesion molecules, and has hence the potential of inducing vascular damage (*Ernst*, 1999). Harbouring of the pathogenicity island and production of the toxin CagA was only moderately correlated to stroke, although a higher correlation was found with stroke located in larger vessels (*Cremonini* et al., 2004). The HSP60 of *H. pylori*, a highly conserved protein, was shown to mediate IL-6 production by macrophages via TLR-2 and TLR-4 (*Gobert* et al., 2004). Infection with *H. pylori* was further as-

sociated with an atherogenic lipid profile (*Hoffmeister* et al., 2001).

Patients with long-lasting infection and atrophic gastritis were shown to have elevated levels of homocystein, probably as a result of vitamin B12 malabsorption (*Santarelli* et al., 2004).

Synergy

Combined seropositivity for *C. pneumoniae* and *H. pylori* was associated with obesity, low socio-economic factors and age (*Ekesbo* et al., 2000). In the ApoE- mouse model, co-infection with *C. pneumoniae* and *H. pylori* synergistically increased expression of VCAM-1 and leukocyte adhesion (*Liuba* et al., 2003; Figure 3). A group of unselected patients with atrial fibrillation had antibodies to *C. pneumoniae* as well as to *H. pylori* (*Olsson* et al., 2002)

Cytomegalovirus

Cytomegalovirus (CMV) belongs to the herpes group of virusses and indeed other members of this group, like *Herpes simplex* 1, have also been implicated in atherosclerosis development. Between 50 and 80% of adults have antibodies to CMV. Infection is usually acquired in

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childhood. Severe infections occur in heart transplantation recipients, and there is strong evidence for a role of CMV in vasculopathy (*Grotton* et al., 1989). CMV appears to directly infect endothelial cells, and can remain latent. Deleterious effects can be mediated by induction of chemokine and other inflammatory compounds (*Streblow* et al., 1999; *Hengel* and *Weber*, 2000). This results in migration of smooth muscle cells, such as monocyte chemotactic protein –1. Because CMV is restricted to the human host related viruses have been used in mouse and rat models of CMV infection. In these models, increased adherence of leukocytes to the aortic intima and accumulation of lipids in the endothelium were found (*Span* et al., 1992).

Periodontal pathogens

Periodontitis is an inflammatory reaction of the tissue surrounding the tooth. It produces few symptoms, progresses slowly and shares a number of features with chronic vascular disease. Some of the recognised species are listed in Table 2. In periodontitis, the lipopolysaccharide $\rightarrow$ Mϕ seems crucial, and peripheral monocyte (Mϕ) individuals secrete 3-10-fold of cytokine mediators as a response to LPS than normal persons (*Beck* et al., 1999).

CONCLUDING REMARKS

There are substantial reports on the association with chronic infection and development of atherosclerosis. It is likely that chronic infections caused by different microbial agents can induce similar vascular pathology, and that the infectious burden, as revealed by a high CFP is related to peripheral artery disease but not a normal or low CFP value (*Nloemenkamp* et al., 2002.). Hence linkage to one certain agent is hampered. During the last decade efforts have been made to standardise diagnostic methods, viz. serology and molecular microbiology methods. This will help to elucidate the issue of microbial infections as a cause of one of the greatest causes of morbidity and mortality. A clear link between infection and atherosclerosis will direct preventive therapies towards microbial disease and effects thereof. However, *C. pneumoniae* located in lymphocytes as well as in monocytes are refractory to antibiotic treatment (*Yamaguchi* et al., *Gieffers* et al., 2001).

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