Any role for *Chlamydia pneumoniae* in ischaemic stroke?

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Any role for *Chlamydia pneumoniae*
in ischaemic stroke?

Manuela Voorend

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Any role for *Chlamydia pneumoniae* in ischaemic stroke?

PROEFSCHRIFT

ter verkrijging van de graad van doctor
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volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
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Voor mijn ouders

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Chapter 1

General introduction

General introduction

Despite extensive research, ischaemic stroke remains one of the main causes of death and a significant contributor to disability in the developed countries, especially in the elderly. The underlying pathogenetic condition in the majority of ischaemic strokes is atherosclerosis. Since the early 90's, atherosclerosis is defined as an inflammatory, immune-mediated disorder, which develops following endothelial injury¹. Endothelial injury may have a number of causes such as elevated and modified low-density lipoprotein (LDL), activity of free radicals, hypertension, and elevated homocysteine. One of the factors implicated in the pathogenesis of atherosclerosis is infection. The micro-organism *Chlamydia pneumoniae* (*C pneumoniae*) has received the most interest in this respect. Extensive research has been done in the area of infections and cardiovascular disease; cerebrovascular disease has received less attention in this respect. In both areas results and opinions about the role of *C pneumoniae* in vascular events have been controversial. In this chapter we will summarize the present data on *C pneumoniae* infection and atherosclerosis, with emphasis on cerebrovascular disease.

Chlamydia pneumoniae

C pneumoniae is an obligate intracellular respiratory micro-organism. *C pneumoniae* infection accounts for approximately 10% of the community acquired pneumonias². Most infections, however, are asymptomatic or cause mild flu-like symptoms. *C pneumoniae* infection causes an acute phase response with the production of cytokines and acute phase proteins. Interleukin-6 (IL-6) is a central mediator of the acute phase response and one of the most important initiators of hepatic production of C-reactive protein (CRP). Serum immunoglobulin M (IgM), IgG and IgA are also produced in reaction to *C pneumoniae* infection. Seroprevalence of these antibodies is common and rises with age². By the age of 20 approximately 50% have detectable antibodies and the prevalence rises to approximately 75% in the elderly. The first infection only causes a time limited immune response, therefore the high prevalence rates in the elderly are explained by reinfection³.

C pneumoniae and atherosclerosis

Cardiovascular disease and peripheral arterial occlusive disease

The first association between *C pneumoniae* infection and cardiovascular disease was established in 1988 when Saikku et al. found IgG and IgA antibodies against *C pneumoniae* more often in males under fifty with acute myocardial infarction or chronic ischaemic heart disease than in healthy controls⁴. Numerous studies followed this initial report producing conflicting evidence. In general it can be said that for every study supporting the initial hypothesis given by Saikku et al., there is one reporting no apparent association between *C pneumoniae* infection and cardiovascular disease. Two meta-analyses were performed on all the available studies, which could not confirm a relationship between the presence of *C pneumoniae* antibodies and cardiovascular disease^{5,6}.

Later studies looked at the association with renal artery stenosis⁷, and stenosis of the arteries of the lower extremities^{8,9}. The conflicting results are partly due to differences in study methods. There were cross sectional case control studies and prospective cohort studies. Furthermore, different criteria were used to establish *C pneumoniae* infection and several authors used evidence of previous and chronic persistent infection interchangeably. Serum antibodies can be detected using a microimmunofluorescence (MIF) test or by using an enzyme linked immunosorbent assay (ELISA). The MIF is the first test and very specific and sensitive, but it is labour intensive and its interpretation is limited to experienced laboratory technicians. The ELISA is an easier test that gives objective (computer generated) results. Regarding the MIF, criteria for past and current *C pneumoniae* infection have been formally deployed¹⁰, but these criteria are often not used in the above mentioned studies. In contrast to this, no criteria are known for *C pneumoniae* infection when the ELISA is used, and the manufacturer of the tests gives cut-off points for seropositivity. In a comparative study between ELISA and MIF, the antibody titres were found to be comparable between the two tests¹¹.

Differences in test methods also account for different results in pathological studies. There are different methods for *C pneumoniae* detection, among them immunohistochemistry, that is used to detect *C pneumoniae* antigens in paraffin sections of formalin fixed tissues, or frozen tissue samples, and Polymerase chain reaction (PCR), a technique used to assess the presence of Chlamydial DNA and can be used to detect chlamydial DNA in tissues. The advantage of immunohistochemistry is that it allows localization of the micro-organism in specific cells tissues. However, the use of different monoclonal antibodies against *C pneumoniae* with different specificity, has yielded widely varying results, and interpretation of the results is subjective¹². PCR is a more

objective technique to detect very small amounts of Chlamydial DNA, but as well as with immunohistochemistry differences in methodology leads to varying results between laboratories¹². The presence of *C pneumoniae* within the atherosclerotic plaque is patchy¹³, so sampling methods strongly influence the risk of false negative results. *C pneumoniae* within the atherosclerotic plaque of coronary arteries was first determined by electron microscopy¹⁴. In later studies *C pneumoniae* was detected within the atherosclerotic plaque of coronary arteries¹⁵⁻¹⁸, the aorta^{19,20} and peripheral arteries^{21,22}. Parallel examination of atherosclerotic and non-atherosclerotic tissue showed that prevalence of *C pneumoniae* within atherosclerotic tissue is higher than in non-atherosclerotic tissue^{23,24}. Prevalence rates of *C pneumoniae* in the atherosclerotic plaque of coronary arteries differed from 0% to 79%^{14-18,25-30}, a variation which is at least partly due to the above mentioned differences in test method.

As mentioned above, *C pneumoniae* infection causes an acute phase response with the production of cytokines and acute phase proteins. IL-6 and CRP levels are higher in patients with atherosclerosis and, both IL-6 levels and CRP levels increase future risk of cardiovascular events in apparently healthy men and women³¹⁻³³.

C pneumoniae infection and atherosclerosis of the cerebral vessels

Ischaemic stroke is defined as an acute focal neurological deficit caused by the occlusion of an intracerebral artery. Occlusion can be due to embolism from either the heart (cardial embolism) or from atherosclerotic plaques in the internal carotid artery or the larger cerebral arteries. Since infection with *C pneumoniae* has been related to cardiovascular disease, it might also play a role in cerebrovascular disease. However, as with cardiovascular disease, the results of the different studies are conflicting.

Ischaemic stroke risk is related to atherosclerosis in the internal carotid artery. Therefore, *C pneumoniae* could contribute to stroke risk by influencing carotid atherosclerosis. Several studies have shown an association between past *C pneumoniae* infection and thickness of the intima media complex (IMT) of the common carotid artery^{34,35}. Carotid IMT is a sign of early carotid atherosclerosis. IMT increases with age, and is related to stroke risk and risk of cardiovascular events³⁶. The presence of *C pneumoniae* antibodies is related to a more rapid increase in IMT³⁷. In contrast to this, other studies have reported no apparent relationship between past *C pneumoniae* infection and early or severe carotid atherosclerosis^{38,39}. The association between *C pneumoniae* antibodies and ischaemic stroke was studied in ten reports⁴⁰⁻⁴⁹. The proportion of studies implying a role of *C pneumoniae* in cerebrovascular disease are similar to that in cardiovascular disease.

Pathological studies show a similar situation, with prevalence rates for *C pneumoniae* DNA in the carotid atherosclerotic plaque varying from 0-83%⁵⁰⁻⁵⁷. Pathological examination of the cerebral vessels can only be performed post-mortem and therefore, the number of studies is limited. Four studies found *C pneumoniae* in the atherosclerotic plaques of the middle cerebral artery, basilar artery and anterior cerebral artery⁵⁸⁻⁶¹. No differences were found between detection of *C pneumoniae* by IHC or PCR⁶¹.

CRP levels and IL-6 levels predict future stroke risk in apparently healthy men and women. Levels of the inflammatory markers high sensitivity CRP (hsCRP) and IL-6 are also related to the IMT of the carotid^{34,62-64} artery. Furthermore, CRP and IL-6 levels are associated with the presence of asymptomatic ischaemic cerebral lesions⁶⁵. The presence of asymptomatic ischaemic cerebral lesions is of importance, since it increases the risk of cognitive decline and dementia⁶⁶, as well as the risk of future stroke⁶⁷.

Mechanisms by which *C pneumoniae* can induce or accelerate atherosclerosis

In vitro, *C pneumoniae* is able to infect endothelial cells, smooth muscle cells and macrophages, and replicate and survive in these cells⁶⁸⁻⁷¹. Reaction to this infection is different for each of these cells. Endothelial infection leads to cell activation in a similar fashion as is seen in the initiation of atherosclerosis⁷². Smooth muscle cells proliferate under the influence of soluble products produced by endothelial cells infected with *C pneumoniae*⁶⁸. Macrophages turn into foam cells⁷³ when infected with *C pneumoniae*. Infection in all of these cells can be persistent, thereby inducing a continuous state of activation in these cells. Infected endothelial cells and smooth muscle cells can produce IL-6, TNF α and IL-1 β ⁷⁴ (see also Figure 1.1). These pro-inflammatory cytokines also play an important role in the process of atherosclerosis, and production could induce a chronic persistent inflammatory state within the vascular wall, thereby enhancing the atherosclerotic process.

Detection of the micro-organism in the atherosclerotic plaque cannot answer the question whether *C pneumoniae* directly influences the atherosclerotic process or is just an innocent bystander. Therefore, animal models were developed to further elucidate the role of *C pneumoniae* infection in atherosclerosis and to determine the route by which *C pneumoniae* infects the vascular wall. Intranasal infection of New Zealand white rabbits showed that *C pneumoniae* infects alveolar macrophages, which subsequently transmigrate through the mucosal barrier. Infected peripheral blood monocytes then act as the vector system within the bloodstream and transmit the infection to the vascular wall⁷⁵. An often-used animal model used for the study of atherosclerosis is the Apolipoprotein E (ApoE) deficient mouse. ApoE deficient

mice spontaneously develop atherosclerosis from which the morphology and location shows similarity to the human situation⁷⁶. Infection with *C pneumoniae* accelerates the process of atherosclerosis in these mice⁷⁶. *C pneumoniae* infection increases T-lymphocyte influx into the vascular wall and induces a shift in type of atherosclerotic plaques to the more severe ones⁷⁷. Furthermore, in the later stages of atherosclerosis, *C pneumoniae* infection causes more unstable atherosclerotic plaques, thereby increasing the risk of a thrombo-embolic complication⁷⁸.

Figure 1.1 Mechanisms by which *C pneumoniae* can induce or accelerate atherosclerosis.

ec = endothelial cell; smc = smooth muscle cell; mφ = macrophage

Treatment options

A rabbit model of atherosclerosis showed that azithromycin, given before *C pneumoniae* infection, reduces atherosclerosis⁷⁹. Secondary prevention studies have investigated the effect of antibiotic treatment on future cardiovascular events. Azithromycin reduced the risk of cardiovascular events 5-fold in a pilot-study of 213 patients⁸⁰, larger studies however, did not show a significant reduction in clinical endpoints, although inflammatory parameters were reduced up to six months after treatment^{81,82}. One study investigated the effect of the antibiotic gatifloxacin on reduction of cardiovascular events and found no effect⁸³. In two studies, the effect of roxithromycin on the risk of

cardiovascular events was studied. One study found a significant reduction in cardiovascular events^{84,85}, whereas the other study did not. A 28 day course of roxithromycin did reduce the progression of peripheral arterial occlusive disease in *C pneumoniae* seropositive patients⁸⁶. The effects of antibiotics were also tested in trials using a surrogate endpoint i.e. a non-clinical endpoint. These endpoints however, reflect processes closely related to the atherosclerotic process, and are therefore useful. Markers of endothelial dysfunction were used as a surrogate endpoint in three studies, one study found a significant reduction in E-selectin and in von Willebrand factor⁸⁷, two other studies could not find an effect of antibiotics on endothelial function^{88,89}. In contrast to the cardiovascular trials, only two reports have been published on antibiotic treatment and stroke prevention. Both studied the effect of antibiotics on carotid atherosclerosis. Melissano et al. studied the presence of *C pneumoniae* DNA in atherosclerotic tissue of the carotid arteries after carotid endarterectomy. *C pneumoniae* DNA was found significantly less frequent in patients who were treated with roxithromycin before surgery. Roxithromycin slows the progression of IMT of the common carotid artery, but does so only temporarily^{90,91}. Until now this is the only clinical trial on secondary prevention of cerebrovascular disease, and no intervention trials have been performed investigating the effect of antibiotic treatment on future cerebrovascular events.

Rationale and outline of the thesis

Studies on the association between *C pneumoniae* infection and cerebrovascular disease have been scarce in comparison to cardiovascular disease. The study results do not point in the same direction, and the association between *C pneumoniae* infection and atherosclerosis remains controversial.

Cerebrovascular disease differs from cardiovascular disease in that it can be divided in two entities: large vessel disease and small vessel disease. Large vessel disease, which is due to atherosclerosis of the larger cerebral vessels, causes mainly larger, usually called territorial infarcts, which often involve the cortex. The risk factors for large vessel disease are rather similar to those of cardiovascular disease in general. Small vessel disease causes small infarcts, less than 15 mm in diameter on cerebral imaging, which are located in the deep brain structures (basal ganglia and internal capsule) or the pons; these are often referred to as lacunar infarcts. Small vessel disease is also the cause of more diffuse damage to the white matter of the brain: leukoaraiosis. The clinical manifestation of either large vessel disease or small vessel disease is consistent over time⁹². *C pneumoniae* could have different influences on the different types of cerebral vessel disease, and identifying a subgroup that is related to *C pneumoniae* infection could have consequences for secondary prevention.

This thesis aims to answer questions on the association between *C pneumoniae* infection and cerebral ischaemia and does so by means of an animal experiment, neuropathological examinations of post mortem brain tissue and four clinical studies on the association between *C pneumoniae* antibody titres, inflammatory parameters, IMT, ischaemic cerebral lesions, and clinical stroke. The aim of chapter 2 was to explore the association between infection with *C pneumoniae*, detected by measuring the presence of *C pneumoniae* antibodies, and ischaemic stroke at young age. In chapter 3 a mouse experiment is described, that aims to explore whether systemic *C pneumoniae* infection can cause cerebral infection, and whether cerebral infection causes brain damage. The effects of atherosclerosis on cerebral infection and cerebral damage was tested by comparing mice with atherosclerosis, i.e. Apolipoprotein E (ApoE) knockout (KO) and ApoE/Low Density Lipoprotein receptor (ApoE/LDLr) KO mouse, both on a C57BL/6J background, to mice without atherosclerosis, i.e. wild type C57BL/6J mice. The effect of age on cerebral infection and cerebral damage was tested by comparing three months old C57BL/6J mice to 8 months old C57BL/6J mice. The aim of chapter 4 was to assess the prevalence of the presence of *C pneumoniae* DNA in cerebral large vessels, small vessels, and brain tissue affected by small vessel disease, in patients who died of various causes. The chapters 5, 6 and 7 are clinical

studies, using a patient population derived from the ACSI study (Azithromycin for the prevention of the progression of Cerebral ischaemic lesions in patients Surviving a non-cardioembolic cerebral Infarct), which investigates the effect of a three-month treatment schedule of azithromycin on ischaemic cerebral damage progression measured at two years. Baseline inflammatory parameters and *C pneumoniae* serology is related to clinical stroke syndrome (large vessel or small vessel disease), the presence of silent brain ischaemia (chapter 5) and the IMT of the common carotid artery (chapter 6). Furthermore IMT will be related to the presence of ipsilateral silent brain ischaemia (chapter 6). The aim of chapter 7 is to study the effect of a three-month course of azithromycin on levels of inflammatory parameters. In chapter 8 a summary of the findings are presented along with implications and possible future plans.

References

1. Ross R. The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature*. 1993;362:801-9.
2. Grayston JT. Infections caused by *Chlamydia pneumoniae* strain twar [see comments]. *Clin Infect Dis*. 1992;15:757-61.
3. Patnode D, Wang S-P, Grayston JT. Persistence of *Chlamydia pneumoniae*, strain twar, micro-immunofluorescent antibody. In: *W R Bodie, H D Caldwell, R P Jones, P-A Mardh, G L Ridgeway, J Schachter, W E Stamm, and M E Ward (ed), Chlamydial infections-1990 Cambridge University Press, Cambridge*. 1990
4. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK, Valtanen V. Serological evidence of an association of a novel *Chlamydia*, twar, with chronic coronary heart disease and acute myocardial infarction. *Lancet*. 1988;2:983-6.
5. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Wong YK, Bernardes Silva M, Ward M. *Chlamydia pneumoniae* igg titres and coronary heart disease: Prospective study and meta-analysis. *British Medical Journal*. 2000;321:208-12.
6. Bloemenkamp DG, Mali WP, Visseren FL, van der Graaf Y. Meta-analysis of sero-epidemiologic studies of the relation between *Chlamydia pneumoniae* and atherosclerosis: Does study design influence results? *Am Heart J*. 2003;145:409-17.
7. van der Ven AJAM, Hommels MJ, Kroon AA, Kessels A, Flobbe K, van Engelshoven J, Bruggeman CA, de Leeuw PW. *Chlamydia pneumoniae* seropositivity and systemic and renovascular atherosclerotic disease. *Arch Intern Med*. 2002;162:786-90.
8. Lindholt JS, Vammen S, Lind I, Fasting H, Henneberg EW. The progression of lower limb atherosclerosis is associated with iga antibodies against *Chlamydia pneumoniae*. *Eur J Vasc Endovasc Surg*. 1999;18:527-9.
9. Krayenbuehl PA, Wiesli P, Maly FE, Vetter W, Schulthess G. Progression of peripheral arterial occlusive disease is associated with *Chlamydia pneumoniae* seropositivity and can be inhibited by antibiotic treatment. *Atherosclerosis*. 2005;179:103-10.
10. Dowell SF, Peeling RW, Boman J, Carlone GM, Fields BS, Guarner J, Hammerschlag MR, Jackson LA, Kuo CC, Maass M, Messmer TO, Talkington DF, Tondella ML, Zaki SR. Standardizing *Chlamydia pneumoniae* assays: Recommendations from the centers for disease control and prevention (USA) and the laboratory centre for disease control (Canada). *Clin Infect Dis*. 2001;33:492-503.
11. Vainas T, De Graaf R, Stassen FR, Kurvers HA, Grauls GE, Kitslaar PJ, Bruggeman CA. *Chlamydia pneumoniae* serology: Comparing a commercial enzyme immunoassay and microimmunofluorescence test in patients with cardiovascular disease. *Apmis*. 2003;111: 363-9.
12. Boman J, Hammerschlag MR. *Chlamydia pneumoniae* and atherosclerosis: Critical assessment of diagnostic methods and relevance to treatment studies. *Clin Microbiol Rev*. 2002;15:1-20.
13. Cochrane M, Pospischil A, Walker P, Gibbs H, Timms P. Distribution of *Chlamydia pneumoniae* DNA in atherosclerotic carotid arteries: Significance for sampling procedures. *J Clin Microbiol*. 2003;41:1454-7.
14. Shor A, Kuo CC, Patton DL. Detection of *Chlamydia pneumoniae* in coronary arterial fatty streaks and atheromatous plaques. *S Afr Med J*. 1992;82:158-61.
15. Kuo CC, Grayston JT, Campbell LA, Goo YA, Wissler RW, Benditt EP. *Chlamydia pneumoniae* (twar) in coronary arteries of young adults (15-34 years old). *Proc Natl Acad Sci U S A*. 1995;92:6911-4.
16. Maass M, Bartels C, Engel PM, Mamat U, Sievers HH. Endovascular presence of viable *Chlamydia pneumoniae* is a common phenomenon in coronary artery disease. *J Am Coll Cardiol*. 1998;31:827-32.
17. Campbell LA, O'Brien ER, Cappuccino AL, Kuo CC, Wang SP, Stewart D, Patton DL, Cummings PK, Grayston JT. Detection of *Chlamydia pneumoniae* twar in human coronary atherectomy tissues [see comments]. *J Infect Dis*. 1995;172:585-8.

18. Kuo CC, Shor A, Campbell LA, Fukushi H, Patton DL, Grayston JT. Demonstration of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries. *J Infect Dis.* 1993;167:841-9.
19. Kuo CC, Gown AM, Benditt EP, Grayston JT. Detection of *Chlamydia pneumoniae* in aortic lesions of atherosclerosis by immunocytochemical stain. *Arterioscler Thromb.* 1993;13:1501-4.
20. Juvonen J, Juvonen T, Laurila A, Alakarppa H, Lounatmaa K, Surcel HM, Leinonen M, Kairaluoma MI, Saikku P. Demonstration of *Chlamydia pneumoniae* in the walls of abdominal aortic aneurysms. *J Vasc Surg.* 1997;25:499-505.
21. Gutierrez J, Linares-Palomino J, Lopez-Espada C, Rodriguez M, Ros E, Piedrola G, del Maroto MC. *Chlamydia pneumoniae* DNA in the arterial wall of patients with peripheral vascular disease. *Infection.* 2001;29:196-200.
22. Kuo CC, Coulson AS, Campbell LA, Cappuccio AL, Lawrence RD, Wang SP, Grayston JT. Detection of *Chlamydia pneumoniae* in atherosclerotic plaques in the walls of arteries of lower extremities from patients undergoing bypass operation for arterial obstruction. *J Vasc Surg.* 1997;26:29-31.
23. Ouchi K, Fujii B, Kudo S, Shirai M, Yamashita K, Gondo T, Ishihara T, Ito H, Nakazawa T. *Chlamydia pneumoniae* in atherosclerotic and nonatherosclerotic tissue. *J Infect Dis.* 2000;181 Suppl 3:S441-3.
24. Kuo CC, Campbell LA. Detection of *Chlamydia pneumoniae* in arterial tissues. *J Infect Dis.* 2000;181 Suppl 3:S432-6.
25. Maass M, Gieffers J, Krause E, Engel PM, Bartels C, Solbach W. Poor correlation between microimmunofluorescence serology and polymerase chain reaction for detection of vascular *Chlamydia pneumoniae* infection in coronary artery disease patients. *Med Microbiol Immunol Berl.* 1998;187:103-6.
26. Thomas M, Wong Y, Thomas D, Ajaz M, Tsang V, Gallagher PJ, Ward ME. Relation between direct detection of *Chlamydia pneumoniae* DNA in human coronary arteries at postmortem examination and histological severity (stary grading) of associated atherosclerotic plaque. *Circulation.* 1999;99:2733-6.
27. Zamorano J, Garcia-Tejada J, Suarez A, Culebras E, Castanon J, Moreno R, Reguillo F, Gil M, Picazo J, Sanchez-Harguindey L. *Chlamydia pneumoniae* in the atherosclerotic plaques of patients with unstable angina undergoing coronary artery bypass grafting: Does it have prognostic implications? *Int J Cardiol.* 2003;90:297-302.
28. Ericson K, Saldeen TG, Lindquist O, Pahlson C, Mehta JL. Relationship of *Chlamydia pneumoniae* infection to severity of human coronary atherosclerosis. *Circulation.* 2000;101:2568-71.
29. Bartels C, Maass M, Bein G, Brill N, Bechtel JF, Leyh R, Sievers HH. Association of serology with the endovascular presence of *Chlamydia pneumoniae* and cytomegalovirus in coronary artery and vein graft disease. *Circulation.* 2000;101:137-41.
30. Weiss SM, Roblin PM, Gaydos CA, Cummings P, Patton DL, Schulhoff N, Shani J, Frankel R, Penney K, Quinn TC, Hammerschlag MR, Schachter J. Failure to detect *Chlamydia pneumoniae* in coronary atheromas of patients undergoing atherectomy. *J Infect Dis.* 1996;173:957-62.
31. Ridker PM, Buring JE, Shih J, Matias M, Hennekens CH. Prospective study of c-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation.* 1998;98:731-3.
32. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med.* 2000;342:836-43.
33. Ridker PM, Rifai N, Stampfer MJ, Hennekens CH. Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation.* 2000;101:1767-72.
34. Hashimoto H, Kitagawa K, Hougaku H, Etani H, Hori M. Relationship between c-reactive protein and progression of early carotid atherosclerosis in hypertensive subjects. *Stroke.* 2004;35:1625-30.

35. Melnick SL, Shahar E, Folsom AR, Grayston JT, Sorlie PD, Wang SP, Szklo M. Past infection by *Chlamydia pneumoniae* strain twar and asymptomatic carotid atherosclerosis. Atherosclerosis risk in communities (aric) study investigators. *Am J Med.* 1999;95:499-504.
36. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK, Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular health study collaborative research group. *N Engl J Med.* 1999;340:14-22.
37. Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Enhanced progression of early carotid atherosclerosis is related to *Chlamydia pneumoniae* (taiwan acute respiratory) seropositivity. *Circulation.* 2001;103:1390-5.
38. Coles KA, Plant AJ, Riley TV, Smith DW, McQuillan BM, Thompson PL. Lack of association between seropositivity to *Chlamydia pneumoniae* and carotid atherosclerosis. *Am J Cardiol.* 1999;84:825-8.
39. Hirashima Y, Kuwayama N, Kubo M, Origasa H, Endo S. *Chlamydia pneumoniae* infection is not involved in carotid artery stenosis. *Atherosclerosis.* 2002;163:165-8.
40. Johnsen SP, Overvad K, Ostergaard L, Tjonneland A, Husted SE, Sorensen HT. *Chlamydia pneumoniae* seropositivity and risk of ischemic stroke: A nested case-control study. *Eur J Epidemiol.* 2005;20:59-65.
41. Ngeh J, Gupta S, Goodbourn C, Panayiotou B, McElligott G. *Chlamydia pneumoniae* in elderly patients with stroke (c-peps): A case-control study on the seroprevalence of *Chlamydia pneumoniae* in elderly patients with acute cerebrovascular disease. *Cerebrovasc Dis.* 2003;15:11-6.
42. Madre JG, Garcia JL, Gonzalez RC, Montero JM, Paniagua EB, Escribano JR, Martinez JD, Cenjor RF. Association between seropositivity to *Chlamydia pneumoniae* and acute ischaemic stroke. *Eur J Neurol.* 2002;9:303-6.
43. Heuschmann PU, Neureiter D, Gesslein M, Craiovan B, Maass M, Faller G, Beck G, Neundoerfer B, Kolominsky-Rabas PL. Association between infection with *helicobacter pylori* and *Chlamydia pneumoniae* and risk of ischemic stroke subtypes: Results from a population-based case-control study. *Stroke.* 2001;32:2253-8.
44. Cook PJ, Honeybourne D, Lip GYH, Beevers DG, Wise R, Davies AH. *Chlamydia pneumoniae* antibody titers are significantly associated with acute stroke and transient cerebral ischemia: The west birmingham stroke project. *Stroke.* 1998;29:404-10.
45. Elkind MS, Lin IF, Grayston JT, Sacco RL. *Chlamydia pneumoniae* and the risk of first ischemic stroke : The northern manhattan stroke study. *Stroke.* 2000;31:1521-5.
46. Glader CA, Stegmayr B, Boman J, Stenlund H, Weinehall L, Hallmans G, Dahlen GH. *Chlamydia pneumoniae* antibodies and high lipoprotein(a) levels do not predict ischemic cerebral infarctions. Results from a nested case-control study in northern sweden. *Stroke.* 1999;30:2013-8.
47. Fagerberg B, Gnarpe J, Gnarpe H, Agewall S, Wikstrand J. *Chlamydia pneumoniae* but not cytomegalovirus antibodies are associated with future risk of stroke and cardiovascular disease: A prospective study in middle-aged to elderly men with treated hypertension. *Stroke.* 1999;30:299-305.
48. Wimmer ML, Sandmann Strupp R, Saikku P, Haberl RL. Association of *Chlamydial* infection with cerebrovascular disease. *Stroke.* 1996;27:2207-10.
49. Voorend M, Faber C, van der Ven A, Kessels F, Bruggeman CA, Lodder J. *Chlamydia pneumoniae* is a likely risk factor for ischemic stroke in young patients. *Journal of stroke and cerebrovascular diseases.* 2004;13:85-91.
50. Maass M, Krause E, Engel PM, Kruger S. Endovascular presence of *Chlamydia pneumoniae* in patients with hemodynamically effective carotid artery stenosis. *Angiology.* 1997;48:699-706.
51. Cochrane M, Pospischil A, Walker P, Gibbs H, Timms P. Discordant detection of *Chlamydia pneumoniae* in patients with carotid artery disease using polymerase chain reaction, immunofluorescence microscopy and serological methods. *Pathology.* 2005;37:69-75.

52. Sessa R, Di Pietro M, Schiavoni G, Santino I, Benedetti-Valentini F, Perna R, Romano S, del Piano M. Chlamydia pneumoniae DNA in patients with symptomatic carotid atherosclerotic disease. *J Vasc Surg.* 2003;37:1027-31.
53. Ong G, Coyle P, AB. A, WG. M, WP. D, O'Neill H, Wyatt D, KB. B, O'Loughlin B, McCaughey C. Non-detection of Chlamydia species in carotid atheroma using generic primers by nested pcr in a population with a high prevalence of Chlamydia pneumoniae antibody. *BMC Infectious Diseases.* 2001;1:12.
54. Prager M, Turel Z, Speidl WS, Zorn G, Kaun C, Niessner A, Heinze G, Huk I, Maurer G, Huber K, Wojta J. Chlamydia pneumoniae in carotid artery atherosclerosis: A comparison of its presence in atherosclerotic plaque, healthy vessels, and circulating leukocytes from the same individuals. *Stroke.* 2002;33:2756-61.
55. LaBiche R, Kozlowski D, Quinn TC, Gaydos C, Azhar S, Ketron G, Sood S, DeGraba TJ. Presence of Chlamydia pneumoniae in human symptomatic and asymptomatic carotid atherosclerotic plaque. *Stroke.* 2001;32:855-60.
56. Yamashita K, Ouchi K, Shirai M, Gondo T, Nakazawa T, Ito H. Distribution of Chlamydia pneumoniae infection in the atherosclerotic carotid artery. *Stroke.* 1998;29:773-8.
57. Chiu B, Viira E, Tucker W, Fong IW. Chlamydia pneumoniae, cytomegalovirus, and herpes simplex virus in atherosclerosis of the carotid artery. *Circulation.* 1997;96:2144-8.
58. Virok D, Kis Z, Karai L, Intzedy L, Burian K, Szabo A, Ivanyi B, Gonczol E. Chlamydia pneumoniae in atherosclerotic middle cerebral artery. *Stroke.* 2001;32:1973-6.
59. Vink A, Poppen M, Schoneveld AH, Roholl PJ, de Kleijn DP, Borst C, Pasterkamp G. Distribution of Chlamydia pneumoniae in the human arterial system and its relation to the local amount of atherosclerosis within the individual. *Circulation.* 2001;103:1613-7.
60. Rassa M, Cazzavillan S, Scagnelli M, Peron A, Bevilacqua PA, Facco M, Bertoloni G, Lauro FM, Zambello R, Bonoldi E. Demonstration of Chlamydia pneumoniae in atherosclerotic arteries from various vascular regions. *Atherosclerosis.* 2001;158:73-9.
61. Wohlschlaeger J, Wimmer ML, Nagler DK, Haberl R, Weis S. Identification of Chlamydia pneumoniae in intracranial and extracranial arteries in patients with stroke and in controls: Combined immunohistochemical and polymerase chain reaction analyses. *Hum Pathol.* 2005;36:395-402.
62. Hashimoto H, Kitagawa K, Hougaku H, Shimizu Y, Sakaguchi M, Nagai Y, Iyama S, Yamanishi H, Matsumoto M, Hori M. C-reactive protein is an independent predictor of the rate of increase in early carotid atherosclerosis. *Circulation.* 2001;104:63-7.
63. Sitzer M, Markus HS, Mendall MA, Liehr R, Knorr U, Steinmetz H. C-reactive protein and carotid intimal medial thickness in a community population. *J Cardiovasc Risk.* 2002;9:97-103.
64. Wang TJ, Nam BH, Wilson PW, Wolf PA, Levy D, Polak JF, D'Agostino RB, O'Donnell CJ. Association of c-reactive protein with carotid atherosclerosis in men and women: The framingham heart study. *Arterioscler Thromb Vasc Biol.* 2002;22:1662-7.
65. Hoshi T, Kitagawa K, Yamagami H, Furukado S, Hougaku H, Hori M. Relations of serum high-sensitivity c-reactive protein and interleukin-6 levels with silent brain infarction. *Stroke.* 2005;36:768-72.
66. Vermeer SE, Prins ND, den Heijer T, Hofman A, Koudstaal PJ, Breteler MM. Silent brain infarcts and the risk of dementia and cognitive decline. *N Engl J Med.* 2003;348:1215-22.
67. de Jong G, van Raak L, Kessels F, Lodder J. Stroke subtype and mortality. A follow-up study in 998 patients with a first cerebral infarct. *J Clin Epidemiol.* 2003;56:262-8.
68. Coombes BK, Mahony JB. Chlamydia pneumoniae infection of human endothelial cells induces proliferation of smooth muscle cells via an endothelial cell-derived soluble factor(s). *Infect Immun.* 1999;67:2909-15.
69. Fryer RH, Schwobe EP, Woods ML, Rodgers GM. Chlamydia species infect human vascular endothelial cells and induce procoagulant activity. *J Invest Med.* 1997;45:168-74.
70. Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA, Quinn TC. Replication of Chlamydia pneumoniae in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun.* 1996;64:1614-20.

71. Kaukoranta Tolvanen SS, Laitinen K, Saikku P, Leinonen M. Chlamydia pneumoniae multiplies in human endothelial cells in vitro. *Microb Pathog.* 1994;16:313-9.
72. Kaukoranta Tolvanen SS, Ronni T, Leinonen M, Saikku P, Laitinen K. Expression of adhesion molecules on endothelial cells stimulated by Chlamydia pneumoniae. *Microb Pathog.* 1996;21:407-11.
73. Kalayoglu MV, Byrne GI. Induction of macrophage foam cell formation by Chlamydia pneumoniae. *J Infect Dis.* 1998;177:725-9.
74. Rodel J, Woytas M, Groh A, Schmidt KH, Hartmann M, Lehmann M, Straube E. Production of basic fibroblast growth factor and interleukin 6 by human smooth muscle cells following infection with Chlamydia pneumoniae. *Infect Immun.* 2000;68:3635-41.
75. Gieffers J, van Zandbergen G, Rupp J, Sayk F, Kruger S, Ehlers S, Solbach W, Maass M. Phagocytes transmit Chlamydia pneumoniae from the lungs to the vasculature. *Eur Respir J.* 2004;23:506-10.
76. Moazed TC, Campbell LA, Rosenfeld ME, Grayston JT, Kuo CC. Chlamydia pneumoniae infection accelerates the progression of atherosclerosis in apolipoprotein e-deficient mice. *J Infect Dis.* 1999;180:238-41.
77. Ezzahiri R, Nelissen Vrancken HJMG, Kurvers HAJM, Stassen FRM, Vliegen I, Grauls GELM, van Pul MML, Kitslaar PJEHM, Bruggeman CA. Chlamydia pneumoniae (Chlamydia pneumoniae) accelerates the formation of complex atherosclerotic lesions in apo e3-leiden mice. *Cardiovasc Res.* 2002;56:269.
78. Ezzahiri R, Stassen FR, Kurvers HA, van Pul MM, Kitslaar PJ, Bruggeman CA. Chlamydia pneumoniae infection induces an unstable atherosclerotic plaque phenotype in ldl-receptor, apoe double knockout mice. *Eur J Vasc Endovasc Surg.* 2003;26:88-95.
79. Muhlestein JB, Anderson JL, Hammond EH, Zhao L, Trehan S, Schwobe EP, Carlquist JF. Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. *Circulation.* 1998;97:633-6.
80. Gupta S, Leatham EW, Carrington D, Mendall MA, Kaski JC, Camm AJ. Elevated Chlamydia pneumoniae antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. *Circulation.* 1997;96:404-7.
81. Muhlestein JB, Anderson JL, Carlquist JF, Salunkhe K, Horne BD, Pearson RR, Bunch TJ, Allen A, Trehan S, Nielson C. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease: Primary clinical results of the academic study. *Circulation.* 2000;102:1755-60.
82. O'Connor CM, Dunne MW, Pfeffer MA, Muhlestein JB, Yao L, Gupta S, Benner RJ, Fisher MR, Cook TD. Azithromycin for the secondary prevention of coronary heart disease events: The wizard study: A randomized controlled trial. *Jama.* 2003;290:1459-66.
83. Cannon CP, Braunwald E, McCabe CH, Grayston JT, Muhlestein B, Giugliano RP, Cairns R, Skene AM. Antibiotic treatment of Chlamydia pneumoniae after acute coronary syndrome. *N Engl J Med.* 2005;352:1646-54.
84. Gurfinkel E. Inflammation, infection, or both in atherosclerosis: The roxis trial in perspective. *J Infect Dis.* 2000;181 Suppl 3:S566-8.
85. Zahn R, Schneider S, Frilling B, Seidl K, Tebbe U, Weber M, Gottwik M, Altmann E, Seidel F, Rox J, Hoffler U, Neuhaus KL, Senges J. Antibiotic therapy after acute myocardial infarction: A prospective randomized study. *Circulation.* 2003;107:1253-9.
86. Wiesli P, Czerwenka W, Meniconi A, Maly FE, Hoffmann U, Vetter W, Schulthess G. Roxithromycin treatment prevents progression of peripheral arterial occlusive disease in Chlamydia pneumoniae seropositive men: A randomized, double-blind, placebo-controlled trial. *Circulation.* 2002;105:2646-52.
87. Parchure N, Zouridakis EG, Kaski JC. Effect of azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of Chlamydia pneumoniae infection. *Circulation.* 2002;105:1298-303.
88. Semaan HB, Gurbel PA, Anderson JL, Muhlestein JB, Carlquist JF, Horne BD, Serebruany VL. The effect of chronic azithromycin therapy on soluble endothelium-derived adhesion molecules in patients with coronary artery disease. *J Cardiovasc Pharmacol.* 2000;36:533-7.

89. Kuvin JT, Gokce N, Holbrook M, Hunter LM, Patel AR, Sliney KA, Craven DE, Grayston JT, Keaney JF, Jr., Karas RH, Vita JA. Effect of short-term antibiotic treatment on Chlamydia pneumoniae and peripheral endothelial function. *Am J Cardiol.* 2003;91:732-5.
90. Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Reduced progression of early carotid atherosclerosis after antibiotic treatment and Chlamydia pneumoniae seropositivity. *Circulation.* 2002;106:2428-33.
91. Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Progression of early carotid atherosclerosis is only temporarily reduced after antibiotic treatment of Chlamydia pneumoniae seropositivity. *Circulation.* 2004;109:1010-5.
92. de Jong G, Kessels F, Lodder J. Two types of lacunar infarcts: Further arguments from a study on prognosis. *Stroke.* 2002;33:2072-6.

Chapter 2

Chlamydia pneumoniae is a likely risk factor for ischaemic stroke in young patients

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Abstract

Recently, *Chlamydia pneumoniae* has been identified as a risk factor for atherosclerosis, cardiovascular disease and stroke. In young patients the causes of stroke are more diverse, and remain unknown in about thirty percent of the cases, despite thorough investigations. To find a possible relationship between *Chlamydia pneumoniae* infection and stroke at young age, we investigated *Chlamydia pneumoniae* antibody titres in 41 patients with ischaemic stroke under the age of fifty and in 55 healthy controls. A positive IgA antibody titre to *Chlamydia pneumoniae* was significantly associated with stroke (crude OR: 2.1; 90% CI 1.1-9.5, $p=0.04$). After adjusting for hypertension, smoking and hypercholesterolaemia, positive Chlamydia antibodies remained associated with stroke (adjusted OR: 2.8, 90% CI 1.1-7.1, $p=0.04$). No significant association between positive IgG antibodies and stroke was found. Since a positive IgA antibody titre may reflect a persistent infection, these data indicate that persistent *Chlamydia pneumoniae* infection may be an independent risk factor for stroke at young age.

Introduction

Five to ten percent of ischaemic stroke occurs in patients younger than fifty years. In most cases, atherosclerosis is the cause of the stroke, but in 30% the cause remains unknown, despite thorough investigations¹. Recent studies showed that in 20–40%, stroke is preceded by an infection. In many cases this is a respiratory infection². *Chlamydia pneumoniae* (*C pneumoniae*) is a recently detected respiratory pathogen. *C pneumoniae* causes respiratory infection, with symptoms that vary from mild upper respiratory infections to severe pneumonia. In most cases however, infection is asymptomatic^{3,4}. Infection with *C pneumoniae* is common: in epidemiological studies the incidence rate of *C pneumoniae* was approximately 50% in middle-aged people⁵. Various studies showed an association between *C pneumoniae* infection and atherosclerotic cardiovascular disease. In 1988, an association between positive IgG antibody titres to *C pneumoniae*, ischaemic chronic heart disease and acute myocardial infarction was established⁶. Since then, numerous studies investigated the relation between positive antibody titres to *C pneumoniae* and ischaemic coronary heart disease. The results of these studies do not always point in the same direction. A recently published prospective study and meta-analysis on the relation between *C pneumoniae* infection and cardiovascular disease did not establish an association⁷. Apart from seroepidemiological studies, various other investigations have been reported. *C pneumoniae* was detected within the atheromatous plaques of coronary arteries^{8,9}, carotid arteries^{10–12}, and the middle cerebral artery¹³. The micro-organism was mainly detected in parts of the artery with severe atherosclerotic changes. One study found no difference between the presence of *C pneumoniae* and the severity of the atherosclerosis¹⁴. In vitro studies show that *C pneumoniae* can infect endothelial cells, smooth muscle cells, monocytes and macrophages, thereby inducing cytokine production, such as tumour necrosis factor alpha (TNF α), interleukin-1 (IL-1) and interleukin-6 (IL-6)^{15,16}. Several animal studies indicate that infection with *C pneumoniae* accelerates the process of atherosclerosis¹⁷, whereas treatment with azithromycin reduced atherosclerotic lesions in a rabbit model¹⁸. Only few investigated the relationship between *C pneumoniae* infection and stroke. Wimmer et al. found positive *C pneumoniae* IgA and IgG antibody titres more often in young patients with stroke or TIA, compared to healthy controls¹⁹. In studies that followed, this association was extended to an elderly population^{20–22}. *C pneumoniae* infection is also related to intima-media thickness (IMT) of the carotid artery²³. A relation between *C pneumoniae* infection and hemodynamically significant carotid artery stenosis could not be found^{24,25}. The aim of this study was to explore a possible relationship between *C pneumoniae* infection and ischaemic stroke in patients under the age of fifty,

in whom no specific cause of the stroke could be detected, thereby leaving atherosclerosis as a possible, or even probable cause of the stroke.

Patients and methods

Patients

Forty-one patients under the age of fifty with ischaemic stroke were included. These patients were included from the ongoing prospective Maastricht Stroke Registry. Fifty-five healthy controls were recruited among hospital and university personnel, and matched for age and sex. Rare causes of stroke, such as dissection, vasculitis, haematological disorders, and stroke due to cardiac embolism were excluded, leaving patients with atherosclerosis as probable cause of the stroke, according to the TOAST criteria²⁶. After approval by the medical ethics committee, written informed consent was obtained from patients and controls.

Routine investigations in the patient group included standard blood tests, fibrinogen, AT III, protein C, protein S, and von Willebrand factor. Further investigations included electrocardiogram, brain CT, non-invasive carotid studies, and echocardiography. Cerebral angiography was performed in twenty-one cases for specific individual reasons. Routine laboratory investigations (Hb, Ht, WBC, platelet count) were normal. Laboratory investigations in the control group included fibrinogen, AT III and von Willebrand factor.

Since vascular risk factors may be different in patients with small-vessel disease and large-vessel disease, we considered patients with lacunar and territorial infarcts separately.

We also investigated the relation between positive antibody titres to *C pneumoniae* and the degree of carotid artery stenosis, defined as a diameter reduction more or less than 50%. Carotid IMT was not recorded.

Measurement of IgG and IgA antibodies

Serum IgA and IgG antibodies to *C pneumoniae* were measured using an enzyme linked immunosorbent assay (ELISA), (Labsystems, Helsinki, Finland). The presence or absence of *C pneumoniae* antibodies was determined by comparing the absorbance value of the sample to a cut-off value, as specified by the manufacturer. Specimens with values equal to or greater than the cut-off value were considered positive for anti-Chlamydia antibodies, for IgA the cut-off value is 8, for IgG 30.

Statistical evaluation

Variables are presented as mean and standard deviations. The association between stroke and positive antibody titres to *C pneumoniae* was analysed by means of crude odds ratios (OR) with 90% confidence intervals (CI). Multivariate logistic regression analysis was subsequently used to determine adjusted odds ratios with 90% CI. Adjusted odds ratios for positive IgG and IgA antibody titres were determined separately, with stroke as dependent variable and smoking, hypertension, hypercholesterolaemia and the respective antibody titres as dependent variables. A one-tailed test was used to test for statistic significance because our hypothesis was unidirectional a priori. For the comparison of the characteristics of patients with and without positive IgA antibodies to *C pneumoniae*, the Student's T-test was used.

Results

Characteristics of patients and controls are summarised in Table 2.1. We found a positive IgA antibody titre in 22 controls (40%) and in 24 patients (59%), $p=0.04$, whereas a positive IgG antibody titre was present in 38 controls (69%) and in 28 patients (68%) (NS). A positive IgA antibody titre to *C pneumoniae* was significantly associated with stroke (crude OR 2.1; 90% CI 1.4-9.5; $p=0.04$). After adjusting for smoking, hypertension and hypercholesterolaemia, IgA remained associated with stroke (adjusted OR 2.8; 90% CI 1.1-7.1, $p=0.04$). In this logistic model the presence of IgG antibodies was not associated with stroke (OR 0.9; 90% CI 0.5-2.0 $p=NS$). The distribution of IgA and IgG antibodies in patients and controls is shown in Figures 2.1 and 2.2.

Table 2.1 Characteristics of young stroke patients and controls (percentages between parenthesis)

	Patients N=41	Controls N=55
Age (mean $\pm$ SD)	43.9 ($\pm$ 6.0)	39.4 ($\pm$ 8.6)
Range	25-50	23-56
Sex	19 male (46)	24 male (44)
Prior stroke/TIA	4 (9)	0
Myocardial infarction	3 (7)	0
Angina pectoris	5 (12)	0
Hypertension	19 (46)	2 (4)
Hypercholesterolaemia	12 (29)	1 (2)
Smoking	28 (68)	16 (29)

Figure 2.1 Distribution of IgA antibody titres in patients and controls.
Figure showing the distribution of IgA antibody titres in patients and controls.
The x-axis marks the cut-off value for IgA seropositivity (= 8)

Figure 2.2 Distribution of IgG antibody titres in patients and controls.
Figure shows the distribution of IgG antibody titres between patients and controls.
The x-axis shows the cut-off value for IgG seropositivity (= 30)

There were no differences in mean values of IgA and IgG antibody titres between the patients and controls. (Figure 2.3) Mean IgA antibody titres: (mean $\pm$ SD) 13.0 $\pm$ 14.5 in patients and 14.8 $\pm$ 23.1 in controls, mean IgG antibody titres: 55.8 $\pm$ 51.3 and 55.2 $\pm$ 60.1 for patients and controls. The patients with positive IgG and IgA antibody titres to *C pneumoniae* did not show statistically significant differences from those patients without a positive antibody titre with respect to age, smoking, hypertension, fibrinogen content and cholesterol (Table 2.2). Patients with a territorial infarct (n=31) more often had a positive IgG antibody titre (n=23; 74%) than did the 10 patients with a lacunar infarct (n=5; 50%) (OR 2.88, 95% CI 0.39-21.08), but the difference was not statistically significant. No significant association was found between a positive IgA antibody titre and more than 50% stenosis of the carotid artery (OR: 1.5; 95% CI 0.24-9.3).

Figure 2.3 Comparison of the mean values for IgA and IgG antibody titers to *C pneumoniae*.
Values for IgA: (mean $\pm$ SD) 13.0 $\pm$ 14.5 in patients and 14.8 $\pm$ 23.1 in controls
Values for IgG: (mean $\pm$ SD) 55.8 $\pm$ 51.3 in patients and 55.2 $\pm$ 60.1 in controls

Table 2.2 Characteristics of patients with and without positive IgA antibodies to *C pneumoniae*.

	Positive IgA antibodies	Negative IgA antibodies	P-value
Age (mean $\pm$ SD)	43.2 $\pm$ 6.2	44.8 $\pm$ 5.6	0.392
Smoking, n (%)	17 (71)	11 (65)	0.678
Hypertension, n (%)	10 (42)	14 (53)	0.477
Serum cholesterol (mmol/l)	5.9 $\pm$ 1.0	6.1 $\pm$ 1.4	0.709
Fibrinogen (g/l)	3.4 $\pm$ 0.9	3.5 $\pm$ 1.1	0.821
ESR (mm/hour)	8.3 $\pm$ 8.9	7.3 $\pm$ 7.2	0.726
WBC ($\times 10^9$ /l)	9.6 $\pm$ 3.0	10.0 $\pm$ 4.1	0.765

Discussion

In this case-control study of young stroke patients, we found positive serum IgA antibody titres against *C pneumoniae* more often in patients than in matched controls, although the mean values of the antibody titre did not differ between the groups. The number of patients with a raised IgG titre against *C pneumoniae* did not differ between the groups. Even though no serological test satisfactory diagnoses chronic persistent *C pneumoniae* infection²⁷, past infection with *C pneumoniae* is presumed when elevated IgG antibodies are present, whereas an elevated IgA antibody titre at one time point is believed to be a marker for chronic persistent *C pneumoniae* infection^{6,20,28}. In our study population, the people who had positive IgA antibody titres also had an elevated IgG antibody titre, indicating that they all had past *C pneumoniae* infections. Therefore, our data suggest that a chronic *C pneumoniae* infection is associated with stroke at young age. *C pneumoniae* has been associated with stroke in earlier reports. At first this relationship was established in a young stroke population. Wimmer et al. found a nearly two-fold increase in the risk of TIA or ischaemic stroke in patients with IgA and IgG antibody titres against *C pneumoniae*¹⁹. This study also included patients with cardio-embolic stroke, whereas we included only patients with atherosclerosis as the most likely cause of the stroke. Some did not find a relationship between positive antibody titres to *C pneumoniae* and stroke, which may have been due to the fact that blood was collected during a *C pneumoniae* epidemic²⁹.

Previous studies found a positive IgA antibody titre associated with fibrinogen level³⁰, smoking³¹, hypertension³², and an altered lipid metabolism³³. Our patients with positive IgG and IgA antibody titres to *C pneumoniae* did not differ from those without positive antibody titres with respect to age, smoking, hypertension, fibrinogen content and cholesterol levels. After adjustment for potential contribution to the association by these factors, the presence of IgA antibody titres remained significantly associated with stroke. These findings suggest that an active *C pneumoniae* infection is an independent contributor to the risk of stroke. Seven of our patients were without hypertension, had no hypercholesterolaemia, and did not smoke. Five of these seven had a positive IgA antibody titre, which further underlines the importance of *C pneumoniae* as an independent risk factor.

Infection with *C pneumoniae* may contribute to the risk of stroke by enhancing carotid artery atherosclerosis. This issue was addressed in several studies. Some found a relationship between *C pneumoniae* infection and increased IMT of the carotid artery²³. Others, however, found no relationship between *C pneumoniae* infection and hemodynamically significant carotid artery atherosclerosis^{24,25}. This may be related to the fact that internal carotid artery wall ulceration without stenosis, of which IMT increase may be a preceding

stage, increases stroke risk, although ulceration and degree of stenosis are closely related³⁴. Our recent animal experiments established that *C pneumoniae* infection enhances plaque formation towards the more unstable types³⁵. We did not find a significant relationship between a positive *C pneumoniae* antibody titre and carotid atherosclerosis, possibly due to the small number of patients in the subgroups.

There are several limitations to our study. The relatively small number of patients implied wide confidence intervals, and therefore larger studies including more patients have to be done in the future. Secondly, the association we detected is a serological association. Serological tests detect antibodies to a specific micro-organism, which indicates that infection with this micro-organism took place at some point in time. However, absolute prove of the micro-organism's actual involvement in the process of atherosclerosis could only come from demonstrating its presence in the vascular wall. Positive antibody titres may not be 100% predictive in that respect, because the presence of *C pneumoniae* is not always demonstrated in the vascular wall of patients with a positive antibody titre³⁶. However, within the limits of these restrictions, our findings suggest that stroke below the age of fifty may be related to a persistent *C pneumoniae* infection.

The idea that a persistent *C pneumoniae* infection contributes to the risk of vascular events such as stroke is appealing from a therapeutic perspective. Although *C pneumoniae* may contribute to the risk of stroke directly, it acts in concordance with the classical risk factors in most cases. Although it is therefore unlikely that antibiotic treatment will reduce the risk of stroke substantially, it is worthwhile investigating, as even a small risk reduction may be clinically significant. In several cardiovascular pilot studies the effect of antibiotic treatment on the risk of cardiovascular events and on inflammatory parameters was studied³⁷⁻³⁹. Two of these studies showed a significant reduction of these events^{37,39}, but one did not³⁸, despite a significant reduction in inflammatory parameters. Larger studies, that included large numbers of patients did not show a reduction of cardiovascular events^{40,41}. A trial on the effect of antibiotic treatment of *C pneumoniae* in stroke should not exclude patients below the age of fifty in whom no specific cause of stroke was detected, as our data indicate that a chronic *C pneumoniae* infection may play a role in these patients. Such a study, with magnetic resonance imaging (MRI) detected increase in (sub) clinical ischaemic events is currently being conducted in our institute.

References

1. Bogousslavsky J, Pierre P. Ischemic stroke in patients under age 45. *Neurol Clin.* 1992;10:113-24.
2. Syrjänen J, Valtonen VV, Iivanainen M, Kaste M, Huttunen JK. Preceding infection as an important risk factor for ischaemic brain infarction in young and middle aged patients. *Br Med J Clin Res Ed.* 1988;296:1156-60.
3. Grayston JT, Campbell LA, Kuo CC, Mordhorst CH, Saikku P, Thom DH, Wang SP. A new respiratory tract pathogen: Chlamydia pneumoniae strain TWAR. *J Infect Dis.* 1990;161:618-25.
4. Grayston JT. Infections caused by Chlamydia pneumoniae strain TWAR [see comments]. *Clin Infect Dis.* 1992;15:757-61.
5. Karvonen M, Tuomilehto J, Pitkaniemi J, Naukkarinen A, Saikku P. Chlamydia pneumoniae IgG antibody prevalence in south-western and eastern Finland in 1982 and 1987. *Int J Epidemiol.* 1994;23:176-84.
6. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK, Valtonen V. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet.* 1988;2:983-6.
7. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Wong YK, Bernardes Silva M, Ward M. Chlamydia pneumoniae IgG titres and coronary heart disease: prospective study and meta-analysis. *British Medical Journal.* 2000;321:208-212.
8. Kuo CC, Shor A, Campbell LA, Fukushi H, Patton DL, Grayston JT. Demonstration of Chlamydia pneumoniae in atherosclerotic lesions of coronary arteries. *J Infect Dis.* 1993;167:841-9.
9. Kuo CC, Grayston JT, Campbell LA, Goo YA, Wissler RW, Benditt EP. Chlamydia pneumoniae (TWAR) in coronary arteries of young adults (15-34 years old). *Proc Natl Acad Sci U S A.* 1995;92:6911-4.
10. Grayston JT, Kuo CC, Coulson AS, Campbell LA, Lawrence RD, Lee MJ, Strandness ED, Wang SP. Chlamydia pneumoniae (TWAR) in atherosclerosis of the carotid artery [see comments]. *Circulation.* 1995;92:3397-400.
11. Chiu B. Multiple infections in carotid atherosclerotic plaques. *Am Heart J.* 1999;138:S534-6.
12. Yamashita K, Ouchi K, Shirai M, Gondo T, Nakazawa T, Ito H. Distribution of Chlamydia pneumoniae infection in the atherosclerotic carotid artery. *Stroke.* 1998;29:773-8.
13. Virok D, Kis Z, Karai L, Intzedy L, Burian K, Szabo A, Ivanyi B, Gonczol E. Chlamydia pneumoniae in atherosclerotic middle cerebral artery. *Stroke.* 2001;32:1973-6.
14. Thomas M, Wong Y, Thomas D, Ajaz M, Tsang V, Gallagher PJ, Ward ME. Relation between direct detection of Chlamydia pneumoniae DNA in human coronary arteries at postmortem examination and histological severity (Stary grading) of associated atherosclerotic plaque. *Circulation.* 1999;99:2733-6.
15. Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA, Quinn TC. Replication of Chlamydia pneumoniae in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun.* 1996;64:1614-20.
16. Kaukoranta Tolvanen SS, Teppo AM, Laitinen K, Saikku P, Linnavuori K, Leinonen M. Growth of Chlamydia pneumoniae in cultured human peripheral blood mononuclear cells and induction of a cytokine response. *Microb Pathog.* 1996;21:215-21.
17. Moazed TC, Campbell LA, Rosenfeld ME, Grayston JT, Kuo CC. Chlamydia pneumoniae infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *J Infect Dis.* 1999;180:238-41.
18. Muhlestein JB, Anderson JL, Hammond EH, Zhao L, Trehan S, Schwobe EP, Carlquist JF. Infection with Chlamydia pneumoniae accelerates the development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model. *Circulation.* 1998;97:633-6.
19. Wimmer ML, Sandmann Strupp R, Saikku P, Haberl RL. Association of chlamydial infection with cerebrovascular disease. *Stroke.* 1996;27:2207-10.

20. Cook PJ, Honeybourne D, Lip GYH, Beevers DG, Wise R, Davies AH. Chlamydia pneumoniae antibody titers are significantly associated with acute stroke and transient cerebral ischemia: the West Birmingham Stroke Project. *Stroke*. 1998;29:404-10.
21. Elkind MS, Lin IF, Grayston JT, Sacco RL. Chlamydia pneumoniae and the risk of first ischemic stroke: The Northern Manhattan Stroke Study. *Stroke*. 2000;31:1521-5.
22. Fagerberg B, Gnarpe J, Gnarpe H, Agewall S, Wikstrand J. Chlamydia pneumoniae but not cytomegalovirus antibodies are associated with future risk of stroke and cardiovascular disease: a prospective study in middle-aged to elderly men with treated hypertension. *Stroke*. 1999;30:299-305.
23. Schmidt C, Hulthe J, Wikstrand J, Gnarpe H, Gnarpe J, Agewall S, Fagerberg B. Chlamydia pneumoniae seropositivity is associated with carotid artery intima-media thickness. *Stroke*. 2000;31:1526-31.
24. Markus HS, Sitzer M, Carrington D, Mendall MA, Steinmetz H. Chlamydia pneumoniae infection and early asymptomatic carotid atherosclerosis. *Circulation*. 1999;100:832-7.
25. Melnick SL, Shahar E, Folsom AR, Grayston JT, Sorlie PD, Wang SP, Szklo M. Past infection by Chlamydia pneumoniae strain TWAR and asymptomatic carotid atherosclerosis. Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Am J Med*. 1999;95:499-504.
26. Gordon DL, Bendixen BH, Adams HP, Jr., Clarke W, Kappelle LJ, Woolson RF. Interphysician agreement in the diagnosis of subtypes of acute ischemic stroke: implications for clinical trials. The TOAST Investigators. *Neurology*. 1993;43:1021-7.
27. Dowell SF, Peeling RW, Boman J, Carlone GM, Fields BS, Guarner J, Hammerschlag MR, Jackson LA, Kuo CC, Maass M, Messmer TO, Talkington DF, Tondella ML, Zaki SR. Standardizing Chlamydia pneumoniae Assays: Recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). *Clin Infect Dis*. 2001;33:492-503.
28. Saikku P, Leinonen M, Tenkanen L, Linnanmaki E, Ekman MR, Manninen V, Manttari M, Frick MH, Huttunen JK. Chronic Chlamydia pneumoniae infection as a risk factor for coronary heart disease in the Helsinki Heart Study [see comments]. *Ann Intern Med*. 1992;116:273-8.
29. Glaser CA, Stegmayr B, Boman J, Stenlund H, Weinehall L, Hallmans G, Dahlen GH. Chlamydia pneumoniae antibodies and high lipoprotein(a) levels do not predict ischemic cerebral infarctions. Results from a nested case-control study in Northern Sweden. *Stroke*. 1999;30:2013-8.
30. Toss H, Gnarpe J, Gnarpe H, Siegbahn A, Lindahl B, Wallentin L. Increased fibrinogen levels are associated with persistent Chlamydia pneumoniae infection in unstable coronary artery disease. *Eur Heart J*. 1998;19:570-7.
31. Hahn DL, Golubjatnikov R. Smoking is a potential confounder of the Chlamydia pneumoniae-coronary artery disease association. *Arterioscler Thromb*. 1992;12:945-7.
32. Cook PJ, Lip GY, Davies P, Beevers DG, Wise R, Honeybourne D. Chlamydia pneumoniae antibodies in severe essential hypertension. *Hypertension*. 1998;31:589-94.
33. Laurila AL, Bloigu A, Nayha S, Hassi J, Leinonen M, Saikku P. Chlamydia pneumoniae antibodies associated with altered serum lipid profile. *Int J Circumpolar Health*. 1998;57 Suppl 1:329-32.
34. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. *N Engl J Med*. 1991;325:445-53.
35. Ezzahiri R, Nelissen Vrancken HJMG, Kurvers HAJM, Stassen FRM, Vliegen I, Grauls GELM, van Pul MML, Kitslaar PJEHM, Bruggeman CA. Chlamydia pneumoniae (Chlamydia pneumoniae) accelerates the formation of complex atherosclerotic lesions in Apo E3-Leiden mice. *Cardiovasc Res*. 2002;56:269.
36. Maass M, Bartels C, Engel PM, Mamat U, Sievers HH. Endovascular presence of viable Chlamydia pneumoniae is a common phenomenon in coronary artery disease. *J Am Coll Cardiol*. 1998;31:827-32.
37. Gurfinkel E. Inflammation, infection, or both in atherosclerosis: the ROXIS trial in perspective. *J Infect Dis*. 2000;181 Suppl 3:S566-8.

38. Anderson JL, Muhlestein JB, Carlquist J, Allen A, Trehan S, Nielson C, Hall S, Brady J, Egger M, Horne B, Lim T. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease and serological evidence for *Chlamydia pneumoniae* infection: The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with *Chlamydia* (ACADEMIC) study. *Circulation*. 1999;99:1540-7.
39. Gupta S. *Chlamydia pneumoniae*, monocyte activation, and azithromycin in coronary heart disease. *Am Heart J*. 1999;138:S539-41.
40. Jackson LA. Description and status of the azithromycin and coronary events study (ACES). *Journal of Infectious Diseases*. 2000;181 Suppl. 3:S579-81.
41. O'Connor CM, Dunne MW, Pfeffer MA, Muhlestein JB, Yao L, Gupta S, Benner RJ, Fisher MR, Cook TD. Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. *Jama*. 2003;290:1459-66.

Chapter 3

Intraperitoneal *Chlamydia pneumoniae* infection enhances microglial activation in the hippocampus of atherosclerotic mice

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Abstract

The presence of *Chlamydia pneumoniae* in murine brain tissue was studied in atherosclerotic and non-atherosclerotic mice, after peritoneal injection. Furthermore, we investigated whether increased permeability of the blood brain barrier (BBB) was implicated in cerebral *Chlamydia pneumoniae* infection and whether intra-cerebral *Chlamydia pneumoniae* infection leads to microglial activation. Using a polymerase chain reaction, *Chlamydia pneumoniae* DNA was found within the brain tissue of 30% of the mice, three, seven and twenty-one days after infection. Atherosclerosis and age does not influence the extent of the cerebral infection. Semi quantitative analyses showed that intra-cerebral *Chlamydia pneumoniae* infection was not accompanied by an altered function of the blood brain barrier. Microglial activation was assessed with immunohistochemistry, quantified in the hippocampus of each infected mouse and compared with mock infected mice. Enhanced microglial activation was found in the atherosclerotic mice. Since microglial activation is a key factor in a number of neuro-inflammatory diseases, and is seen in chronic cerebral ischaemia, *Chlamydia pneumoniae* infection might play a role in these diseases.

Introduction

Chlamydia pneumoniae (*C pneumoniae*) infection has been implicated in the development and progression of atherosclerosis, cardiovascular and cerebrovascular disease¹⁻³. *C pneumoniae* is a respiratory pathogen, causing respiratory infections, which often are asymptomatic⁴. From the respiratory tissue, *C pneumoniae* disseminates through infection of monocytes⁴, and through this route is able to infect endothelial cells⁵. Endothelial cells infected with *C pneumoniae* show an increased expression of adhesion molecules, and undergo morphological changes leading to a higher permeability⁶. Infection of cerebral endothelial cells, therefore, can lead to an increased permeability of the blood brain barrier (BBB). In fact, it has been shown that in vitro *C pneumoniae* infection of human brain endothelial cells promotes transendothelial entry of monocytes, through an up regulation of adhesion molecules⁷. The ability of *C pneumoniae* to enter the brain after systemic infection in vivo, however, has not been described before.

Intra-cerebral presence of *C pneumoniae* has been implicated in neuro-inflammatory disorders, and it has been stipulated that the presence of this micro-organism causes low-grade inflammation of the brain, subsequently leading to the release of neurotoxic agents, like tumour necrosis factor alpha (TNF α). Although intra-cerebral presence of *C pneumoniae* has been described, it is still unknown whether *C pneumoniae* is just an innocent bystander or plays an active role in neuro-inflammation.

Key factor in the pathogenesis of neuro-inflammatory disorders is activation of microglial cells⁸. Microglia, the primary immune effector cells of the central nervous system, respond to traumatic injury or the presence of pathogens in the brain⁸. Microglial activation is also seen in ischaemic cerebral lesions, caused by cerebrovascular atherosclerosis, or diffuse cerebral hypoperfusion⁹. In this animal experiment we studied if *C pneumoniae* could be detected in cerebral tissue after an intraperitoneal infection, and whether increased permeability of the BBB was implicated in cerebral *C pneumoniae* infection. Subsequently, we studied whether intra-cerebral *C pneumoniae* infection leads to microglial activation. Furthermore, since cerebral atherosclerosis and age influence BBB function, we studied whether atherosclerosis and/or age influences the development of a cerebral infection and microglial activation. In addition, we studied if intra-cerebral *C pneumoniae* infection alters BBB function.

Materials and methods

Chlamydia pneumoniae

C pneumoniae strain TWAR 2043 (ATCC) was cultured on HEp-2 cells as previously described¹⁰.

Bacterial titres were determined by titration in HEp-2 cells and stained with a monoclonal antibody directed against *C pneumoniae*, RR 402 (DAKO, Glostrup, Denmark), followed by fluorescein isothiocyanate (FITC) conjugated rabbit-anti-mouse serum (DAKO, Glostrup, Denmark). Titres were expressed as number of inclusion forming units (IFU) per millilitre.

Animals

For this study, mice with atherosclerosis, i.e. Apolipoprotein E (ApoE) knockout (KO) and ApoE/Low Density Lipoprotein receptor (ApoE/LDLr) KO mouse, both on a C57BL/6J background were used, and compared to mice without atherosclerosis, i.e. wild type C57BL/6J mice. The ApoE/LDLr KO mice were donated by the department of Internal Medicine (head: Prof. dr. JW van der Meer) of the University Hospital Nijmegen (Nijmegen, the Netherlands), the ApoE KO mice were provided from the own breeding of the Central Animal Facility of the Maastricht University. C57BL/6J mice used as control mice were obtained from Charles Rivers (USA). All the mice were fed standard chow diet. The mice were kept under specific pathogen free (SPF) conditions, at the Central Animal Facilities of the Maastricht University, the Netherlands.

Twenty-one mice of each group were infected intraperitoneally with 3×10^7 IFU *C pneumoniae*. ApoE/LDLr KO and ApoE KO mice were infected at eight months of age. To assess the influence of age on *C pneumoniae* infection and microglial activation, two groups of C57BL/6J were used, aged three and eight months at the time of infection. The same number of mice were mock infected with a sucrose-phosphate-glucose solution (SPG). SPG is the medium in which *C pneumoniae* is conserved.

The mice were sacrificed, three, seven and twenty-one days after infection ($n=7$ per time point). At the time of sacrifice, the mice were anaesthetized with pentobarbital. After exsanguination through the apex of the heart, and perfusion with Phosphate Buffered Saline (PBS), the brains were removed. A small frontal section was used for *C pneumoniae* detection by the polymerase chain reaction (PCR). The remaining tissue was fixed in 10% formol and embedded in paraffin for sectioning and immunohistochemical staining. From the ApoE KO mice only material for PCR was available, and these mice were not used for microglial activation.

Microglial activation

As the primary antibody, the F4/80 monoclonal antibody (BMA Biomedicals AG, Augst, Switzerland) was used. The antibody is expressed by most macrophages and macrophage precursors, and is also expressed on activated macrophages and microglial cells¹¹. Twelve μm thick paraffin embedded sections were pre-incubated with 2% BSA in PBS to block aspecific staining, after which the primary antibody was put on the slides, for one hour at 37°C. After washing in PBS, the slides were incubated with the secondary antibody: biotinylated goat anti-mouse serum (DAKO, Glostrup, Denmark). Immunostaining was performed by the avidin-biotin alkaline phosphatase method (DAKO, Glostrup, Denmark). Fast red was used as a chromogen, after which the sections were counterstained with haematoxylin. The number of activated (F4/80 positive) microglial cells in the hippocampus of each mouse was counted, as described by Long et al.¹², using the Analysis[®] system (Soft imaging systems, Munster, Germany). Since the measured surface of the hippocampus could not be standardized, the number of cells was corrected for the surface of hippocampus (number of cells * 1000000/total area of hippocampus measured).

Detection of *C pneumoniae* by PCR

To detect the presence of *C pneumoniae* in the murine brains, a sensitive PCR was used (detection ability of one copy of DNA). The following sequences were used as primers: sense CPC, 5'-TTA TTA ATT GAT GGT ACA ATA-3'; antisense CPD, 5'-ATC TAC GGC AGT AGT ATA GTT-3'¹³. PCR was performed in a total volume of 50 μl , containing 5 μg DNA. DNA was extracted from brain tissue with the Wizard Genomic DNA purification kit (Promega, Germany). Amplification was done by thermal cycling starting at 37°C for ten minutes and 95°C for fifteen minutes. 20 cycles of "Touchdown PCR" were performed from 60°C to 50°C, followed by 40 cycles of one minute at 94°C, 50°C, and 72°C.

The PCR products were resolved in a 2% agarose gel, stained with ethidium bromide, and photographed.

Detection of *C pneumoniae* by immunohistochemistry

To localize *C pneumoniae* antigens within the murine brain, immunohistochemistry (IHC) was performed. For this several anti *C pneumoniae* antibodies were used: mouse anti-*C pneumoniae* antibody (RR 402, DAKO Glostrup, Denmark), a polyclonal anti-*C pneumoniae* antibody, and a rat anti-*C pneumoniae* antibody that was developed in our own laboratory. For each antibody the procedure was as follows: twelve μm thick paraffin embedded

sections were pre-incubated with PBS containing 2% BSA to block aspecific staining, after which the primary antibody was put on the slides. After washing in PBS, the slides were incubated with the secondary biotinylated antibody. Immunostaining was performed by the avidin-biotin alkaline phosphatase method (DAKO, Glostrup, Denmark). Fast red was used as a chromogen, after which the sections were counterstained with haematoxylin. As positive controls, HEp-2 cells in vitro infected with *C. pneumoniae* were used.

C. pneumoniae antibodies

To ensure proper infection of the mice, a microimmunofluorescence (MIF) test (Labsystems, Helsinki, Finland) was performed to detect *C. pneumoniae* antibodies in the mice twenty-one days after inoculation, as described previously¹⁴.

Permeability of the BBB

Permeability of the BBB was evaluated by measuring the presence of immunoglobulin G in the brain, as described previously¹⁵, using a specific mouse IgG antibody. The slides were incubated with mouse specific IgG (DAKO) for one hour at room temperature. Staining of the sections was done using horseradish peroxidase (DAKO, Glostrup, Denmark). Where cerebral IgG was present, the colour intensity of the area's coloured by the IgG staining was assessed semiquantitatively.

Statistics

A log scale was used because of the non-linear distribution of the values of microglial activation. A linear regression model was used to assess the difference in microglial activation in Chlamydia PCR positive mice versus Chlamydia PCR negative mice, Chlamydia PCR positive mice versus mock-infected mice and Chlamydia PCR negative mice versus mock infected mice. Furthermore, we compared microglial activation in the ApoE/LDLr KO mice versus eight months old C57BL/6J mice and three months old C57BL/6J mice, respectively. Results are given in log average values of the number of activated microglial cells and the standard error of mean.

Results

Detection of *C pneumoniae*

C pneumoniae IgG antibodies were found in all *C pneumoniae* infected mice twenty-one days after infection.

Five infected ApoE/LDLr KO mice died before scheduled sacrifice, three that were scheduled to be sacrificed seven days after infection and two that were scheduled to be sacrificed twenty-one days after infection. These mice were excluded from the study. The causes of death of these mice were unknown, macroscopic examination of the brains showed no abnormalities. PCR was not performed in these mice, because exact time of death could not be established and DNA might already be degraded in these mice. In total, *C pneumoniae* DNA was detected in the brain tissue of 24 out of 79 infected mice (30.0%). As shown in Figure 3.1 and Table 3.1, there is a marked difference in infection pattern in the ApoE/LDLr KO mice, compared the other types of mice. In the ApoE KO and C57BL/6J, the infection rate is the highest three days after infection, with a decline afterwards. The ApoE/LDLr KO however, show a low infection rate three days after infection, which rises at the other time points. In total, three days after infection 42.8% of the brains contained *C pneumoniae* DNA, this decreased to 24% and 23.1% positivity for the time points seven and twenty-one days after infection, respectively. Immunohistochemical staining could not confirm the presence of *C pneumoniae* in the murine brain tissue.

Figure 3.1 Distribution of the proportions of PCR positive brains of ApoE KO, ApoE/LDLr KO, three months old C57BL/6J (C57BL/6J, 3m), eight months old C57BL/6J (C57BL/6J, 8m) mice three, seven and twenty-one days after infection.

Table 3.1 Distribution of the presence of *C. pneumoniae* DNA in the brain tissue of ApoE KO, ApoE/LDLr KO, three months old C57BL/6J (C57BL/6J, 3m), eight months old C57BL/6J (C57BL/6J, 8m) mice three, seven and twenty-one days after infection. Results are given as number of positive mice/total mice analysed (percentage positives).

	ApoE KO	ApoE/LDLr KO	C57BL/6J, 3 m	C57BL/6J, 8m	Total
3 days p.i.	4/7 (54)	1/7 (14)	3/7 (43)	4/7 (57)	12/28 (42.8)
7 days p.i.	0/7 (0)	1/4 (25)	3/7 (43)	2/7 (28)	6/25 (24)
21 days p.i.	1/7 (14)	3/5 (60)	0/7 (0)	2/7 (28)	6/26 (23.1)

Microglial activation

Microglial activation was assessed in ApoE/LDLr KO mice, three month old C57BL/6J mice and eight months old C57BL/6J mice. In each of these groups, microglial activation was compared in Chlamydia PCR positive mice versus Chlamydia PCR negative mice, Chlamydia PCR positive mice versus mock-infected mice and Chlamydia PCR negative mice versus mock-infected mice. The highest number of activated microglial cells was found in the Chlamydia infected PCR positive ApoE/LDLr KO mice. There were significantly less activated microglial cells in the mock infected ApoE/LDLr KO mice compared with the Chlamydia infected PCR positive mice (-0.394, 95% CI: -0.716/-0.073) p-value: 0.017. Chlamydia infected PCR negative mice showed less microglial activation than Chlamydia infected PCR positive mice, but this was not statistically significant, (-0.252 (95% CI: -0.598/0.093), p-value 0.150. No difference in microglial activation was found between the Chlamydia infected PCR negative ApoE/LDLr KO mice and the mock infected ApoE/LDLr KO mice (-0.142, 95% CI: -0.133/0.417), p-value: 0.308. C57BL/6J mice showed significantly less activated microglial cells than the ApoE/LDLr KO mice, in both the three month old (-0.953 95% CI: -1.252/-0.655) and eight months old mice (-0.989 95% CI: -1.276/-0.702), p-value for both 0.000.

C57BL/6J mice showed no differences in microglial activation between the Chlamydia infected PCR positive, Chlamydia infected PCR negative and mock infected mice, nor was there a difference in microglial activation between the three months old and eight months old mice.

These data are summarized in Figure 3.2.

BBB permeability

No cerebral IgG was observed in the infected and mock injected ApoE/LDLr KO mice, nor was there enhanced permeability of the BBB in the C57BL/6J mice. Semi quantitative analyses therefore, was not performed¹⁵.

Figure 3.2 Differences in microglial activation in eight months old ApoE/LDLr KO mice (left), three months old C57BL/6J (middle) and eight months old C57BL/6J mice (right), divided into chlamydia PCR positive mice, chlamydia PCR negative mice, and mock infected mice.

* $p=0.017$ for the difference in microglial activation in infected PCR positive ApoE/LDLr KO mice compared to mock infected ApoE/LDLr KO mice.

Discussion

The presence of *C pneumoniae* DNA in murine brain tissue was established after intraperitoneal injection, proving haematogenic dissemination of *C pneumoniae* into the brain. Little et al. already showed that intranasal infection with *C pneumoniae* leads to the presence of the micro-organism within pulmonary tissue, the heart and the brain¹⁶. Aged animals are more susceptible to pulmonary infection than younger animals. In our study we focused on the effect of age on intra-cerebral *C pneumoniae* infection, as well as the effect of atherosclerosis. Infection rate was not influenced by these factors, but the ApoE/LDLr KO mice showed an infection pattern that differed from the ApoE KO and C57BL/6J mice, with the highest amount of positive brains twenty-one days after infection, whereas the ApoE KO and C57BL/6J mice show the highest positivity rate three days after infection. ApoE/LDLr mice show extensive atherosclerosis throughout the vascular tree with advanced lesions already present in 16 week old mice¹³. This extensive atherosclerosis can lead to generalized hypoperfusion, slowing down the haematogenic spread of the infection. Furthermore, hypoperfusion or increased monocyte recruitment into atherosclerotic areas could delay recruitment of monocytes into inflammatory areas, thereby prolonging cerebral presence of *C pneumoniae*.

Although all *C pneumoniae* infected mice seroconverted, indicating that sufficient infection had taken place, not all mice developed cerebral *C pneumoniae* infection. Only 42.8% of the murine brains were positive three days after infection, a number that decreased in time. This finding is in agreement with earlier experiments performed in our laboratory, showing the presence of *C pneumoniae* DNA in 100% of the spleens and lungs during the first week after infection, compared to only 33% of the carotid vessels¹³. This suggests variable susceptibility of various organs to *C pneumoniae* infection, a phenomenon also described by others¹⁴.

Cerebral *C pneumoniae* infection enhances microglial activation in the hippocampus of atherosclerotic mice. By measuring the number of activated microglial cells in *C pneumoniae* infected mice, we found a significant increase in number of activated microglial cells in the brains of *C pneumoniae* PCR positive ApoE/LDLr KO mice, compared to mock-infected mice. There were no differences in microglial activation between the *C pneumoniae* infected *C pneumoniae* PCR negative mice and to the mock-infected mice. Furthermore we observed a higher number of activated microglia in atherosclerotic mice compared to non-atherosclerotic mice.

The microglial activation is found in the acute phase of the infection, i.e. during the first week after inoculation and remains for at least three weeks after infection. Microglial activation is associated with a number of neurodegenerative diseases, such as Alzheimer's disease (AD). The characterized amyloid depositions in the AD brain are surrounded by clustered activated microglia, in an attempt to phagocytose and degrade amyloid¹⁷. These activated microglia are responsible for the neuronal injury and neuronal loss seen in AD, by means of the production of neurotoxic agents such as inflammatory cytokines, complement factors and acute phase proteins^{18,19}. An identical pathogenetic mechanism can be assumed for neuronal damage seen in chronic cerebral ischaemia, as in these circumstances microglial activation is also a prominent feature. The role of *C pneumoniae* in this can be double edged, by playing a role in the vascular changes and by cerebral *C pneumoniae* infection and subsequent microglial activation. The association between cerebral atherosclerosis and microglial activation has never been described before. Microglial activation is seen after focal cerebral ischaemia induced by middle cerebral artery occlusion²⁰. In humans activated microglia were demonstrated in autopsied brains from patients with Binswanger's disease²¹, which is characterized by diffuse ischaemic white matter damage due to hypoperfusion.

PCR cannot localize the micro-organism within the tissue, so it remains unclear which cells within the sample contain the chlamydial DNA. The detected *C pneumoniae* DNA can be present in brain tissue, as well as in the endothelial cells of cerebral blood vessels. In an attempt to localize *C pneumoniae* within

the brain tissue we used IHC but we were not able to detect positive cells, although the positive controls used in our tests showed presence of intracellular *C pneumoniae*. Our PCR is very sensitive and can detect up to one copy of DNA, so one explanation for the negative IHC might be that only a small amount of *C pneumoniae* is present within the brain tissue.

Studies performed in our laboratory have proved that intraperitoneal *C pneumoniae* injection results in dissemination to the vessel wall and various organs, indicating general infection¹³. Since our infection method was the same as in these studies we can assume that this general infection also took place in our mice, even though we only tested the presence of cerebral *C pneumoniae*. However, significant enhancement of microglial activation only occurs in infected PCR positive mice, indicating that the microglial activation observed is an effect of cerebral *C pneumoniae* infection, as opposed to a general inflammatory response.

C pneumoniae infection did not alter the integrity of the BBB. Neither the infected nor the mock-infected mice showed a higher permeability of the BBB, in both ApoE/LDLr knockout and C57BL/6J mice. Monocytes can be transported over an intact BBB under physiological conditions²². Intracellular micro-organisms, like *Streptococcus suis*, can cross the BBB and enter the brain via the so-called "Trojan horse mechanism"²³. *C pneumoniae* is known to reside in the peripheral blood monocyte²⁴, and has recently been detected in leucocytes²⁵, and as such could use this Trojan horse mechanism to enter the brain over an intact BBB. Once inside the brain, *C pneumoniae* is responsible for enhancement of microglial activation, which, through release of neurotoxic agents can lead to neuronal injury and loss.

In conclusion: Intraperitoneal *C pneumoniae* injection leads to intra-cerebral *C pneumoniae* infection. This process is not accompanied by or caused by an enhanced permeability of the BBB. Intra-cerebral *C pneumoniae* infection causes enhanced microglial activation in ApoE/LDLr KO mice. Very small amounts of *C pneumoniae* DNA might already be responsible for this effect. Since microglial activation has been indicated as a key factor in neurodegenerative and neuro-inflammatory disorders such as AD and MS, infection with *C pneumoniae* might contribute to these processes. Whether this is a process unique for *C pneumoniae* remains to be tested in further experiments.

References

1. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK, Valtonen V. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet*. 1988;2:983-6.
2. Cook PJ, Honeybourne D, Lip GY, Beevers DG, Wise R, Davies P. Chlamydia pneumoniae antibody titres are significantly associated with acute stroke and transient cerebral ischaemia: the West Birmingham Stroke Project. *Stroke*. 1998;29:404-10.
3. Elkind MS, Lin IF, Grayston JT, Sacco RL. Chlamydia pneumoniae and the risk of first ischaemic stroke : The Northern Manhattan Stroke Study. *Stroke*. 2000;31:1521-5.
4. Grayston JT. Infections caused by Chlamydia pneumoniae strain TWAR. *Clin Infect Dis*. 1992;15:757-61.
5. Rosenfeld ME, Blessing E, Lin TM, Moazed TC, Campbell LA, Kuo C. Chlamydia, inflammation, and atherogenesis. *J Infect Dis*. 2000;181 Suppl 3:S492-7.
6. Persidsky Y. Model systems for studies of leukocyte migration across the blood - brain barrier. *J Neurovirol*. 1999;5:579-90.
7. MacIntyre A, Abramov R, Hammond CJ, Hudson AP, Arking EJ, Little CS, Appelt DM, Balin BJ. Chlamydia pneumoniae infection promotes the transmigration of monocytes through human brain endothelial cells. *J Neurosci Res*. 2003;71:740-50.
8. Gonzalez-Scarano F, Baltuch G. Microglia as mediators of inflammatory and degenerative diseases. *Annu Rev Neurosci*. 1999;22:219-40.
9. Tomimoto H, Aikiguchi I. [The role of immunologic reactions in the pathogenesis in Binswanger's disease; a clue to therapeutic approach]. *Rinsho Shinkeigaku*. 1999;39:56-8.
10. Roblin PM, Dumornay W, Hammerschlag MR. Use of HEP-2 cells for improved isolation and passage of Chlamydia pneumoniae. *J Clin Microbiol*. 1992;30:1968-71.
11. Warschkau H, Kiderlen AF. A monoclonal antibody directed against the murine macrophage surface molecule F4/80 modulates natural immune response to *Listeria monocytogenes*. *J Immunol*. 1999;163:3409-16.
12. Long JM, Kalehua AN, Muth NJ, Hengemihle JM, Jucker M, Calhoun ME, Ingram DK, Mouton PR. Stereological estimation of total microglia number in mouse hippocampus. *J Neurosci Methods*. 1998;84:101-8.
13. Ezzahiri R, Stassen FR, Kurvers HA, van Pul MM, Kitslaar PJ, Bruggeman CA. Chlamydia pneumoniae infection induces an unstable atherosclerotic plaque phenotype in LDL-receptor, ApoE double knockout mice. *Eur J Vasc Endovasc Surg*. 2003;26:88-95.
14. Ezzahiri R, Nelissen Vrancken HJMG, Kurvers HAJM, Stassen FRM, Vliegen I, Grauls GELM, van Pul MML, Kitslaar PJEHM, Bruggeman CA. Chlamydia pneumoniae (Chlamydia pneumoniae) accelerates the formation of complex atherosclerotic lesions in Apo E3-Leiden mice. *Cardiovasc Res*. 2002;56:269.
15. Mulder M, Blokland A, van den Berg DJ, Schulten H, Bakker AH, Terwel D, Honig W, de Kloet ER, Havekes LM, Steinbusch HW, de Lange EC. Apolipoprotein E protects against neuropathology induced by a high-fat diet and maintains the integrity of the blood-brain barrier during aging. *Lab Invest*. 2001;81:953-60.
16. Little CS, Hammond CJ, MacIntyre A, Balin BJ, Appelt DM. Chlamydia pneumoniae induces Alzheimer-like amyloid plaques in brains of BALB/c mice. *Neurobiol Aging*. 2004;25:419-29.
17. Rogers J, Strohmeier R, Kovelowski CJ, Li R. Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. *Glia*. 2002;40:260-9.
18. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeier R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. Inflammation and Alzheimer's disease. *Neurobiol Aging*. 2000;21:383-421.
19. Eikelenboom P, Bate C, Van Gool WA, Hoozemans JJ, Rozemuller JM, Veerhuis R, Williams A. Neuroinflammation in Alzheimer's disease and prion disease. *Glia*. 2002;40:232-9.

20. Buttini M, Appel K, Sauter A, Gebicke-Haerter PJ, Boddeke HW. Expression of tumor necrosis factor alpha after focal cerebral ischaemia in the rat. *Neuroscience*. 1996;71:1-16.
21. Rosenberg GA, Sullivan N, Esiri MM. White matter damage is associated with matrix metalloproteinases in vascular dementia. *Stroke*. 2001;32:1162-8.
22. Qing Z, Sewell D, Sandor M, Fabry Z. Antigen-specific T cell trafficking into the central nervous system. *J Neuroimmunol*. 2000;105:169-78.
23. Williams AE, Blakemore WF. Pathogenesis of meningitis caused by *Streptococcus suis* type 2. *J Infect Dis*. 1990;162:474-81.
24. Kuo CC, Jackson LA, Campbell LA, Grayston JT. *Chlamydia pneumoniae* (TWAR). *Clin Microbiol Rev*. 1995;8:451-61.
25. Berger M, Schroder B, Daeschlein G, Schneider W, Busjahn A, Buchwalow I, Luft FC, Haller H. *Chlamydia pneumoniae* DNA in non-coronary atherosclerotic plaques and circulating leukocytes. *J Lab Clin Med*. 2000;136:194-200.

Chapter 4

Absence of *Chlamydia pneumoniae*,
Cytomegalovirus and Herpes Simplex Virus
type 1 in cerebral large vessels, small vessels
and brain tissue in clinically unmanifested
atherosclerosis of the brain

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Abstract

Aim of the study

To explore whether *Chlamydia pneumoniae*, Cytomegalovirus and Herpes Simplex Virus type 1 could be detected in large and small cerebral arteries, as well as in an area of brain parenchyma where white matter lesions (leukoaraiosis) can be found, in patients with clinically unmanifested cerebrovascular atherosclerosis.

Methods and results

Arterial specimens from the basilar artery and middle cerebral artery, and brain samples from the basal ganglia and periventricular white matter were obtained. Neuropathological changes were assessed in Haematoxylin/Eosin stained sections. Polymerase chain reaction (PCR) was performed on paraffin embedded sections. Subsequently, we performed immunohistochemical staining on samples, which were found positive in PCR. We failed to detect *Chlamydia pneumoniae*, CMV, or HSV-1, in any of the cerebral large vessels. In the brain tissue, we found only one case positive for CMV, and one for *Chlamydia pneumoniae*.

Conclusions

Our findings suggest a limited role for *Chlamydia pneumoniae*, CMV and HSV-1 in cerebral large and small vessel atherosclerosis.

Introduction

Atherosclerosis is an inflammatory disease. The 'response to injury' hypothesis proposes endothelial dysfunction as the initiating factor, followed by influx of monocyte-derived macrophages and T-lymphocytes¹. Over the past decade there has been much speculation about the role of micro-organisms in the activation and maintenance of this inflammatory response. The micro-organisms most extensively investigated in this context are *Chlamydia pneumoniae* (*C pneumoniae*) and Cytomegalovirus (CMV)²⁻⁴. Some reports have described the role of Herpes Simplex Virus type 1 (HSV-1) as well^{5,6}. *C pneumoniae* and CMV both reside in the peripheral blood monocyte, from which they can spread into the atherosclerotic plaque^{7,8}. For both *C pneumoniae* and CMV, the relationship between infection and atherosclerosis was first established in the late 80's^{9,10}. Two studies showed that patients suffering from cardiovascular disease more often had antibodies against the micro-organisms than a matched control group. After these initial reports various studies followed, using different techniques to document an infection, like serology, immunohistochemistry (IHC) and polymerase chain reaction (PCR) (reviewed in²). The results of these studies varied, and not all confirmed the association. Pathological studies have shown the presence of *C pneumoniae*¹¹, CMV¹² and HSV-1^{13,14}, in arterial specimens from various sites. One study showed the presence of all three micro-organisms in the same carotid arterectomy sample¹⁵. Most of the aforementioned studies focused on the role of these micro-organisms in cardiovascular disease and their presence in the atherosclerotic plaques of coronary and carotid arteries. There are only a few reports on the relationship between infection with these micro-organisms and cerebrovascular disease (CVD)¹⁶⁻¹⁹. Even less attention has been paid to the actual presence of these micro-organisms in the large cerebral vessels, with only a few reports on the presence of *C pneumoniae* in these vessels²⁰⁻²². In the study by Rassu et al., the presence of CMV was studied as well, the presence of HSV-1 has never been investigated in this context.

Factors involved in CVD may differ from those in cardiovascular disease. CVD may broadly be divided in two entities: large vessel disease (LVD) and small vessel disease (SVD). LVD, which is due to atherosclerosis of the larger cerebral vessels, causes mainly larger, usually called territorial infarcts, which often involve the cortex. The risk factors for large vessel disease are similar to those of cardiovascular disease in general. SVD causes small infarcts, less than 15 mm in diameter on cerebral imaging, which are located in the deep brain structures (basal ganglia and internal capsule) or the pons; these are often referred to as lacunar infarcts. SVD is also the cause of more diffuse damage to the white matter of the brain: leukoaraiosis. The clinical manifestation of either LVD or SVD is consistent over time²³. Therefore, it is

rational to investigate potential causes or factors that sustain these two types of cerebrovascular diseases separately. The role of *C pneumoniae*, CMV and HSV-1 in SVD has never been investigated in this respect.

The aim of this study was to investigate whether the presence of *C pneumoniae*, CMV and HSV-1 could be established in the large and small cerebral arteries, as well as in brain parenchyma where leukoaraiosis can be found, in cases with pathological evidence of cerebrovascular atherosclerosis.

Materials and methods

Autopsy specimens

Included in the study were autopsy specimens from ten men and nine women, ranging from 33 to 80 years of age (median 70). Patient characteristics are summarized in Table 4.1. At autopsy the brain was removed, and immersed in 10% buffered formaldehyde for at least two weeks. The large cerebral vessels were dissected after which the brain was cut in frontal sections. The material was embedded in paraffin and used for routine neuropathological examination. For this study, specimens were obtained from the areas supplied by small vessels, i.e., the basal ganglia and the periventricular white matter. Arterial specimens included the distal 3 cm of the basilar artery (BA) and the proximal 3 cm of the middle cerebral artery (MCA), directly after it branches from the internal carotid artery. Arterial sites showing macroscopic signs of atherosclerosis were also included. The large vessels were embedded longitudinally. Paraffin embedded material was used for Haematoxylin/Eosin (HE) staining, immunohistochemical staining and polymerase chain reaction (PCR). Ten 5 μ m sections were cut, followed by a 50 μ m section. This was serially continued until all material was used. The first of each 5 μ m section was used for HE staining; the following 5 μ m section was used for IHC. The 50 μ m sections were used for PCR; three 50 μ m sections were pooled in one serum tube and stored at 4°C until further processing.

Serology

Post-mortem serum samples were taken from each case. The sera were kept at -20°C until further processing. *C pneumoniae* serology was performed using a microimmunofluorescence test (Labsystems, Helsinki, Finland). Samples were considered positive when titres were 1/32 or higher.

CMV and HSV-1 antibodies were aimed to detect using commercially available Enzyme Linked Immunoabsorbent Assays used in routine diagnosis of these

antibodies (Serion, Germany). Samples were considered positive when titres were >30.

Table 4.1 Case characteristics

Case	Sex	Age	LVD	SVD	Cause of death
1	F	80.0	+	NA	Hemorrhagic stroke
2	M	51.0	0	NA	Hepatic encephalopathy
3	M	87.0	++	+	Ischaemic stroke
4	M	73.0	+++	NA	Cerebellar hematoma
5	F	57.0	+	NA	Gastrointestinal bleeding
6	M	74.0	++	+	Cardiac arrest
7	M	62.0	+	+	Respiratory arrest
8	M	63.0	+++	+++	Hemorrhagic stroke
9	M	47.0	+	++	Cerebral metastasis
10	M	39.0	+	0	Sepsis
11	M	81.0	+++	+	Ischaemic stroke
12	F	70.0	++	+	Non-hodgkin lymphoma
13	F	33.0	+	+	Myocardial infarction
14	M	79.0	+++	++	Hemorrhagic stroke
15	F	86.0	+++	+	Post op bleeding
16	M	69.0	++	+++	Myocardial ischaemia
17	F	86.0	+++	+	Meningitis
18	M	65.0	+	+	Myocardial ischaemia
19	M	79.0	++	0	Aortic dissection

LVD: + mild atherosclerosis: intimal fibrosis; ++ moderate atherosclerosis: fibro-atheromatous plaques; +++ severe atherosclerosis: fibro-atheromatous plaques with calcification.

SVD: + Mild: concentric vessel wall thickening in small arteries and arterioles but with mild or minimal luminal narrowing ++ moderate: significant luminal narrowing, but with the lumen spanning more than half the total external diameter; +++ severe: the internal vessel diameter was less than half of the external diameter NA: not assessed.

Tissue preparation and PCR analysis

Prior to DNA isolation, the sections were deparaffinized by adding 700 µl of xylol. After this they were vibrated for one minute using the Vortex® and centrifuged for ten minutes at 40000 rpm in a Micro 22R® rotator. This procedure was repeated. After deparaffination, the sections were rehydrated by adding 70% ethanol. Again, the samples were vibrated using the Vortex® for one minute and centrifuged for ten minutes at 40000 rpm in the Micro 22R® rotator. This was also done twice. Following deparaffination, DNA was extracted from brain tissue with the Wizard Genomic DNA purification kit (Promega, Germany). Then, the purified DNA was subjected to PCR aiming to detect either *C pneumoniae*, CMV or HSV-1 DNA.

All PCR's were performed in a total volume of 50 μ l, containing 1 μ g purified DNA, 0.1 mM of each dNTP, 0.5 μ M of each primer:

- *C. pneumoniae* CPC, 5'-TTA TTA ATT GAT GGT ACA ATA-3'; antisense CPD, 5'-ATC TAC GGC AGT AGT ATA GTT-3';
- CMV: sense 5' GCG GGA GAT GTG GAT GGC TTG TAT TAA GGA 3'-antisense 5'-GCA GAC TCT CAG AGG ATC GGC CCC C-3';
- HSV-1: sense 5'-GCA TCG TCG AGG TGG AC-3'; antisense 5'-CCT GCC ACT TGG TCA TG-3' DNA

Amplification was carried out in a Perkin Elmer 9600 thermal cycler, using the following cycling conditions:

- *C. pneumoniae*: 37°C for ten minutes and 95°C for fifteen minutes. 20 cycles of "Touchdown PCR" were performed from 60°C to 50°C, followed by 40 cycles of 1 minute at 94°C, 50°C, and 72°C.
- CMV: 37°C for ten minutes and 95°C for fifteen minutes. PCR was performed in 50 cycles of 30 seconds at 95°C, 70°C and 72°C.
- HSV-1: 37°C for ten minutes and 95°C for fifteen minutes. PCR was performed in 50 cycles of 30 seconds at 94°C, 69°C and 72°C.

After this the PCR products were kept overnight at 15°C. The PCR products were resolved in a 2% agarose gel, stained with ethidium bromide, and photographed.

Histology and immunohistochemistry

Five μ m paraffin sections were used for HE staining, performed using standard procedures. The morphological changes were evaluated on routinely processed 5 μ m HE sections. The evaluation of the vascular changes was performed by the neuropathologist (BK), who was blinded for case characteristics. Cerebral large vessel atherosclerosis was graded as mild (+) moderate (++) , and severe (+++) when intimal fibrosis, fibro-atheromatous plaques, or fibro-atheromatous plaques with calcification were present, respectively. The changes in the small cerebral vessels were recorded as mild (+), moderate (++) , or severe (+++) in each HE stained block from the basal ganglia or periventricular white matter. Mild SVD was defined as concentric vessel wall thickening in small arteries and arterioles but with mild or minimal luminal narrowing, moderate SVD implied significant luminal narrowing, but with the lumen spanning more than half the total external diameter. In severe SVD the internal vessel diameter was less than half of the external diameter²⁴. Apart from this the presence of lacunar infarcts was recorded.

Since the PCR is the most sensitive technique to detect the presence of micro-organisms, we first used the PCR to detect CMV, *C. pneumoniae* and HSV-1. In cases that were positive by PCR, IHC was performed, to identify the specific cells infected with each micro-organism. For *C. pneumoniae*, the RR402 mouse

monoclonal antibody (against a major outer membrane protein MOMP of *C pneumoniae*) (DAKO, Denmark) was used as the primary antibody (dilution 1:50). This antibody is specific for *C pneumoniae*²⁵. Normal mouse ascites was used as a negative control. Five μm thick paraffin embedded sections were pre-incubated with 2% Bovine Serum Antigen (BSA) in PBS to block aspecific staining, after which the slides were incubated with the primary antibody at 37°C, for one hour. After washing in Phosphate Buffered Saline (PBS), the sections were incubated with the secondary antibody: biotinylated goat anti-mouse serum (DAKO, Denmark). Immunostaining was performed by the avidin-biotin alkaline phosphatase method (DAKO, Denmark). Fast red was used as a chromogen, after which the sections were counterstained with haematoxylin. Infected Hep-2 cells were used as a positive control; mock infected Hep-2 cells were used as negative control. The same method was used for the immunohistochemical staining of CMV using an antibody against the E13 gene of human CMV as the primary antibody (Argene Biosoft, France) (dilution 1:50).

Results

Cerebral large vessels

From all cases, either the BA or MCA was available for assessment of cerebral large vessel atherosclerosis. Both vessels could be examined in 14/19 cases. In five cases cerebral vessels other than the BA or MCA showed macroscopic signs of atherosclerosis; these were three vertebral artery samples, one anterior cerebral artery, and the intracranial part of one internal carotid artery, respectively. In all but one case microscopic atherosclerotic changes were seen in at least one of the arteries. The case without atherosclerotic changes was a 51-year old male who had died from hepatic encephalopathy. In total, 43 arterial specimens from 19 patients could be investigated. Four of these samples (9%) were without atherosclerotic changes. In the remaining 91% atherosclerotic changes varied from intima fibrosis (35%), and fibro-atheromatous plaques (35%), to fibro-atheromatous plaques with calcification (21%). Territorial cerebral infarction as a result of large vessel atherosclerosis was seen in two cases. More severe atherosclerosis was observed in cases that had died from vascular causes, but this difference was not significant. Using the PCR method, we failed to establish the presence of *C pneumoniae*, CMV, or HSV-1 in any of the cerebral large vessels. No immunohistochemical staining was done on the cerebral large vessel specimens, since we only performed IHC on specimens positive in PCR.

Cerebral small vessels, basal ganglia, periventricular white matter

In 15/19 cases cerebral small vessel samples and brain tissue were available for neuropathological examination. SVD was present in either the basal ganglia or periventricular white matter in 13 of these. SVD was mostly observed in the basal ganglia, with some degree of SVD (changes to the cerebral small vessels or lacunar infarction) present in 9/15 cases. Signs of SVD in the periventricular white matter were found in 5/15 cases. When SVD was present, it was graded as mild, with concentric vessel wall thickening in small arteries and arterioles but with mild or minimal luminal narrowing, in 71 percent of the samples. Lacunar infarcts were found in 7/15 basal ganglia, and in 2/15 of periventricular white matter samples. We found only one case positive for CMV, and one for *C. pneumoniae*. In the first case, which had died of basilar artery thrombosis, CMV was found in the basal ganglia. In the second case, which died from a post-operative bleeding after a laparotomy, *C. pneumoniae* was found in the periventricular white matter. IHC showed the presence of *C. pneumoniae* in the PCR positive sample. *C. pneumoniae* was present in ganglion cells and underneath the basal membrane, probably in myo-fibrocytes of the medial lamina of the arteriole. By IHC the presence of CMV in the PCR positive sample could not be confirmed. Since PCR did not show any HSV-1 DNA in any of the samples, IHC for HSV-1 was not performed.

Serology

IgG antibodies against *C. pneumoniae* were found in 15/19 cases, HSV-1 in 16 and, IgG antibodies against CMV in 18.

Both cases that were found positive in PCR had antibodies against CMV and *C. pneumoniae*.

Discussion

Cerebral large vessel disease

We failed to establish the presence of *C. pneumoniae*, CMV or HSV-1 DNA in large vessel samples of patients who died from various causes, but who all had signs of atherosclerosis in at least one of the cerebral arteries, varying from intima fibrosis to fully developed atherosclerotic plaques with calcifications.

Considering these findings, our results do not support the hypothesis that these micro-organisms play a conditional role in the generation of cerebral atherosclerosis, be it large or small vessel disease. However, our data cannot rule out that these micro-organisms do play a role in clinically manifest large vessel

disease, since the majority of our patient group was asymptomatic; only two out of nineteen patients had had a clinical ischemic stroke.

The presence of micro-organisms in atherosclerotic plaques is patchy. Therefore, detection rates vary between studies²⁶. Even if the vessels are embedded and processed longitudinally, and are of considerable length like in our study, not all the material is used in the PCR. Therefore, although the PCR can detect up to one copy of DNA, false negative results may occur as a result of a "sampling error". This patchy presence of micro-organisms is also responsible for the differences in results between IHC and PCR²⁷. In one study, that used IHC to detect *C pneumoniae* in the cerebral vessels of asymptomatic patients, *C pneumoniae* was found in only two percent, even though these specimens showed various signs of atherosclerosis²⁰. When PCR was used to detect *C pneumoniae* in fully developed atherosclerotic lesions, the micro-organism was found in one third of the vessels²¹.

For PCR, frozen sections as well as formalin fixed sections are used. Deparaffinization of formalin fixed sections, which is needed to allow isolation of DNA from the material, might result in loss of DNA. However, Gass et al. showed that correctly processed material even when embedded in paraffin should provide enough DNA to establish the presence of the micro-organism²⁸. The presence of CMV DNA in atherosclerotic tissue was first reported in a study using formalin fixed sections; CMV was found in 94 percent of the samples²⁹. Studies on the presence of CMV in atherosclerotic tissue showed a prevalence varying from 0%³⁰ to 94%²⁹. Using frozen sections or paraffin embedded tissue for PCR analysis, does not influence the prevalence of CMV. Therefore, it is unlikely that the absence of micro-organisms in our samples is due to the fact that we used formalin fixed sections.

Cerebral small vessel disease

We detected *C pneumoniae* DNA in the brain tissue of one out of 19 cases, and found CMV DNA in one other case. We could not detect HSV-1 DNA in any of the samples. PCR is considered the gold standard for detection of micro-organisms, but it has several disadvantages. Since PCR techniques become more and more sensitive, even very small amounts of DNA can be detected, leaving the question open for the clinical relevance of some findings. Our PCR's are among the most sensitive, with *C pneumoniae* being detected from up to one copy of DNA and both CMV and HSV-1 from up to 10 copies of DNA. Another disadvantage is that PCR does not localize the micro-organism within the tissue. In an attempt to localize the micro-organisms we used immuno-histochemistry on the samples positive in PCR, *C pneumoniae* was found in the basal ganglia, more specifically in ganglion cells and underneath

the basal membrane of a small arteriole. The CMV presence could not be confirmed by IHC.

Studies on the prevalence of *C pneumoniae*, CMV and HSV-1 in brain tissue, detected these micro-organisms in 95³¹, 94³² and 67³³ percent. However, the brain samples tested in these studies came from frontal and temporal cortex, whereas all the patients had clinically manifest dementia. No studies have been reported on the presence of *C pneumoniae*, CMV or HSV-1 in the basal ganglia or the periventricular white matter, the areas of the brain supplied by small vessels.

Our results do not point at an important role of the micro-organisms in the generation of SVD, neither as a cause of lacunar infarcts, nor as that of leukoaraiosis. On the other hand, our results cannot deny any role of these micro-organisms in eventual sustenance or acceleration of evident small vessel disease, which can only be accomplished with studying cases with clinically manifest small vessel cerebrovascular disease. However, with the early case fatality rate in small vessel stroke being low, such study would not be easy to perform. Although signs of small vessel disease were found in 13 of our 15 cases, none had clinically manifest disease.

Patients presenting with clinically apparent SVD can be divided in two subtypes with different prognosis. If at first clinical stroke one or more 'silent' lacunar infarcts and/or leukoaraiosis are present, prognosis for recurrent stroke and death are worse²³. It is hypothesized that there are two entities of SVD, with different underlying pathogenesis; micro-atheromatosis in single symptomatic stroke and arteriolosclerosis in the other subtype. There is evidence that in arteriolosclerosis there is a leak in the blood brain barrier, with subsequent leakage of plasma components into the brain, causing neuronal damage³⁴. Hypothetically, blood components, but also micro-organisms such as *C pneumoniae*, CMV or HSV-1 could infiltrate the brain tissue in such patients. It is still unclear whether the presence of these micro-organisms merely reflects a BBB dysfunction, or whether these micro-organisms are a cause of BBB dysfunction. Our series was too small to consider the two lacunar stroke subtypes separately, and therefore cannot deny any differential effect of the micro-organisms in the clinically manifest two types of cerebral small vessel disease.

In contrast to our findings with PCR and IHC, antibodies against *C pneumoniae*, CMV and HSV-1 were found in the majority of the cases. This concurs with the prevalence of these antibodies in a general population. Positive antibody titres do not predict the presence of micro-organisms in the vascular wall of cases, whereas the opposite is also true³⁵.

Conclusions

We were unable to detect *C pneumoniae*, CMV or HSV-1 in atherosclerotic large cerebral vessels, despite the presence of moderate to severe atherosclerotic changes in these vessels. Except for one case, in which *C pneumoniae* and CMV were found in a sample of basal ganglia and periventricular white matter, this was also the case with respect to the cerebral small vessels. Our findings suggest a limited role for *C pneumoniae*, CMV and HSV-1 in large and small vessel atherosclerosis. Whether these micro-organisms play a significant role in symptomatic cerebral LVD and different types of SVD needs to be determined in further studies.

References

1. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999;340:115-26.
2. de Boer OJ, van der Wal AC, Becker AE. Atherosclerosis, inflammation, and infection. *J Pathol*. 2000;190:237-43.
3. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Wong YK, Bernardes Silva M, Ward M. Chlamydia pneumoniae igg titres and coronary heart disease: Prospective study and meta-analysis. *British Medical Journal*. 2000;321:208-12.
4. Bruggeman C. Does cytomegalovirus play a role in atherosclerosis? *Herpes*. 2000;7:51-4.
5. Ridker PM, Hennekens CH, Stampfer MJ, Wang F. Prospective study of herpes simplex virus, cytomegalovirus, and the risk of future myocardial infarction and stroke. *Circulation*. 1998;98:2796-9.
6. Sorlie PD, Adam E, Melnick SL, Folsom A, Skelton T, Chambless LE, Barnes R, Melnick JL. Cytomegalovirus/herpesvirus and carotid atherosclerosis: The alic study. *J Med Virol*. 1994;42:33-7.
7. May AE, Redecke V, Gruner S, Schmidt R, Massberg S, Miethke T, Ryba B, Prazeres Da Costa C, Schomig A, Neumann FJ. Recruitment of chlamydia pneumoniae-infected macrophages to the carotid artery wall in noninfected, nonatherosclerotic mice. *Arterioscler Thromb Vasc Biol*. 2003;23:789-94.
8. Guetta E, Guetta V, Shibutani T, Epstein SE. Monocytes harboring cytomegalovirus: Interactions with endothelial cells, smooth muscle cells, and oxidized low-density lipoprotein. Possible mechanisms for activating virus delivered by monocytes to sites of vascular injury. *Circ Res*. 1997;81:8-16.
9. Adam E, Melnick JL, Probstfield JL, Petrie BL, Burek J, Bailey KR, McCollum CH, DeBaakey ME. High levels of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis. *Lancet*. 1987;2:291-3.
10. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK, Valtonen V. Serological evidence of an association of a novel chlamydia, twar, with chronic coronary heart disease and acute myocardial infarction. *Lancet*. 1988;2:983-6.
11. Taylor Robinson D, Thomas BJ. Chlamydia pneumoniae in atherosclerotic tissue. *J Infect Dis*. 2000;181 Suppl 3:S437-40.
12. Radke PW, Merkelbach Bruse S, Messmer BJ, vom Dahl J, Dorge H, Naami A, Vogel G, Handt S, Hanrath P. Infectious agents in coronary lesions obtained by endarterectomy: Pattern of distribution, coinfection, and clinical findings. *Coron Artery Dis*. 2001;12:1-6.
13. Espinola Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp H, Rippin G, Hafner G, Pfeifer U, Meyer J. Are morphological or functional changes in the carotid artery wall associated with chlamydia pneumoniae, helicobacter pylori, cytomegalovirus, or herpes simplex virus infection? *Stroke*. 2000;31:2127-33.
14. Yamashiroya HM, Ghosh L, Yang R, Robertson AL, Jr. Herpesviridae in the coronary arteries and aorta of young trauma victims. *Am J Pathol*. 1988;130:71-9.
15. Chiu B, Viira E, Tucker W, Fong IW. Chlamydia pneumoniae, cytomegalovirus, and herpes simplex virus in atherosclerosis of the carotid artery. *Circulation*. 1997;96:2144-8.
16. Cook PJ, Honeybourne D, Lip GYH, Beevers DG, Wise R, Davies AH. Chlamydia pneumoniae antibody titers are significantly associated with acute stroke and transient cerebral ischemia: The west birmingham stroke project. *Stroke*. 1998;29:404-10.
17. Elkind MS, Lin IF, Grayston JT, Sacco RL. Chlamydia pneumoniae and the risk of first ischaemic stroke : The northern manhattan stroke study. *Stroke*. 2000;31:1521-5.
18. Fagerberg B, Gnarpe J, Gnarpe H, Agewall S, Wikstrand J. Chlamydia pneumoniae but not cytomegalovirus antibodies are associated with future risk of stroke and cardiovascular disease: A prospective study in middle-aged to elderly men with treated hypertension. *Stroke*. 1999;30:299-305.
19. Wimmer ML, Sandmann Strupp R, Saikku P, Haberl RL. Association of chlamydial infection with cerebrovascular disease. *Stroke*. 1996;27:2207-10.

20. Vink A, Poppen M, Schoneveld AH, Roholl PJ, de Kleijn DP, Borst C, Pasterkamp G. Distribution of chlamydia pneumoniae in the human arterial system and its relation to the local amount of atherosclerosis within the individual. *Circulation*. 2001;103:1613-7.
21. Virok D, Kis Z, Karai L, Intzedy L, Burian K, Szabo A, Ivanyi B, Gonczol E. Chlamydia pneumoniae in atherosclerotic middle cerebral artery. *Stroke*. 2001;32:1973-6.
22. Rattu M, Cazzavillan S, Scagnelli M, Peron A, Bevilacqua PA, Facco M, Bertoloni G, Lauro FM, Zambello R, Bonoldi E. Demonstration of chlamydia pneumoniae in atherosclerotic arteries from various vascular regions. *Atherosclerosis*. 2001;158:73-9.
23. de Jong G, Kessels F, Lodder J. Two types of lacunar infarcts: Further arguments from a study on prognosis. *Stroke*. 2002;33:2072-6.
24. Lammie GA, Brannan F, Slattery J, Warlow C. Nonhypertensive cerebral small-vessel disease. An autopsy study. *Stroke*. 1997;28:2222-9.
25. Grayston JT, Campbell LA, Kuo CC, Mordhorst CH, Saikku P, Thom DH, Wang SP. A new respiratory tract pathogen: Chlamydia pneumoniae strain twar. *J Infect Dis*. 1990;161:618-25.
26. Cochrane M, Pospischil A, Walker P, Gibbs H, Timms P. Distribution of chlamydia pneumoniae DNA in atherosclerotic carotid arteries: Significance for sampling procedures. *J Clin Microbiol*. 2003;41:1454-7.
27. Mygind T, Birkelund S, Falk E, Christiansen G. Evaluation of real-time quantitative pcr for identification and quantification of chlamydia pneumoniae by comparison with immunohistochemistry. *J Microbiol Methods*. 2001;46:241-51.
28. Gass P, Kiessling M, Schafer P, Mester C, Schmitt HP, Kuhn JE. Detection of human cytomegalovirus DNA in paraffin sections of human brain by polymerase chain reaction and the occurrence of false negative results. *J Neurol Neurosurg Psychiatry*. 1993;56:211-4.
29. Hendrix M, Daemen M, Bruggeman C. Cytomegalovirus nucleic acid distribution within the human vascular tree. *Am J Pathol*. 1991;138:563-7.
30. Watt S, Aesch B, Lanotte P, Tranquart F, Quentin R. Viral and bacterial DNA in carotid atherosclerotic lesions. *Eur J Clin Microbiol Infect Dis*. 2003;22:99-105.
31. Balin BJ, Gerard HC, Arking EJ, Appelt DM, Branigan PJ, Abrams JT, Whittum Hudson JA, Hudson AP. Identification and localization of chlamydia pneumoniae in the alzheimer's brain. *Med Microbiol Immunol (Berl)*. 1998;187:23-42.
32. Lin WR, Wozniak MA, Wilcock GK, Itzhaki RF. Cytomegalovirus is present in a very high proportion of brains from vascular dementia patients. *Neurobiol Dis*. 2002;9:82-7.
33. Lin WR, Wozniak MA, Cooper RJ, Wilcock GK, Itzhaki RF. Herpesviruses in brain and alzheimer's disease. *J Pathol*. 2002;197:395-402.
34. Wardlaw JM, Sandercock PA, Dennis MS, Starr J. Is breakdown of the blood brain barrier responsible for lacunar stroke, leukoaraiosis and dementia? *Stroke*. 2003;34:806-12.
35. Maass M, Gieffers J, Krause E, Engel PM, Bartels C, Solbach W. Poor correlation between microimmunofluorescence serology and polymerase chain reaction for detection of vascular chlamydia pneumoniae infection in coronary artery disease patients. *Med Microbiol Immunol Berl*. 1998;187:103-6.

Chapter 5

Silent ischaemic brain lesions; absence of correlation with *Chlamydia pneumoniae* antibodies and cytokine responses

Abstract

Aim of the study

To investigate whether *Chlamydia pneumoniae* antibody levels, C-reactive protein and levels of TNF α , IL-6, IL-1 β , IL-10 and IFN γ are related to the presence of silent ischaemic brain lesions on MRI in symptomatic stroke patients.

Patients and methods

Patients were derived from a study investigating the effect of a three-month course of azithromycin on progression of silent ischaemic brain lesions. Levels of high sensitivity CRP (hsCRP) and IL-6 were assessed on baseline serum samples of 149 patients obtained at randomisation, as was the *Chlamydia pneumoniae* IgG antibody titre. Cytokine levels were assessed in the supernatant of whole blood stimulated with *Chlamydia pneumoniae* antigen. Baseline brain MRI was scored on the presence of asymptomatic lacunar infarction and leukoaraiosis.

Results

No differences were found in cytokine levels, hsCRP and *Chlamydia pneumoniae* serology, between patients with and without silent brain ischaemia, or between patients with a cortical or lacunar infarct.

Conclusions

Serum inflammatory parameters and *Chlamydia pneumoniae* serology are not correlated with the presence of silent brain ischaemia on MRI, nor does it distinguish between patients with a cortical or lacunar infarct.

Introduction

Inflammation is a key factor in the initiation and progression of atherosclerosis¹. Inflammatory markers such as high sensitivity C-reactive protein (hsCRP) and Interleukin-6 (IL-6) in serum of apparently healthy men and women predict the risk of future cardiovascular disease or ischaemic cerebrovascular disease^{2,3}. Moreover, hsCRP and IL-6 levels are associated with carotid atherosclerosis^{4,5}. Infections, specifically infection with *Chlamydia pneumoniae* (*C pneumoniae*) have also been implicated in the process of atherosclerosis⁶.

C pneumoniae infection of monocytes, macrophages and endothelial cells induces the production of several inflammatory and anti-inflammatory cytokines that are implicated in the process of atherosclerosis and its clinical sequelae. When *C pneumoniae* is added to peripheral blood monocytes in vitro, production of tumour necrosis factor alpha (TNF α) and IL-6 is induced⁷. Serological studies have shown a relationship between the presence of IgG and IgA antibodies against *C pneumoniae* and ischaemic stroke⁸⁻¹¹, and *C pneumoniae* has been isolated from atherosclerotic plaques of cerebral vessels¹²⁻¹⁴. Neurological symptoms may only be the 'tip of the iceberg' of cerebral atherosclerosis. Even on CT almost 30 percent of ischaemic stroke patients have so-called silent ischaemic lesions, which are (mostly lacunar) infarcts, ischaemic white matter lesions, or both, which progress over time despite secondary prevention measures^{15,16}. It is unknown whether *C pneumoniae* infection contributes to this chronic, progressive process of cerebrovascular atherosclerosis. Therefore, we explored whether the presence of silent ischaemic brain lesions (SBL) on magnetic resonance imaging (MRI) of the brain relates to *C pneumoniae* infection, possibly mediated by inflammatory markers that are implicated in cerebral atherosclerosis. We studied lacunar and atherothrombotic ischaemic stroke separately in this respect.

Patients and methods

Patients

The patients in this study have been randomised in the ACSI study (Azithromycin for the prevention of the progression of Cerebral ischaemic lesions in patients Surviving a non-cardioembolic cerebral Infarct), which investigates the effect of a three-month treatment schedule of azithromycin on ischaemic cerebral damage progression measured at two years. At baseline patients have MRI, whereas blood was drawn for the assessment of hsCRP, IL-6 and *C pneumoniae* serology. Inclusion ended September 2003, when the

projected 170 patients had been included. In four of these patients no MRI was performed because of claustrophobia, from another two MRI was of bad quality, so that the presence of asymptomatic ischaemic lesions could not be assessed. In 15 patients serum samples were not available. Therefore, from a total of 149 patients sufficient MRI data and measured baseline serum parameters (hsCRP, IL-6 and IgG antibodies against *C pneumoniae*) were available for analysis. Baseline whole blood samples were stimulated with *C pneumoniae* in 75 of these 149 patients. The samples were stimulated for 24 or 48 hours as described by Netea et al.¹⁷, followed by measurement of TNF α , IL-6, IL-10, IL-1 beta (IL-1 β), and interferon gamma (IFN γ).

MRI

The MRI protocol included T2 and FLAIR weighted axial images of the brain. The following parameters were scored: location and type of symptomatic lesion; the presence and number of asymptomatic lacunar infarcts in the basal ganglia, internal capsule or pons; the presence and severity of periventricular hyperintensities and white matter hyperintensities in the centrum semi ovale¹⁸. The MRI's had been scored without knowledge of clinical information. Silent ischaemic brain lesions (SBL) were analysed as a dichotome variable: presence or absence of one or more silent ischaemic lesions, of white matter lesions (extending peri-ventricular lesions or confluent lesions in the centrum semi ovale), or both¹⁸.

Definition of lacunar and cortical infarcts

Lacunar infarct: an acute stroke syndrome with a MRI lesion compatible with the occlusion of a single perforating artery, consisting of a subcortical (basal ganglia, internal capsule, brainstem) small sharply demarcated hypodense lesion with a diameter less than 15 millimetre. If no such lesion was visible we used the established criteria of unilateral motor- and/or sensory signs that involved the whole of at least two of the three body parts (face, arm, leg) without disturbance of consciousness, visual fields, language or other cortical functions. We distinguished four lacunar syndromes: pure motor stroke (PMS), sensorimotor stroke (SMS), pure sensory stroke (PSS) and atactic hemiparesis/dysarthria -clumsy -hand syndrome (AH/DCHS)¹⁹.

Cortical infarct: an acute stroke syndrome with MRI findings compatible with an infarct involving the cortex. If no such lesion was present we used established clinical criteria consisting of unilateral motor and/or sensory symptoms in combination with signs of disturbance of consciousness, visual fields, language or other cortical functions. Also, patients with an isolated mono-paresis were included as well as patients with incomplete involvement of two body parts, or

with isolated cortical dysfunction (mostly aphasia) Apart from this, patients had no evidence of an existing source of cardiac embolism.

C-reactive protein

Venous blood samples drawn on randomisation for the trial were immediately centrifuged for 10 minutes at 1200 rpm and at 4°C. Serum was stored at -20°C until analysis. HsCRP was determined with the IMMULITE CRP method (Diagnostic Product Corporation, Los Angeles, USA). This assay provides a detection limit of 0.10 mg/l and has been approved by the Food and Drug Administration for clinical use in the United States.

Serum Interleukin-6

Levels of IL-6 were determined using an enzyme linked immuno absorbent assay (ELISA, eBiosciences, San Diego, USA). ELISA's were performed as instructed by the manufacturer. Briefly, 96-wells plates were coated with antigen and kept overnight at 4°C. The following day, the wells were blocked using the tests assay diluent, after which the standards and patient samples were added and incubated for one hour. A biotinylated capture antigen, horseradish peroxidase for colouring the samples and H₂SO₄ as stop solution, followed this.

Stimulated cytokines

From 75 consecutive patients, whole blood was stimulated with *C pneumoniae* antigen as follows: 200 µl of whole blood was added to 750 µl of RPMI medium and 50 µl of *C pneumoniae* antigen (3×10^6 infection forming units (IFU), dilution 1:20). Mock stimulated samples consisted of 200 µl of whole blood added to 800 µl of RPMI medium. The samples were stimulated for 24 hours (TNF α , IL-6 and IL-1 β) or 48 hours (IL-10 and IFN γ), as described by Netea et al.⁷. Cytokine levels were measured using ELISA (eBiosciences, San Diego, USA), with the method as described above for IL-6.

Measurement of IgG antibodies against *C pneumoniae*

Levels of serum IgG antibodies against *C pneumoniae* were measured using an ELISA, (LabSystems, Helsinki, Finland) as described previously²⁰. The presence or absence of *C pneumoniae* antibodies was determined by comparing the absorbance value of the sample to a cut-off value, as specified by the manufacturer. Specimens with values equal to or greater than the cut-off value were considered positive for anti-Chlamydia antibodies. For IgG the cut-off was 30.

Statistics

Since hsCRP, IL-6, and anti *C pneumoniae* IgG concentrations were skewed, a non-parametric test was used for comparison of the mean values in the groups with and without SBL. Serology values were dichotomised to assess a relationship between the levels of the different serum parameters and the presence of SBL in logistic regression analyses.

Results

Patient population

Of the 149 included patients 81 (54%) had silent ischaemic lesions. Table 5.1 shows characteristics of patients with and without silent lesions. Patients with SBL were significantly older than patients without SBL. There were 104 lacunar and 45 territorial infarct patients, of whom 54 (52%) and 27 (60%) had silent ischaemic lesions, respectively.

Table 5.1 Patient characteristics of patients with and without silent brain ischemia (SBL). Numbers indicate percentages, unless otherwise indicated. The numbers in the parenthesis indicate standard deviations.

	SBL -	SBL +	P-value
Number of patients	68	81	
Lacunar infarcts	48	52	0.36
Cortical infarcts	40	60	0.27
Male	75	61	0.29
Age (years)	61.2 (9.4)	65.8 (8.8)	0.003
Smoking	38	41	0.45
Hypertension	40	50	0.41
Infection	25	24	0.98
Seropositive for <i>C pneumoniae</i>	84	83	0.79
PAOD	7	4	0.98
Hypercholesterolaemia	21	21	0.98
DM	3	7	0.61
Statin therapy	62	48	0.09
Cardiovascular disease ^a	10	12	0.98
hsCRP (mg/l)	8.4 (18.2)	5.9 (8.1)	0.29
IL-6 (pg/ml)	6.4 (26.3)	2.8 (9.7)	0.27
IgG antibody titre (EIA)	138.5 (116.6)	128.9 (103.0)	0.59

^a History of angina pectoris, myocardial infarction or coronary by-pass surgery

Baseline serum markers

There were no differences in mean hsCRP levels, serum IL-6 levels and IgG antibody titres against *C pneumoniae* between patients with or without SBL, and patients with a cortical or lacunar infarct (data summarized in Table 5.1 and Figures 5.1 and 5.2). Linear regression analysis showed no relationship between the levels of the serum parameters and the presence of SBL. When patients with lacunar infarct were analysed separately, we also found no differences between those with or without silent ischaemic lesions.

Figure 5.1 Mean log values of hsCRP, IgG and IL-6 in patients with and without SBL.

Figure 5.2 Mean log values of hsCRP, IgG and IL-6 in patients with cortical or lacunar infarct.

IgG antibodies against *C pneumoniae*

Logistic regression analysis did not show a difference in the presence of positive IgG antibody titres between patients with and without SBL. (Crude OR for the presence of IgG antibodies: 1.30, 95% CI: 0.55-3.16, $p=0.54$)

The presence of IgG antibodies against *C pneumoniae* did not differ between atherothrombotic and lacunar infarcts (crude OR: 1.10, 95% CI: 0.44-2.95, $p=0.79$)

C pneumoniae antibody titre was not related to hsCRP and IL-6 levels, mean values between seropositive and seronegative patients were similar. (3.9 ± 3.7 vs. 5.5 ± 6.6 , $p=0.31$ for hsCRP, and 2.1 ± 3.7 vs. 6.1 ± 23.8 , $p=0.42$ for IL-6).

Stimulated cytokines

Mean values of $\text{TNF}\alpha$, IL-6, IL-1 β , IL-10 and IFN γ , did not differ between patients with or without SBL, even when analysed separately for lacunar infarct patients (Table 5.2 and figure 5.3). Levels of cytokines did not differ between patients with a cortical infarct and a lacunar infarct (figure 5.4). There were neither correlations between levels of hsCRP and the levels of the stimulated cytokines, nor was there any relationship between *C pneumoniae* antibody titres and cytokine levels.

Table 5.2 Levels of inflammatory parameters in whole blood stimulated with *C pneumoniae* antigen in patients with and without silent brain ischemia (SBL).^a

	SBL -	SBL +	P-value
Number of patients	36	39	
$\text{TNF}\alpha$ (pg/ml)	9.6 (39.8)	36.1 (120.5)	0.18
IL-6 (pg/ml)	88.7 (90.6)	76.7 (77.0)	0.93
IL-1 β (pg/ml)	64.4 (103.5)	96.3 (148.2)	0.34
IL-10 (pg/ml)	25.7 (54.2)	23.0 (57.0)	0.99
IFN γ (pg/ml)	17.6 (68.6)	36.5 (91.2)	0.65

^a Values are given as mean and standard deviation

Figure 5.3 Mean log values of $\text{TNF}\alpha$, IL-6, IL-1 β , IL-10 and IFN γ in patients with and without SBL.

Figure 5.4 Mean log values of TNF α , IL-6, IL-1 β , IL-10 and IFN γ in patients with cortical or lacunar infarct.

Discussion

In our 149 patients, of whom 81 had silent lesions, we were unable to detect differences between patients with or without "silent" ischaemic lesions for a number of inflammatory markers. Also, when lacunar stroke patients were considered separately, no differences emerged. We also could neither detect a correlation between the presence of serum *C pneumoniae* antibodies and silent lesions, nor with any of the inflammatory markers. Our findings argue against a prominent role of *C pneumoniae* infection in the genesis or sustenance of the vascular pathology that underlies silent ischaemic brain lesions. They also do not sustain the idea that infection with this microorganism influences the process of endothelial activation in patients with such lesions.

Our study has some limitations. First, our series was selected because of the in- and exclusion criteria (and willingness) to participate in a clinical trial. Without doubt this resulted in a younger and less severely affected population as is evident from the overrepresentation of lacunar stroke patients and the younger age than expected for stroke in general. Nevertheless, 54 percent of our patients had silent lesions on MRI. Using MRI over CT increases the sensitivity of lesion detection, but the specificity with regard to the severity of the underlying pathology of such lesions may be lower than with CT. Therefore, we cannot exclude the possibility that differences might have emerged in case our population been older and contained more severely affected patients, which means those with a territorial infarct. On the other hand, the most interesting distinction is that between patients with or without silent lesions within a lacunar stroke population because of the issue of phenotyping different types of cerebral small vessel disease²¹. Silent ischaemic brain lesions are

most strongly associated with lacunar stroke. Secondly, variation in statin therapy may be of influence on the levels of various markers. We were unable to account for duration or intensity of such treatment in our series. A third limitation is the rather small sample size of our study, but in any case it makes any substantial effects of *C pneumoniae* infection unlikely.

There is some uncertainty in the literature as to the underlying vascular pathology of silent ischaemic lesions, but the available evidence points at so-called lipohyalinosis or also called arteriolosclerosis, as the pathology in most cases^{22,23}. Arteriolosclerosis is primarily a hyperplasia of the medial vascular layer, rather than an endothelial affliction. However, this stage may progress to that in which there is ultimately loss of vascular wall integrity, and inflammatory processes may eventually obstruct the vessel lumen²³. In contrast, small vessel atheromatosis is primarily an inflammatory process from the start with early luminal obstruction, causing symptomatic lacunar infarcts. Consequently, the two types of small vessel disease may differ in terms of endothelial activation and consequently, serum titres of markers participating in this process. However, the extent of silent vascular damage and its stage of progression may be of influence on the degree of endothelial activation in both types of vascular pathology, and the absence of difference in inflammatory markers between our stroke patients with or without silent lesions may be due to the fact that all patients were symptomatic, viz. suffered from atherosclerosis, which might have masked any difference caused by the cerebral vessels alone. Hassan et al, however, were able to detect a lower serum level of the endothelial marker tissue factor pathway inhibitor in 63 lacunar stroke patients with leukoaraiosis compared to 47 without, whereas both groups had elevated levels compared to controls²⁴. This suggests that some markers may be more specifically activated, depending on extent and progression of endothelial damage, in which stroke study populations may differ.

To arrive at a genetics-based explanation of cerebral small vessel disease, differences on a molecular level between different subtypes of this disease should be explored. So far *C pneumoniae* serology nor any of the markers we measured seem to be of value in discriminating between different small vessel phenotypes, nor between cerebral large or small vessel disease. The issue should be further explored in well-defined, large patient series, whereas eventual differences should be valid with regard to differences in prognosis.

References

1. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med.* 1999;340:115-26.
2. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med.* 1997;336:973-9.
3. Ridker PM, Buring JE, Shih J, Matias M, Hennekens CH. Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation.* 1998;98:731-3.
4. Winbeck K, Kukla C, Poppert H, Klingelhof J, Conrad B, Sander D. Elevated C-reactive protein is associated with an increased intima to media thickness of the common carotid artery. *Cerebrovasc Dis.* 2002;13:57-63.
5. Yamagami H, Kitagawa K, Nagai Y, Hougaku H, Sakaguchi M, Kuwabara K, Kondo K, Masuyama T, Matsumoto M, Hori M. Higher levels of interleukin-6 are associated with lower echogenicity of carotid artery plaques. *Stroke.* 2004;35:677-81.
6. Campbell LA, Kuo CC. *Chlamydia pneumoniae*—an infectious risk factor for atherosclerosis? *Nat Rev Microbiol.* 2004;2:23-32.
7. Netea MG, Selzman CH, Kullberg BJ, Galama JM, Weinberg A, Stalenhoef AF, Van der Meer JW, Dinarello CA. Acellular components of *Chlamydia pneumoniae* stimulate cytokine production in human blood mononuclear cells. *Eur J Immunol.* 2000;30:541-9.
8. Wimmer ML, Sandmann Strupp R, Saikku P, Haberl RL. Association of chlamydial infection with cerebrovascular disease. *Stroke.* 1996;27:2207-10.
9. Cook PJ, Honeybourne D, Lip GYH, Beevers DG, Wise R, Davies AH. *Chlamydia pneumoniae* antibody titres are significantly associated with acute stroke and transient cerebral ischemia: the West Birmingham Stroke Project. *Stroke.* 1998;29:404-10.
10. Elkind MS, Lin IF, Grayston JT, Sacco RL. *Chlamydia pneumoniae* and the risk of first ischemic stroke: The Northern Manhattan Stroke Study. *Stroke.* 2000;31:1521-5.
11. Fagerberg B, Gnarpe J, Gnarpe H, Agewall S, Wikstrand J. *Chlamydia pneumoniae* but not cytomegalovirus antibodies are associated with future risk of stroke and cardiovascular disease: a prospective study in middle-aged to elderly men with treated hypertension. *Stroke.* 1999;30:299-305.
12. Virok D, Kis Z, Karai L, Intzedy L, Burian K, Szabo A, Ivanyi B, Gonczol E. *Chlamydia pneumoniae* in atherosclerotic middle cerebral artery. *Stroke.* 2001;32:1973-6.
13. Vink A, Poppen M, Schoneveld AH, Roholl PJ, de Kleijn DP, Borst C, Pasterkamp G. Distribution of *Chlamydia pneumoniae* in the human arterial system and its relation to the local amount of atherosclerosis within the individual. *Circulation.* 2001;103:1613-7.
14. Rassa M, Cazzavillan S, Scagnelli M, Peron A, Bevilacqua PA, Facco M, Bertoloni G, Lauro FM, Zambello R, Bonaldi E. Demonstration of *Chlamydia pneumoniae* in atherosclerotic arteries from various vascular regions. *Atherosclerosis.* 2001;158:73-9.
15. van Zanten M, Boiten J, Kessels F, Lodder J. Significant progression of white matter lesions and small deep (lacunar) infarcts in patients with stroke. *Arch Neurol.* 1996;53:650-55.
16. Boon A, Lodder J, Heuts-van Raak L, Kessels F. Silent brain infarcts in 755 consecutive patients with a first-ever supratentorial ischemic stroke. Relationship with index-stroke subtype, vascular risk factors, and mortality. *Stroke.* 1994;25:2384-90.
17. Netea MG, Selzman CH, Kullberg BJ, Galama JM, Weinberg A, Stalenhoef AF, Van der Meer JW, Dinarello CA. Acellular components of *Chlamydia pneumoniae* stimulate cytokine production in human blood mononuclear cells. *Eur J Immunol.* 2000;30:541-9.
18. Schmidt R, Scheltens P, Erkinjuntti T, Pantoni L, Markus HS, Wallin A, Barkhof F, Fazekas F. White matter lesion progression: a surrogate endpoint for trials in cerebral small-vessel disease. *Neurology.* 2004;63:139-44.
19. de Jong G, van Raak L, Kessels F, Lodder J. Stroke subtype and mortality. a follow-up study in 998 patients with a first cerebral infarct. *J Clin Epidemiol.* 2003;56:262-8.

20. Vainas T, De Graaf R, Stassen FR, Kurvers HA, Grauls GE, Kitslaar PJ, Bruggeman CA. Chlamydia pneumoniae serology: comparing a commercial enzyme immunoassay and microimmunofluorescence test in patients with cardiovascular disease. *Apmis*. 2003;111: 363-9.
21. Staals J, Lodder J. Hypertension and Lacunar Infarction. Chapter 19 in: Advanced therapy in hypertension and vascular disease. Mohler ER, Townsend RR, Editors. BC Decker Inc. Publisher. 2005.
22. de Jong G, Kessels F, Lodder J. Two types of lacunar infarcts: further arguments from a study on prognosis. *Stroke*. 2002;33:2072-6.
23. Lammie G. Pathology of lacunar infarction. in: *Subcortical stroke Oxford University Press*. 2002:37-47.
24. Hassan A, Hunt BJ, O'Sullivan M, Parmar K, Bamford JM, Briley D, Brown MM, Thomas DJ, Markus HS. Markers of endothelial dysfunction in lacunar infarction and ischaemic leukoaraiosis. *Brain*. 2003;126:424-32.

Chapter 6

Is the intima-media thickness of the common carotid artery related to ipsilateral silent brain ischaemia, stroke syndrome, and inflammatory parameters?

Abstract

Aim of the study

To establish whether carotid intima-media thickness is different in cortical versus lacunar infarcts, and whether carotid intima-media thickness is related to the presence of silent brain ischaemia, in stroke patients. Furthermore, we investigated the correlation between inflammatory parameters and carotid intima-media thickness.

Patients and methods

Carotid intima-media thickness was measured with B-mode ultrasound, in 74 patients derived from a study investigating the effect of a three-month course of azithromycin on progression of silent ischaemic brain lesions. Levels of high-sensitivity CRP (hsCRP) and IL-6 were assessed on serum samples obtained at randomisation, as was the *Chlamydia pneumoniae* IgG antibody titre. Cytokine levels were assessed in the supernatant of whole blood stimulated with *Chlamydia pneumoniae* antigen. Baseline brain MRI was scored on the presence of asymptomatic lacunar infarction and leukoaraiosis.

Results

No correlations were found between inflammatory levels, *Chlamydia pneumoniae* serology and carotid intima-media thickness. Mean values of carotid intima-media thickness did not differ between cortical infarcts and lacunar infarcts, nor were they different between patients with and without silent brain ischaemia.

Conclusions

In a population of patients with a symptomatic stroke, carotid intima-media thickness values do not differ between patients with and without silent ischaemic brain lesions on MRI, or between patients with a cortical and lacunar syndrome. Furthermore, carotid intima-media thickness is not related to *Chlamydia pneumoniae* serology, or inflammatory parameters.

Introduction

Common carotid intima-media thickness (CCA-IMT) can be assessed non-invasively by B-mode ultrasonography, and is used as an indicator of early carotid atherosclerosis. CCA-IMT increases with age and is associated with the classical risk factors for cardiovascular disease¹⁻⁴. Increases in CCA-IMT are associated with an increased risk of myocardial infarction and stroke⁵⁻⁷. Therefore, CCA-IMT is regarded as a valid indicator of generalized atherosclerosis. Inflammation is a key factor in atherosclerosis development⁸. Inflammatory parameters such as C-reactive protein (CRP) are elevated in patients with generalized atherosclerosis⁹, and CRP levels are associated with an increased risk of stroke and myocardial infarction in apparently healthy men and women^{10,11}. CRP levels are also associated with higher CCA-IMT^{12,13}. Anti-*Chlamydia pneumoniae* (*C pneumoniae*) IgG antibodies are related to CCA-IMT levels in patient without clinical evidence of stroke^{14,15}, and *C pneumoniae* seroprevalence is associated with clinical stroke^{5,7,16-20}. In approximately 30 percent of stroke patients so-called silent brain lesions (SBL) are seen on CT, consisting of lacunar infarcts, ischaemic white matter lesions or both. These lesions progress over time, despite sufficient secondary prevention^{21,22}. SBL increase the risk of cognitive decline²³, and the presence of SBL is associated with a higher risk of future stroke²⁴. The relation between CCA-IMT and the risk of stroke has only been investigated in population based studies^{5,7}, and data on the relation between CCA-IMT and stroke subtypes are scarce^{25,26}. We therefore sought to find out whether CCA-IMT is related to stroke subtype, the presence of ipsilateral SBL on MRI, and to serum inflammatory parameters and *C pneumoniae* serology.

Patients and methods

The patients in this study were randomised in the ACSI study (Azithromycin for the prevention of the progression of Cerebral ischaemic lesions in patients Surviving a non-cardioembolic cerebral Infarct), which investigates the effect of a three-month treatment schedule of azithromycin on ischaemic cerebral damage progression on magnetic resonance imaging (MRI) of the brain measured at two years, in patients with a symptomatic stroke. Patients with a symptomatic stroke were recruited in two hospitals, the University Hospital Maastricht, and one affiliated clinic. Three months after the stroke, the patients were randomised to receive either azithromycin 500 mg or a matching placebo once daily for 3 days, then once weekly for twelve weeks. At baseline patients have MRI, whereas blood was drawn for the assessment of high-sensitivity CRP (hsCRP), IL-6 and *C pneumoniae* serology. CCA-IMT could only be

assessed in the University Hospital Maastricht, therefore, baseline CCA-IMT was assessed in eighty-six patients from the ACSI study. Twelve of these patients were left out from the analysis, because they had an atherosclerotic plaque in the common carotid artery. So CCA-IMT data were available of seventy-four patients.

CCA-IMT measurements

With high resolution B-mode ultrasonography (SONOS 5500; Philips, Eindhoven, the Netherlands) a longitudinal image of the common carotid artery in the late diastolic phase is obtained. The thickness of the double line pattern of the far wall is estimated manually, and expressed as the mean of 4 separate measurements. The settings of the ultrasound system are identical for all measurements. Special attention is paid to only acquire images that do not exceed the dynamic range of the system.

MRI

The MRI protocol included T2 and FLAIR weighted axial images of the brain. The following parameters were scored: location and type of symptomatic lesion; the presence and number of asymptomatic lacunar infarcts in the basal ganglia, internal capsule or pons; the presence and severity of periventricular hyperintensities and white matter hyperintensities in the centrum semi ovale²⁷. The MRI's had been scored without knowledge of clinical information. Silent ischaemic brain lesions (SBL) were analysed as a dichotome variable: presence or absence of one or more silent ischaemic lesions, of white matter lesions (extending peri-ventricular lesions or confluent lesions in the centrum semi ovale), or both. Separate variables were made for right-sided SBL, left sided SBL, and overall presence of SBL.

C-reactive protein

Venous blood samples drawn on randomisation for the trial were immediately centrifuged for 10 minutes at 1200 rpm and at 4°C. Serum was stored at -20°C until analysis. CRP was determined highly sensitive (hsCRP) with the IMMULITE CRP method (Diagnostic Product Corporation, Los Angeles, USA). This assay provides a detection limit of 0.10 mg/l and has been approved by the Food and Drug Administration for clinical use in the United States.

Stimulated cytokines

Stimulation of mononuclear cells with *C pneumoniae* induces the production of the inflammatory cytokines TNF α , IL-6, IL-1 β , IL-10 and IFN γ ²⁸. For this

purpose, whole blood was stimulated with *C pneumoniae* antigen as follows: 200 μ l of whole blood was added to 750 μ l of RPMI medium and 50 μ l of *C pneumoniae* antigen (3×10^6 infection forming units (IFU), dilution 1:20). Control samples consisted of 200 μ l of whole blood where 800 μ l of RPMI medium was added to. The samples were incubated at 37° C for 24 hours (TNF α , IL-6 and IL-1 β) or 48 hours (IL-10 and IFN γ), as described by Netea et al.²⁸. After this, the samples were centrifuged and the supernatant was collected and stored for further analysis. Cytokine levels were determined using an enzyme linked immune absorbent assay (ELISA) (eBiosciences, San Diego, USA). ELISA's were performed as instructed by the manufacturer. Briefly, 96-wells plates were coated with antigen and kept overnight at 4°C. The following day, the wells were blocked using the tests assay diluent, after which the standards and patient samples were added and incubated for one hour. A biotinylated capture antigen, HRP for colouring the samples and H₂SO₄ as stop solution, followed this.

Measurement of IgG antibodies against *C pneumoniae*

Levels of serum IgG antibodies against *C pneumoniae* were measured using an ELISA, as described before²⁹ (Labsystems, Helsinki, Finland). The presence or absence of *C pneumoniae* antibodies was determined by comparing the absorbance value of the sample to a cut-off value, as specified by the manufacturer. Specimens with values equal to or greater than 30 were considered positive for anti-Chlamydia antibodies. We also recorded the absolute levels of the IgG antibody titre.

Statistics

Non-parametric tests were used to assess mean CCA-IMT values in patients with and without silent brain lesions. Mean levels of right CCA-IMT were assessed in patients with and without right-sided SBL, left sided CCA-IMT in patients with and without left sided SBL. Furthermore, a variable was made with the highest CCA-IMT found in the left or right CCA, and mean values of the highest CCA-IMT were assessed in patients with and without asymptomatic lacunar infarction, leukoaraiosis or both. Spearman correlations tests were used to assess correlations between CCA-IMT and levels of hsCRP, TNF α , IL-6, IL-1 β , IL-10, IFN γ and levels of IgG antibodies.

Results

The patients in this study did not differ from the remainder of the ACSI population, with respect to age, risk factors or seroprevalence for IgG antibodies. There were no differences in mean CCA-IMT with regard to the presence of ipsilateral SBL on the right side (0.76 ± 0.18 vs. 0.76 ± 0.19) or the left side (0.82 ± 0.25 vs. 0.77 ± 0.17). Furthermore, when we compared the highest mean CCA-IMT values of both sides in patients with and without SBL, no differences were found (0.85 ± 0.19 vs. 0.86 ± 0.21). These results are summarized in Table 6.1.

Table 6.1 Mean CCA-IMT values in millimetre (SD in parenthesis) related to asymptomatic ischaemic brain lesions, SBL + = lesions present, SBL - = no lesions

	SBL +	SBL -	P-value
Right CCA-IMT (mm)	0.76 (0.18)	0.76 (0.19)	0.71
Left CCA-IMT (mm)	0.82 (0.25)	0.77 (0.17)	0.62
Highest CCA-IMT values of both left and right CCA (mm)	0.85 (0.19)	0.86 (0.21)	0.87

CCA-IMT was associated with age (p -value <0.05). There were no correlations between CCA-IMT levels and levels of $\text{TNF}\alpha$, IL-6, IL-1 β , IL-10, IFN γ , hsCRP and IgG antibody titre (table 6.2). There were no differences in CCA-IMT levels between patients with *C pneumoniae* antibody titre >30 (cut-off for seropositivity) and those with titres <30 (0.083 ± 0.016 vs. 0.093 ± 0.030 , $p=0.194$).

Table 6.2 Top: Spearman correlation coefficients of the correlation between baseline serum levels of IL-6, hsCRP, and IgG antibody titre and common carotid intima-media thickness (CCA-IMT)
Bottom: Spearman correlation coefficients of the correlation between cytokine levels in the supernatant of mononuclear cells stimulated with *C pneumoniae* antigen and CCA-IMT.

Baseline serum	CCA-IMT	P-value
HsCRP	-0.007	0.954
Serum IL-6	0.072	0.547
IgG antibody titre	-0.064	0.589
Mononuclear cells stimulated with <i>C pneumoniae</i> antigen		
$\text{TNF}\alpha$	-0.074	0.530
IL-6	0.229	0.056
IL-1 β	0.002	0.988
IL-10	-0.060	0.613
IFN γ	-0.104	0.378

Discussion

CCA-IMT, inflammation and SBL

In this series of patients with a symptomatic stroke, the CCA-IMT was not related to inflammatory parameters (hsCRP, IL-6, IL-10, IL-1 β , IFN γ , and TNF α). Furthermore the CCA-IMT could not distinguish between a cortical or lacunar syndrome, and no relation was found between CCA-IMT levels and the presence of ischaemic SBL on MRI.

When the relation between CRP levels and CCA-IMT was studied in community based cohort studies, results were conflicting. In two community based studies of over a 1000 individuals without clinical signs of atherosclerosis, CRP was related to CCA-IMT in univariate analysis^{13,30}. After correcting for cardiovascular risk factors, the association either weakened¹³, or was no longer present³⁰. In a smaller study, including 186 patients, the association was found to be weak, and confined to smokers³¹. Leinonen et al. studied the relation between CCA-IMT and CRP in 239 patients with diabetes type 2, and 78 healthy controls³². CCA-IMT was found to be higher in diabetes patients, than in controls, as were CRP levels. In the diabetes group however, CRP levels and CCA-IMT did not correlate. The association between CCA-IMT and CRP does not seem to be very strong, since it has only been proven in large cohort studies, and is closely related to cardiovascular risk factors. Furthermore, in a small selected group of 239 patients with known cardiovascular risk factors³², or with stroke as in our series, the two parameters do not correlate. In the acute phase of stroke, inflammatory parameters rise, thereby obscuring any relationship between CCA-IMT and inflammatory markers. Our patients, however, did not enter the study within three months after stroke, thereby eliminating this confounding factor. The results of our study therefore indicate that within a selected group of patients with a symptomatic stroke, CCA-IMT levels and levels of inflammatory parameters do not correlate. We can of course not exclude a weak association between the two because of the rather small sample size, but at least CCA-IMT does not seem to be a significant marker of endothelial activation in symptomatic cerebrovascular disease patients.

A few studies report on the association between CCA-IMT and stroke^{5,7}, but in these studies no distinction was made between ischaemic and hemorrhagic stroke, or between cortical and lacunar strokes. CCA-IMT is considered as a valid sign for early carotid atherosclerosis, and as such should be more related to ischaemic stroke than to hemorrhagic stroke. This issue was addressed in two studies, which found that CCA-IMT is higher in patients with ischaemic stroke compared to patients with hemorrhagic stroke^{33,34}. Different ischaemic stroke subtypes, i.e. cortical and lacunar stroke might be differently associated

with CCA-IMT, due to differences in vascular pathology. When cardiac embolism and rare causes such as dissection are ruled out as the cause of a cortical stroke, carotid atherosclerosis remains one of the main causes. Lacunar infarcts are less frequently associated with carotid atherosclerosis. Therefore, CCA-IMT might be closer related to cortical stroke. In two case control studies CCA-IMT was measured in ischaemic stroke patients, compared to controls, and to subtypes of stroke^{25,26}. One study, including approximately 300 patients²⁵, found that CCA-IMT values were higher in patients with a cortical stroke, compared to a lacunar stroke; cardiac risk factors were identical between the groups. Another study identified stroke type, according to the cause (atherothrombotic, cardioembolic, lacunar, dissection, other rare causes of stroke), and found no differences between these subtypes, in a population of approximately 1000 patients. In our series we did not find any differences in mean CCA-IMT between patients with a cortical and lacunar stroke. CCA-IMT values between cortical and lacunar patients were almost identical, thereby making a clinical relevant association unlikely, even though it was tested in a small population.

Future risk of stroke increases when SBL are present on MRI at first clinical presentation. SBL are closely related to lacunar infarcts, and their underlying pathology is arteriolosclerosis rather than atherosclerosis. Arteriolosclerosis is primarily a hyperplasia of the medial vascular layer, rather than an endothelial mediated vascular lesion. One would expect CCA-IMT not to be related with conditions due to arteriolosclerosis. However, vascular risk factors are identical in patients with and without SBL²⁴, and the severity of SBL might merely reflect more severe generalized vascular pathology. In our patient population, vascular risk factors were not different between patients with and without SBL, and accordingly, we could not find any differences in CCA-IMT values in patients with and without SBL.

Chlamydia pneumoniae serology and CCA-IMT

We did not find an association between the presence of anti-*C pneumoniae* antibodies and CCA-IMT. Mean CCA-IMT values were identical in seropositive and seronegative patients, whereas also, the levels of the anti *C pneumoniae* IgG antibody titres did not correlate with CCA-IMT values. The association between *C pneumoniae* antibodies and CCA-IMT was mainly investigated in patients without clinical evidence of stroke. A positive association was found in patients with cardiovascular risk factors¹⁴, but without any clinical signs of cardiovascular disease, and in patients with cardiovascular and peripheral arterial occlusive disease, without stroke¹⁵. Seroprevalence of anti-*C pneumoniae* IgG antibodies is higher in stroke patients, than in healthy controls, and seroprevalence in our population was 81%, compared to 58%¹⁴

and 34%¹⁵ respectively, in the above mentioned studies. One study investigated CCA-IMT progression over time in stroke patients with and without *C pneumoniae* antibodies, and found CCA-IMT progression to be related to *C pneumoniae* seropositivity³⁵. Increases in CCA-IMT increase the risk of future cardiovascular disease and stroke^{5,36-40}. An increase in CCA-IMT between 0.15 and 0.2 mm increases the relative risk of stroke^{5,6}. At this moment, follow up data are lacking in our study, but will be obtained in a later phase. So our data do not exclude an association between baseline levels of inflammation and enhanced CCA-IMT progression.

To conclude, CCA-IMT values are not different in patients with a cortical or lacunar stroke, nor do they differ between patients with and without SBL on MRI, in a population of patients with a symptomatic stroke. Furthermore, CCA-IMT is not related to *C pneumoniae* serology, or inflammatory parameters.

References

1. Heiss G, Sharrett AR, Barnes R, Chambless LE, Szklo M, Alzola C. Carotid atherosclerosis measured by B-mode ultrasound in populations: associations with cardiovascular risk factors in the ARIC study. *Am J Epidemiol.* 1991;134:250-6.
2. Burke GL, Evans GW, Riley WA, Sharrett AR, Howard G, Barnes RW, Rosamond W, Crow RS, Rautaharju PM, Heiss G. Arterial wall thickness is associated with prevalent cardiovascular disease in middle-aged adults. The Atherosclerosis Risk in Communities (ARIC) Study. *Stroke.* 1995;26:386-91.
3. Allan PL, Mowbray PJ, Lee AJ, Fowkes FG. Relationship between carotid intima-media thickness and symptomatic and asymptomatic peripheral arterial disease. The Edinburgh Artery Study. *Stroke.* 1997;28:348-53.
4. Salonen R, Salonen JT. Progression of carotid atherosclerosis and its determinants: a population-based ultrasonography study. *Atherosclerosis.* 1990;81:33-40.
5. O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK, Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N Engl J Med.* 1999;340:14-22.
6. Salonen JT, Salonen R. Ultrasonographically assessed carotid morphology and the risk of coronary heart disease. *Arterioscler Thromb.* 1991;11:1245-9.
7. Bots ML, Hoes AW, Koudstaal PJ, Hofman A, Grobbee DE. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. *Circulation.* 1997;96:1432-7.
8. Ross R. Atherosclerosis is an inflammatory disease. *Am Heart J.* 1999;138:S419-20.
9. Van Der Meer IM, De Maat MP, Hak AE, Kilian AJ, Del Sol AI, Van Der Kuip DA, Nijhuis RL, Hofman A, Witteman JC. C-reactive protein predicts progression of atherosclerosis measured at various sites in the arterial tree: the Rotterdam Study. *Stroke.* 2002;33:2750-5.
10. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med.* 1997;336:973-9.
11. Ridker PM, Buring JE, Shih J, Matias M, Hennekens CH. Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation.* 1998;98:731-3.
12. Hashimoto H, Kitagawa K, Hougaku H, Etani H, Hori M. Relationship between C-reactive protein and progression of early carotid atherosclerosis in hypertensive subjects. *Stroke.* 2004;35:1625-30.
13. Sitzer M, Markus HS, Mendall MA, Liehr R, Knorr U, Steinmetz H. C-reactive protein and carotid intimal medial thickness in a community population. *J Cardiovasc Risk.* 2002;9:97-103.
14. Schmidt C, Hulthe J, Wikstrand J, Gnarp H, Gnarp J, Agewall S, Fagerberg B. Chlamydia pneumoniae seropositivity is associated with carotid artery intima-media thickness. *Stroke.* 2000;31:1526-31.
15. Espinola Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp H, Rippin G, Hafner G, Pfeifer U, Meyer J. Are morphological or functional changes in the carotid artery wall associated with Chlamydia pneumoniae, Helicobacter pylori, cytomegalovirus, or herpes simplex virus infection? *Stroke.* 2000;31:2127-33.
16. Cook PJ, Honeybourne D, Lip GYH, Beevers DG, Wise R, Davies AH. Chlamydia pneumoniae antibody titers are significantly associated with acute stroke and transient cerebral ischemia: the West Birmingham Stroke Project. *Stroke.* 1998;29:404-10.
17. Elkind MS, Lin IF, Grayston JT, Sacco RL. Chlamydia pneumoniae and the risk of first ischemic stroke: The Northern Manhattan Stroke Study. *Stroke.* 2000;31:1521-5.
18. Fagerberg B, Gnarp J, Gnarp H, Agewall S, Wikstrand J. Chlamydia pneumoniae but not cytomegalovirus antibodies are associated with future risk of stroke and cardiovascular disease: a prospective study in middle-aged to elderly men with treated hypertension. *Stroke.* 1999;30:299-305.

19. Voorend M, Faber C, van der Ven A, Kessels F, Bruggeman CA, Lodder J. Chlamydia pneumoniae is a likely risk factor for ischemic stroke in young patients. *Journal of stroke and cerebrovascular diseases*. 2004;13:85-91.
20. Wimmer ML, Sandmann Strupp R, Saikku P, Haberl RL. Association of chlamydial infection with cerebrovascular disease. *Stroke*. 1996;27:2207-10.
21. van Zanten M, Boiten J, Kessels F, Lodder J. Significant progression of white matter lesions and small deep (lacunar) infarcts in patients with stroke. *Arch Neurol*. 1996;53:650-55.
22. Boon A, Lodder J, Heuts-van Raak L, Kessels F. Silent brain infarcts in 755 consecutive patients with a first-ever supratentorial ischemic stroke. Relationship with index-stroke subtype, vascular risk factors, and mortality. *Stroke*. 1994;25:2384-90.
23. Vermeer SE, Prins ND, den Heijer T, Hofman A, Koudstaal PJ, Breteler MM. Silent brain infarcts and the risk of dementia and cognitive decline. *N Engl J Med*. 2003;348:1215-22.
24. de Jong G, Kessels F, Lodder J. Two types of lacunar infarcts: further arguments from a study on prognosis. *Stroke*. 2002;33:2072-6.
25. Cupini LM, Pasqualetti P, Diomedi M, Vernieri F, Silvestrini M, Rizzato B, Ferrante F, Bernardi G. Carotid artery intima-media thickness and lacunar versus nonlacunar infarcts. *Stroke*. 2002;33:689-94.
26. Touboul PJ, Elbaz A, Koller C, Lucas C, Adrai V, Chedru F, Amarenco P. Common carotid artery intima-media thickness and brain infarction : the Etude du Profil Genetique de l'Infarctus Cerebral (GENIC) case-control study. The GENIC Investigators. *Circulation*. 2000;102:313-8.
27. Schmidt R, Scheltens P, Erkinjuntti T, Pantoni L, Markus HS, Wallin A, Barkhof F, Fazekas F. White matter lesion progression: a surrogate endpoint for trials in cerebral small-vessel disease. *Neurology*. 2004;63:139-44.
28. Netea MG, Selzman CH, Kullberg BJ, Galama JM, Weinberg A, Stalenhoef AF, Van der Meer JW, Dinarello CA. Acellular components of Chlamydia pneumoniae stimulate cytokine production in human blood mononuclear cells. *Eur J Immunol*. 2000;30:541-9.
29. Vainas T, De Graaf R, Stassen FR, Kurvers HA, Grauls GE, Kitslaar PJ, Bruggeman CA. Chlamydia pneumoniae serology: comparing a commercial enzyme immunoassay and microimmunofluorescence test in patients with cardiovascular disease. *Apmis*. 2003;111:363-9.
30. Folsom AR, Pankow JS, Tracy RP, Arnett DK, Peacock JM, Hong Y, Djousse L, Eckfeldt JH. Association of C-reactive protein with markers of prevalent atherosclerotic disease. *Am J Cardiol*. 2001;88:112-7.
31. Hak AE, Stehouwer CD, Bots ML, Polderman KH, Schalkwijk CG, Westendorp IC, Hofman A, Witteman JC. Associations of C-reactive protein with measures of obesity, insulin resistance, and subclinical atherosclerosis in healthy, middle-aged women. *Arterioscler Thromb Vasc Biol*. 1999;19:1986-91.
32. Leinonen ES, Hiukka A, Hurt-Camejo E, Wiklund O, Sarna SS, Mattson Hulten L, Westerbacka J, Salonen RM, Salonen JT, Taskinen MR. Low-grade inflammation, endothelial activation and carotid intima-media thickness in type 2 diabetes. *J Intern Med*. 2004;256:119-27.
33. Vemmos KN, Tsigoulis G, Spengos K, Papamichael CM, Zakopoulos N, Daffertshofer M, Lekakis JP, Mavrikakis M. Common carotid artery intima-media thickness in patients with brain infarction and intracerebral haemorrhage. *Cerebrovasc Dis*. 2004;17:280-6.
34. Tsigoulis G, Vemmos KN, Spengos K, Papamichael CM, Cimboneriu A, Zis V, Zakopoulos N, Mavrikakis M. Common carotid artery intima-media thickness for the risk assessment of lacunar infarction versus intracerebral haemorrhage. *J Neurol*. 2005.
35. Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Enhanced progression of early carotid atherosclerosis is related to Chlamydia pneumoniae (Taiwan acute respiratory) seropositivity. *Circulation*. 2001;103:1390-1395.
36. Chambless LE, Heiss G, Folsom AR, Rosamond W, Szklo M, Sharrett AR, Clegg LX. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: the Atherosclerosis Risk in Communities (ARIC) Study, 1987-1993. *Am J Epidemiol*. 1997;146:483-94.

37. Cao JJ, Thach C, Manolio TA, Psaty BM, Kuller LH, Chaves PH, Polak JF, Sutton-Tyrrell K, Herrington DM, Price TR, Cushman M. C-reactive protein, carotid intima-media thickness, and incidence of ischemic stroke in the elderly: the Cardiovascular Health Study. *Circulation*. 2003;108:166-70.
38. Hollander M, Hak AE, Koudstaal PJ, Bots ML, Grobbee DE, Hofman A, Witteman JC, Breteler MM. Comparison between measures of atherosclerosis and risk of stroke: the Rotterdam Study. *Stroke*. 2003;34:2367-72.
39. Rosvall M, Janzon L, Berglund G, Engstrom G, Hedblad B. Incidence of stroke is related to carotid IMT even in the absence of plaque. *Atherosclerosis*. 2005;179:325-31.
40. Wattanakit K, Folsom AR, Chambless LE, Nieto FJ. Risk factors for cardiovascular event recurrence in the Atherosclerosis Risk in Communities (ARIC) study. *Am Heart J*. 2005;149:606-12.

Chapter 7

Azithromycin does not change *Chlamydia pneumoniae* stimulated cytokine production in mononuclear cells of patients with ischaemic cerebral lesions

Abstract

Aim of the study

To investigate the effect of azithromycin on cytokine production of mononuclear blood cells stimulated with *Chlamydia pneumoniae*.

Patients and methods

Patients were derived from a study investigating the effect of a three-month course of azithromycin on progression of silent ischaemic brain lesions. The patients were randomised to receive either azithromycin 500 mg or a matching placebo once daily for 3 days, then once weekly for twelve weeks. Whole blood samples of 75 consecutive patients were stimulated with *Chlamydia pneumoniae* and levels of $\text{TNF}\alpha$, IL-6, IL-1 β , IL-10, and IFN γ were assessed in the supernatant, using an enzyme linked immuno absorbent assay. To assess the ratio between the pro-inflammatory T helper 1 cells and the anti-inflammatory T helper 2 cells, the IFN γ /IL-10 ratio was calculated.

Results

Stimulation of mononuclear cells with *Chlamydia pneumoniae* induces cytokine production in these cells. Cytokine levels and IFN γ /IL-10 ratio at three months post-treatment did not differ between the patients treated with azithromycin or placebo, nor did the changes in cytokine levels.

Conclusions

A three-month course of azithromycin does not influence cytokine production in mononuclear cells of ischaemic stroke patients, stimulated with *Chlamydia pneumoniae*.

Introduction

Since the early 90's, atherosclerosis is defined as an inflammatory, immunological disorder, developing after endothelial injury¹. The inflammatory cytokines Interferon gamma ($\text{IFN}\gamma$), Interleukin-6 (IL-6), Tumour necrosis factor alpha ($\text{TNF}\alpha$), and IL-1 β play a role in the inflammatory response related with atherosclerosis. T-lymphocytes are present in all stages of the atherosclerotic plaque. T-lymphocytes can be divided in the pro-inflammatory T helper (Th) cells 1 and Th2 cells. The Th1 cell induces macrophage activation, leading to the production of $\text{IFN}\gamma$, a potent inflammatory cytokine. IL-10 induces Th2 activation, thereby exerting an anti-inflammatory effect by blocking production of IL-1 β and $\text{TNF}\alpha$ ². In atherogenic mouse models, atherosclerotic lesion development can be stopped by pharmacological blocking of Th1 cells, or through IL-10 stimulation³. In humans with unstable angina pectoris, lymphocyte activation is towards production of $\text{IFN}\gamma$, more than in patients with stable angina pectoris⁴, which indicates that inflammation plays a role in the thrombo-embolic events leading to myocardial infarction.

One of the factors implicated in the pathogenesis of atherosclerosis is infection with intracellular micro-organisms such as the gram-negative bacteria *Chlamydia pneumoniae* (*C pneumoniae*). Sero-epidemiological studies reported an association between the presence of anti-*Chlamydia* antibodies and cardiovascular or cerebrovascular events⁵⁻¹⁰. *C pneumoniae* is able to infect, replicate and survive in the cells of the vascular wall¹¹⁻¹⁴. Low-grade infection of human endothelial results in endothelial dysfunction and the expression of adhesion molecules¹⁵. Persistent infection of endothelial cells and monocytes in vitro, leads to production of all the above mentioned cytokines¹⁶⁻¹⁸. Incubation of human mononuclear cells with *C pneumoniae* induces the production of the pro-inflammatory cytokines IL-6, $\text{TNF}\alpha$ and IL-1 β ¹⁹, and IL-10²⁰. Azithromycin has anti-microbial and anti-inflammatory effects²¹, therefore, we expect that treatment with azithromycin results in a diminished inflammatory state.

Our group is performing a double blind, placebo controlled trial investigating the effect of a three-month course of azithromycin on the progression of cerebrovascular lesions in patients surviving a non-cardioembolic ischaemic stroke. The aim of the present study was to investigate whether a three-month course of azithromycin changes cytokine production by mononuclear cells stimulated with *C pneumoniae* antigen, in an ex-vivo experiment.

Patients and methods

Patients

The patients in this study had been randomised in the ACSI study (Azithromycin for the prevention of the progression of Cerebral ischaemic lesions in patients Surviving a non-cardioembolic cerebral Infarct), which investigates the effect of a three-month treatment schedule of azithromycin on ischaemic cerebral damage progression on magnetic resonance imaging (MRI) measured at two years. The patients were randomised to receive either azithromycin 500 mg or a matching placebo once daily for 3 days, then once weekly for twelve weeks. Blood was drawn at randomisation and at three months, after finishing the course of study medication. Because the final follow-up of the ACSI trial is not yet completed, deblinding of the patients was not preferred. Therefore, the patients were assigned to two groups (A and B), without deblinding the allocated treatment. At the time of data analysis for this study, it was not known which group was verum or placebo.

Stimulation of cytokine production

From 75 consecutive patients, whole blood was stimulated with *C pneumoniae* antigen as follows: 200 μ l of whole blood was added to 750 μ l of RPMI medium and 50 μ l of *C pneumoniae* antigen (3×10^6 infection forming units (IFU), dilution 1:20). Mock stimulated samples consisted of 200 μ l of whole blood added to 800 μ l of RPMI medium. The samples were stimulated for 24 hours (TNF α , IL-6 and IL-1 β) or 48 hours (IL-10 and IFN γ), as described by Netea et al.¹⁹. After this, the samples were centrifuged and supernatant was collected and stored at -80°C for further analysis.

Measurement of cytokines

Levels of cytokines were determined using an enzyme linked immuno absorbent assay (ELISA) (eBiosciences, San Diego, USA). ELISA's were performed as instructed by the manufacturer. Briefly, 96-wells plates were coated with coating antigen and kept overnight at 4°C . The following day, the wells were blocked using the tests assay diluent, after which the standards and patient samples were added and stimulated for one hour. A biotinylated capture antigen, horseradish peroxidase for colouring the samples and H_2SO_4 as stop solution, followed this.

C-reactive protein

Venous blood samples drawn on randomisation for the trial were immediately centrifuged for 10 minutes at 1200 rpm and at 4°C. Serum was stored at -20°C until analysis. CRP was determined highly sensitive (hsCRP) with the IMMULITE CRP method (Diagnostic Product Corporation, Los Angeles, USA). This assay provides a detection limit of 0.10 mg/L and has been approved by the Food and Drug Administration for clinical use in the United States.

Measurement of IgG antibodies against *C pneumoniae*

Levels of serum IgG antibodies against *C pneumoniae* were measured using an ELISA, as described before²². (Labsystems, Helsinki, Finland). The presence or absence of *C pneumoniae* antibodies was determined by comparing the absorbance value of the sample to a cut-off value, as specified by the manufacturer. Specimens with values equal to or greater than 30 were considered positive for anti-Chlamydia antibodies.

Statistics

Since the levels of the cytokines were skewed, non-parametric tests were used. Mean levels of cytokines produced in *C pneumoniae* stimulated cells were compared to mean levels of cytokines produced by mock stimulated cells, at baseline, as well as after three months. We assessed differences in cytokines at three months between patients treated with azithromycin or placebo. A variable was made to assess the differences in cytokine levels after three months compared with baseline values (levels at three months – levels at baseline). We used a non-parametric test to assess the mean differences these changes, in the azithromycin group and the placebo group. In a linear regression model associations were tested between changes in cytokine levels after three months, with sex, current smoking, hypertension, age, cardiovascular disease, hypercholesterolaemia, peripheral arterial occlusive disease, the presence of *C pneumoniae* antibodies and baseline hsCRP levels. A p-value of <0.05 was considered significant. Results were given as mean values and the standard deviation.

Results

The patient groups were designated group A and B, without knowledge which one of these groups is verum or placebo. The patients in this study did not differ from the remainder of the ACSI population, with respect to age and cardiovascular risk factors; only seroprevalence for IgG antibodies was higher

in the study group (89% vs. 81%), but this was not statistically significant. Patient characteristics are listed in Table 7.1. At baseline, as well as at three months cytokine levels of the *C pneumoniae* stimulated cells were significantly higher than in the mock stimulated cells (Table 7.2). There were no significant differences in levels of $\text{TNF}\alpha$, IL-6, IL-1 β , IL-10, IFN γ and IFN γ /IL-10 ratio in the supernatant of *C pneumoniae* stimulated cells, between the two patient groups (Table 7.3). Furthermore, we found no significant differences in changes in the levels of $\text{TNF}\alpha$, IL-6, IL-1 β , IL-10 IFN γ and IFN γ /IL-10 ratio, in the supernatant of *C pneumoniae* stimulated cells, between the two patient groups, after three months of treatment. (Table 7.4). No differences were found in cytokine levels, and differences in cytokine levels, in the supernatant of mock stimulated cells, between the two patient groups (data not shown).

In a multiple linear regression model using the cytokines as the dependent variable and sex, current smoking, hypertension, age, cardiovascular disease, hypercholesterolaemia, peripheral arterial occlusive disease, allocated treatment, *C pneumoniae* serology and baseline hsCRP as independent variables, we only found an association between differences in $\text{TNF}\alpha$ levels at three months and baseline *C pneumoniae* serology.

Table 7.1 Baseline characteristics between the two treatment groups.

Characteristics ^a	A (N = 39)	B (N = 36)
Age (years) ^b	62.41 $\pm$ 8.74	62.35 $\pm$ 9.87
Sex (male)	25 (64)	26 (72)
Smoking	16 (41)	9 (25)
Hypertension	19 (51)	14 (40)
Diabetes	1 (2.6)	1 (2.8)
Hypercholesterolaemia	10 (26)	7 (19)
Cardiovascular disease	5 (13)	2 (6)
Peripheral arterial disease	4 (10.3)	0 (0)
<i>C pneumoniae</i> antibodies	39 (100)	28 (78)
HsCRP (mg/l) ^b	3.45 $\pm$ 3.57	3.84 $\pm$ 5.21

^a values are expressed as number (percentage); ^b values are expressed as mean $\pm$ standard deviation

Table 7.2 Cytokine levels in supernatant of mononuclear cells stimulated with *C pneumoniae* antigen or mock stimulated (all patients combined).

Cytokines ^a	<i>C pneumoniae</i> stimulated	RPMI stimulated	P-value
TNF α (pg/ml)			
Baseline	22.66 $\pm$ 89.22	0.36 $\pm$ 1.71	< 0.05
3 months	62.45 $\pm$ 150.20	0.28 $\pm$ 1.63	< 0.01
IL-6 (pg/ml)			
Baseline	105.28 $\pm$ 74.33	4.94 $\pm$ 25.89	< 0.01
3 months	82.53 $\pm$ 75.40	11.38 $\pm$ 39.11	< 0.01
IL-1 β (pg/ml)			
Baseline	91.36 $\pm$ 131.71	3.86 $\pm$ 26.92	< 0.01
3 months	115.26 $\pm$ 163.44	4.09 $\pm$ 12.17	< 0.01
IL-10 (pg/ml)			
Baseline	23.11 $\pm$ 53.98	0.40 $\pm$ 1.77	< 0.01
3 months	29.62 $\pm$ 61.17	0.35 $\pm$ 2.84	< 0.01
IFN γ (pg/ml)			
Baseline	48.66 $\pm$ 109.47	0.65 $\pm$ 3.81	< 0.01
3 months	86.07 $\pm$ 167.04	1.05 $\pm$ 4.98	< 0.01

^a values are expressed as mean $\pm$ standard deviation; p-values for the comparison of *C pneumoniae* stimulated and mock stimulated cells.

Table 7.3 Cytokine levels of the *C pneumoniae* stimulated cells of patient in groups A and B, three months after treatment with either azithromycin or placebo.

Cytokines ^a	Treatment groups		P-value
	A	B	
TNF α (pg/ml)	57.02 $\pm$ 147.66	68.33 $\pm$ 154.78	0.64
IL-6 (pg/ml)	78.08 $\pm$ 79.79	87.85 $\pm$ 71.01	0.68
IL-1 β (pg/ml)	122.75 $\pm$ 174.50	107.57 $\pm$ 153.70	0.99
IL-10 (pg/ml)	26.93 $\pm$ 60.98	32.55 $\pm$ 62.15	0.54
IFN γ (pg/ml)	48.50 $\pm$ 82.29	126.16 $\pm$ 221.82	0.87
IFN γ /IL-10 ratio	0.07 $\pm$ 0.24	0.36 $\pm$ 1.12	0.73

^a values are expressed as mean $\pm$ standard deviation; p-value for the comparison of the treatment groups

Table 7.4 Differences in changes of levels ^b of cytokines levels of the *C pneumoniae* stimulated in both treatment groups.

Cytokines ^a	A	B	P-value
TNF α (pg/ml)	42.65 $\pm$ 25.49	36.69 $\pm$ 34.17	0.88
IL-6 (pg/ml)	-22.21 $\pm$ 15.50	-22.42 $\pm$ 18.37	0.76
IL-1 β (pg/ml)	45.99 $\pm$ 33.68	24.10 $\pm$ 34.09	0.65
IL-10 (pg/ml)	5.56 $\pm$ 11.18	4.87 $\pm$ 16.17	0.97
IFN γ (pg/ml)	32.01 $\pm$ 23.99	34.58 $\pm$ 79.90	0.98
IFN/IL-10 ratio	0.04 $\pm$ 0.05	0.24 $\pm$ 0.26	0.65

^a values are expressed as mean $\pm$ standard deviation; ^b changes in levels are: levels at three months – levels at baseline, negative values indicate that levels at three months were lower than levels at baseline.

Discussion

This is the first study to relate cytokine production by mononuclear cells stimulated ex-vivo with *C pneumoniae*, of ischaemic stroke patients, after treatment with azithromycin or placebo. When we compared cytokine levels and the IFN γ /IL-10 ratio after three months in both treatment groups, we found no differences. Because of the small population size, we also compared cytokine levels between the two groups over time, i.e. before and after treatment, and found no differences. We conclude that azithromycin does not influence cytokine production in mononuclear cells stimulated with *C pneumoniae* antigen.

Linear regression analysis, when adjusted for vascular risk factors, age, treatment and *C pneumoniae* serology, only showed that the difference in change in TNF α levels over time, was related to *C pneumoniae* serology. The difference between TNF α levels at three months and baseline was greater in patients without *C pneumoniae* antibodies. A possible explanation for this might be the fact that the distribution of *C pneumoniae* antibodies between the two groups was disproportionate, with 100% seropositivity in group A versus 78% seropositivity in group B. Several studies have shown that TNF α levels are higher in patients with *C pneumoniae* antibodies, than in patients without *C pneumoniae* antibodies²³⁻²⁵. In our population, however, baseline TNF α levels between seropositive and seronegative patients were not different, nor were TNF α levels after treatment. The relation of TNF α levels with *C pneumoniae* serology might therefore be purely coincidental.

If *C pneumoniae* infection plays a specific role in cytokine production, mononuclear cells of patients with *C pneumoniae* antibodies might be more triggered to produce cytokines when stimulated with *C pneumoniae*. Treatment of *C pneumoniae* infection with azithromycin, might subsequently reduce the trigger to cytokine production, leading to lower levels of these cytokines. In our study population, 89% of the patients were seropositive, and no differences in cytokine levels after three months between the two treatment groups were found. A limitation in this fact is that our sample size is small. However, cytokine levels between the two treatment groups were almost identical after three months, thereby making an association unlikely, even though it was tested in a small population. The effect of azithromycin on levels of inflammation in ischaemic stroke patients has never been tested before. In three small studies, with sample sizes smaller than in our study, the effect of azithromycin on endothelial function and activation was tested. One of these studies found a positive effect of azithromycin on endothelial function²⁶, whereas the other two did not^{27,28}. All in all, our results do not plead for a specific role of *C pneumoniae* infection on cytokine production of mononuclear cells. Cytokine production of mononuclear cells might be triggered as the result

of a more aspecific effect of inflammation in general as opposed to a specific effect of *C pneumoniae*. The role of inflammation in atherosclerosis is underlined by the fact that T-lymphocytes present in the atherosclerotic plaque are mostly the inflammatory Th1 type, with only a small number of anti-inflammatory Th2 cells²⁹. Azithromycin has anti-inflammatory effects as well as antimicrobial effects²¹, and attenuation of the inflammatory response in general might therefore affect cytokine production, and the Th1/Th2 balance, which is reflected in the IFN γ /IL-10 ratio. However, the IFN γ /IL-10 ratio did not differ between patients treated with azithromycin or placebo, nor was there a difference in change of the ratio after treatment, suggesting that azithromycin does not influence the inflammatory response, after three months. In the ACADEMIC study, treatment of patients with cardiovascular disease with azithromycin in a dose similar to our study, did not reduce levels of IL-6 and IL-1 β after three months, but reduced levels were seen after six months, suggesting a more delayed effect of azithromycin in levels of inflammation³⁰.

In summary: stimulation of mononuclear cells with *C pneumoniae* leads to cytokine production of these cells. A three-month course of azithromycin does not influence cytokine production in mononuclear cells of ischaemic stroke patients, stimulated with *C pneumoniae*, nor does it influence the IFN γ /IL-10 ratio, after three months. Therefore, we conclude that induction of cytokine production is not affected by treatment with azithromycin.

References

1. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 1993;362:801-9.
2. Daugherty A, Rateri DL. T lymphocytes in atherosclerosis: the yin-yang of Th1 and Th2 influence on lesion formation. *Circ Res*. 2002;90:1039-40.
3. Laurat E, Poirier B, Tupin E, Caligiuri G, Hansson GK, Bariety J, Nicoletti A. In vivo downregulation of T helper cell 1 immune responses reduces atherogenesis in apolipoprotein E-knockout mice. *Circulation*. 2001;104:197-202.
4. Liuzzo G, Goronzy JJ, Yang H, Kopecky SL, Holmes DR, Frye RL, Weyand CM. Monoclonal T-cell proliferation and plaque instability in acute coronary syndromes. *Circulation*. 2000;101:2883-8.
5. Saikku P, Leinonen M, Mattila K, Ekman MR, Nieminen MS, Makela PH, Huttunen JK, Valtanen V. Serological evidence of an association of a novel Chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet*. 1988;2:983-6.
6. Cook PJ, Honeybourne D, Lip GYH, Beevers DG, Wise R, Davies AH. Chlamydia pneumoniae antibody titers are significantly associated with acute stroke and transient cerebral ischemia: the West Birmingham Stroke Project. *Stroke*. 1998;29:404-10.
7. Elkind MS, Lin IF, Grayston JT, Sacco RL. Chlamydia pneumoniae and the risk of first ischemic stroke: The Northern Manhattan Stroke Study. *Stroke*. 2000;31:1521-5.
8. Fagerberg B, Gnarpe J, Gnarpe H, Agewall S, Wikstrand J. Chlamydia pneumoniae but not cytomegalovirus antibodies are associated with future risk of stroke and cardiovascular disease: a prospective study in middle-aged to elderly men with treated hypertension. *Stroke*. 1999;30:299-305.
9. Voorend M, Faber C, van der Ven A, Kessels F, Bruggeman CA, Lodder J. Chlamydia pneumoniae is a likely risk factor for ischemic stroke in young patients. *Journal of stroke and cerebrovascular diseases*. 2004;13:85-91.
10. Wimmer ML, Sandmann Strupp R, Saikku P, Haberl RL. Association of chlamydial infection with cerebrovascular disease. *Stroke*. 1996;27:2207-10.
11. Coombes BK, Mahony JB. Chlamydia pneumoniae infection of human endothelial cells induces proliferation of smooth muscle cells via an endothelial cell-derived soluble factor(s). *Infect Immun*. 1999;67:2909-15.
12. Fryer RH, Schwobe EP, Woods ML, Rodgers GM. Chlamydia species infect human vascular endothelial cells and induce procoagulant activity. *J Invest Med*. 1997;45:168-74.
13. Gaydos CA, Summersgill JT, Sahney NN, Ramirez JA, Quinn TC. Replication of Chlamydia pneumoniae in vitro in human macrophages, endothelial cells, and aortic artery smooth muscle cells. *Infect Immun*. 1996;64:1614-20.
14. Kaukoranta Tolvanen SS, Laitinen K, Saikku P, Leinonen M. Chlamydia pneumoniae multiplies in human endothelial cells in vitro. *Microb Pathog*. 1994;16:313-9.
15. Kaukoranta Tolvanen SS, Ronni T, Leinonen M, Saikku P, Laitinen K. Expression of adhesion molecules on endothelial cells stimulated by Chlamydia pneumoniae. *Microb Pathog*. 1996;21:407-11.
16. Rodel J, Woytas M, Groh A, Schmidt KH, Hartmann M, Lehmann M, Straube E. Production of basic fibroblast growth factor and interleukin 6 by human smooth muscle cells following infection with Chlamydia pneumoniae. *Infect Immun*. 2000;68:3635-41.
17. Gaydos CA. Growth in vascular cells and cytokine production by Chlamydia pneumoniae. *J Infect Dis*. 2000;181 Suppl 3:S473-8.
18. Caspar-Bauguil S, Puissant B, Nazzari D, Lefevre JC, Thomsen M, Salvayre R, Benoist H. Chlamydia pneumoniae induces interleukin-10 production that down-regulates major histocompatibility complex class I expression. *J Infect Dis*. 2000;182:1394-401.
19. Netea MG, Selzman CH, Kullberg BJ, Galama JM, Weinberg A, Stalenhoef AF, Van der Meer JW, Dinarello CA. Acellular components of Chlamydia pneumoniae stimulate cytokine production in human blood mononuclear cells. *Eur J Immunol*. 2000;30:541-9.

20. Geng Y, Shane RB, Berencsi K, Gonczol E, Zaki MH, Margolis DJ, Trinchieri G, Rook AH. Chlamydia pneumoniae inhibits apoptosis in human peripheral blood mononuclear cells through induction of IL-10. *J Immunol.* 2000;164:5522-9.
21. Amsden GW. Anti-inflammatory effects of macrolides--an underappreciated benefit in the treatment of community-acquired respiratory tract infections and chronic inflammatory pulmonary conditions? *J Antimicrob Chemother.* 2005;55:10-21.
22. Vainas T, De Graaf R, Stassen FR, Kurvers HA, Grauls GE, Kitslaar PJ, Bruggeman CA. Chlamydia pneumoniae serology: comparing a commercial enzyme immunoassay and microimmunofluorescence test in patients with cardiovascular disease. *Apmis.* 2003;111:363-9.
23. Bruunsgaard H, Ostergaard L, Andersen-Ranberg K, Jeune B, Pedersen BK. Proinflammatory cytokines, antibodies to Chlamydia pneumoniae and age-associated diseases in Danish centenarians: is there a link? *Scand J Infect Dis.* 2002;34:493-9.
24. Schumacher A, Seljeflot I, Lerkerod AB, Sommervoll L, Otterstad JE, Arnesen H. Chlamydia LPS and MOMP seropositivity are associated with different cytokine profiles in patients with coronary heart disease. *Eur J Clin Invest.* 2005;35:431-7.
25. Blanc P, Corsi AM, Gabbuti A, Peduzzi C, Meacci F, Olivieri F, Lauretani F, Francesco M, Ferrucci L. Chlamydia pneumoniae seropositivity and cardiovascular risk factors: The InCHIANTI Study. *J Am Geriatr Soc.* 2004;52:1626-31.
26. Parchure N, Zouridakis EG, Kaski JC. Effect of Azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of Chlamydia pneumoniae infection. *Circulation.* 2002;105:1298-303.
27. Kuvin JT, Gokce N, Holbrook M, Hunter LM, Patel AR, Sliney KA, Craven DE, Grayston JT, Keaney JF, Jr., Karas RH, Vita JA. Effect of short-term antibiotic treatment on Chlamydia pneumoniae and peripheral endothelial function. *Am J Cardiol.* 2003;91:732-5.
28. Semaan HB, Gurbel PA, Anderson JL, Muhlestein JB, Carlquist JF, Horne BD, Serebruany VL. The effect of chronic azithromycin therapy on soluble endothelium-derived adhesion molecules in patients with coronary artery disease. *J Cardiovasc Pharmacol.* 2000;36:533-7.
29. Benagiano M, Azzurri A, Ciervo A, Amedei A, Tamburini C, Ferrari M, Telford JL, Baldari CT, Romagnani S, Cassone A, D'Elia MM, Del Prete G. T helper type 1 lymphocytes drive inflammation in human atherosclerotic lesions. *Proc Natl Acad Sci U S A.* 2003;100:6658-63.
30. Anderson JL, Muhlestein JB, Carlquist J, Allen A, Trehan S, Nielson C, Hall S, Brady J, Egger M, Horne B, Lim T. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease and serological evidence for Chlamydia pneumoniae infection: The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with Chlamydia (ACADEMIC) study. *Circulation.* 1999;99:1540-7.

Chapter 8

General discussion

General discussion

Although the role of *C pneumoniae* infection in atherosclerosis and clinical vascular events has been extensively investigated, final judgement has not passed yet. It is still unknown whether the presence of *C pneumoniae* is coincidental, or if there is a causal relationship.

This thesis addresses the question whether there is a role for *C pneumoniae* infection in ischaemic stroke, by means of an animal experiment, neuropathological examinations of post mortem brain tissue and four clinical studies on the association between *C pneumoniae* antibody titres, inflammatory parameters, common carotid artery intima-media thickness and ischaemic cerebral lesions.

In 1886, Koch postulated his criteria to prove the involvement of a micro-organism in a specific disease, which were: 1) the pathogen must be present in (nearly) all cases of the disease, 2) the pathogen must be isolated from the host with the disease, and grown in pure culture, 3) the specific disease must be produced when a pure culture of the pathogen is inoculated in a healthy susceptible host, 4) the bacteria must be recoverable from the experimentally infected host. Koch's postulates have some limitations, for instance the effect of therapy is not taken into account, but they can still be used as a footing to discuss the role of *C pneumoniae* in ischaemic stroke.

Initial evidence of infection with *C pneumoniae* in individuals with ischaemic stroke comes from serological studies. The presence of Immunoglobulin (Ig) G and IgA was assessed in a stroke population and compared to healthy controls¹⁻⁷. In a small population of young people with ischaemic stroke, we found anti-*C pneumoniae* IgA antibodies more often in stroke patients than in healthy controls. In the past, the presence of IgA antibodies was believed to reflect chronic persistent *C pneumoniae* infection, but it has not been validated as such yet⁸. Criteria have been posted only for acute infection (IgM>1:16, or a four fold increase in IgG) and presumed past infection (IgG>1:16), using the microimmunofluorescence (MIF) test. Using these criteria, seroprevalence is very high, and it rises with age⁹. In the population of young people with stroke (mean age 44 years), seroprevalence for IgG was 69%, compared to 81% in the population of the ACSI trial (mean age 64 years). As the presence of antibodies only reflects past infection, proving the role of *C pneumoniae* in clinical ischaemic stroke also requires detection of the presence of the micro-organism in the vascular wall of the cerebropetal or cerebral vessels. Positive antibody titres do not reflect vascular infection, as the presence of *C pneumoniae* is not always demonstrated in the vascular wall of patients with a positive antibody titre¹⁰. Collection of atherosclerotic material during life is nearly impossible, and is only available from the internal carotid artery fragments obtained during carotid endarterectomy, this reflects only a small

selected group of stroke patients, as approximately 10% of the ischaemic stroke population requires carotid endarterectomy. The presence of *C pneumoniae* in the larger intracerebral vessels can only be investigated post-mortem as described in chapter 4. We failed to establish the presence of *C pneumoniae* in large intracerebral vessel samples, although all the vessels had signs of atherosclerosis varying from intima fibrosis to fully developed atherosclerotic plaques with calcifications. *C pneumoniae* was only found in one sample of the basal ganglia. A limitation to these results might be that the cases were not stroke patients. However, another study on the presence of *C pneumoniae* in cerebral arteries found no differences between stroke patients and controls¹¹. Further reports on the presence of *C pneumoniae* in intracerebral vessels have been scarce, in the mere three reports prevalence rates varied from 0-66%¹²⁻¹⁴. The presence of *C pneumoniae* within the vessel wall was reported to be very patchy, and consequently, false negative results could emerge¹⁵. Differences in methodology between these studies account in part for the variable results. However, even taken that into account, the results of the serological and pathological studies do not meet Koch's first postulate of infection. Proving the second postulate is difficult in the case of *C pneumoniae*, as the micro-organism is very hard to culture. However, in one study *C pneumoniae* was isolated from a atherosclerotic coronary artery and successfully cultured¹⁶, in a later study, *C pneumoniae* was isolated from 16% of coronary artery specimens¹⁷.

For obvious reasons, Koch's third and fourth postulate (the specific disease must be produced when a pure culture of the pathogen is inoculated in a healthy susceptible host, and the bacteria must be recoverable from the experimentally infected host) can only be investigated in animal studies. *C pneumoniae* infection accelerates atherosclerosis progression in Apolipoprotein E deficient mice¹⁸, and early atherosclerotic lesions are seen in the aorta of rabbits infected with *C pneumoniae*¹⁹. In the infected animals the presence of *C pneumoniae* could be established in the vascular wall by polymerase chain reaction and immunohistochemistry. In our study we proved that intraperitoneal injection of *C pneumoniae* leads to cerebral infection, thereby proving haematogenic dissemination of *C pneumoniae* into the brain. In the mouse type with extensive atherosclerosis, cerebral *C pneumoniae* infection leads to enhanced microglial activation. Microglial activation has been indicated as a key factor in neurodegenerative and neuroinflammatory disorders such as Alzheimer's disease and Multiple Sclerosis²⁰ and is associated with chronic cerebral ischaemia. Therefore, it can be hypothesized that *C pneumoniae* infection might play a role in these conditions. These observations indicate a role for *C pneumoniae* infection in the initiation and acceleration of the atherosclerotic process and (ischaemic) cerebral damage in an animal model. Extrapolation to the human situation,

however, is difficult. Furthermore, although extensive atherosclerosis is found in these animals, a thrombo-embolic event such as myocardial infarction or cerebral infarction has not been investigated in these animal models. With animal models, therefore the third and fourth of Koch's postulates may be proved, but as said before, extrapolation to the human situation is virtually impossible. Therefore it is difficult to assess causal relationship of *C pneumoniae* infection and stroke, using Koch's postulates. Furthermore, the role of *C pneumoniae* in stroke may not be a specific quality of *C pneumoniae*, as the risk of ischaemic stroke is associated with infection in general, especially infections occurring several weeks before the stroke²¹. However, as opposed to other micro-organisms, *C pneumoniae* has the ability to remain inside the host in a chronic state, thereby causing a continuous state of low grade inflammation. This leads to increased levels of inflammatory agents such as C-reactive protein (CRP) and inflammatory cytokines such as Interleukin-6 (IL-6), TNF α , or IL-1 β . Increased levels of CRP and IL-6 are associated with increased stroke risk in apparently healthy people^{22,23}, and CRP levels are higher in stroke patients compared to healthy controls²⁴. However, different types of stroke can be identified, with different types of vascular pathology and different risk of recurrent stroke²⁵. If levels of inflammatory agents differ between the different types of stroke this might have implications for eventual secondary prevention. Our study of CRP and different cytokines in a stroke population revealed no differences in inflammatory parameters between cortical infarcts and lacunar infarcts. Furthermore, levels of inflammation were not associated with the presence of silent ischaemic brain lesions. So, even though CRP and IL-6 levels might identify patients at risk of stroke, they cannot differentiate between types of stroke, and might not be able to identify patients with a higher risk of recurrent stroke. So there seems to be no specific role of *C pneumoniae* in cerebral atherosclerosis. In large cohort trials, CRP levels have been associated with carotid intima-media thickness (IMT)^{26,27}. Other studies report an association between the presence of anti Chlamydia antibodies and IMT. The IMT is regarded as an early sign of carotid atherosclerosis, and *C pneumoniae* could also contribute to stroke risk through this mechanism. The relation between IMT and risk of stroke however has been investigated in large cohort studies and the usefulness of IMT levels in the individual patient has not been established yet, nor is it evident whether IMT is a useful predictive parameter in stroke patients. In our stroke population we could not relate IMT to CRP or any of the tested cytokines, nor to *C pneumoniae* serology. Furthermore IMT values did not differ between stroke types. So, again using a marker of generalized atherosclerosis, we were unable to detect any specific role of *C pneumoniae*. However, if a role for *C pneumoniae* infection in stroke is still assumed, treatment with antibiotics against *C pneumoniae* should be effective in the

secondary prevention of stroke. This has been tested for cardiovascular disease and peripheral arterial occlusive disease. The available evidence points in different directions, with some studies finding a reduction in clinical cardiovascular events in patients treated with antibiotics^{28,29}, whereas others found no differences between treatment or placebo³⁰⁻³². Studies that reported a reduction in vascular events were small with a limited duration of follow up. Two larger studies, including over 10000 patients, reported no significant reduction in clinical vascular events in patients treated for either three months³⁰ or one year³¹. The effect of antibiotic treatment can also be tested using non-clinical endpoints, i.e. surrogate endpoints. These trials require a smaller sample size and are therefore easier to perform. Most of these studies found no effect of antibiotic treatment in endothelial markers such as adhesion molecules^{33,34}; one study found a reduction in E-selectin accompanied by improved endothelial function³⁵. Carotid IMT is often used as a surrogate endpoint in clinical trials. The antibiotic roxithromycin has been proven to reduce progression of carotid IMT at two years of follow up, and therefore might lower future risk of stroke and cardiovascular events in stroke patients seropositive for *C pneumoniae*³⁶. This effect, however, proved to be temporarily, at three and four years of follow up increase in carotid IMT was equal to the situation before therapy³⁷. Inflammatory parameters such as CRP and IL-6 have been used as a secondary endpoint in the ACADEMIC trial, which only found a decrease in inflammatory parameters three months after the end of the study medication³⁸. In our stroke population we investigated whether treatment with azithromycin influences the ability of mononuclear cells to produce cytokines after stimulation with *C pneumoniae*. Azithromycin did not change cytokine levels after a three-month treatment course. Several considerations have to be made in assessing the effect of antibiotics on clinical vascular events such as stroke. *C pneumoniae* has proven to be very resistant to antibiotic treatment in vitro³⁹, and long term treatment with antibiotics do not fully eliminate *C pneumoniae* from circulating human monocytes⁴⁰. So even long term treatment with antibiotics such as in the ACES study³¹ (one year) might not be sufficient to fully eradicate *C pneumoniae*. In fact, the addition in secondary prevention from antibiotics might only come when they are used life long, such as platelet aggregation inhibitors and statins, raising the problem of antibiotic resistance. Furthermore, in animal studies the effect of azithromycin is only seen when the antibiotic is administered just after infection, something that is not possible in the human situation.

This thesis addresses the question whether there is a role for *C pneumoniae* infection in ischaemic stroke, in almost any aspect. Our animal study showed that *C pneumoniae* has the ability to enter the brain via haematogenic dissemination and that, in susceptible animals, this leads to microglial

activation, which is seen in areas of chronic cerebral ischaemia⁴¹. However, post mortem examination of 20 human brains showed *C pneumoniae* in only one sample of the basal ganglia and in none of the large cerebral vessels. In the four clinical studies, we found IgA antibodies against *C pneumoniae* more often in stroke patients than in healthy controls. No differences were found in IgG antibodies and the overall prevalence of IgG antibodies was high. An even higher seroprevalence was found in the older stroke population, and in this stroke population, we could not find a relationship between *C pneumoniae* serology, inflammatory parameters and stroke subtype. Furthermore, within our stroke population we found no correlation between inflammatory levels, *C pneumoniae* serology and carotid IMT, which is considered an early sign of carotid atherosclerosis. A three-month course of azithromycin does not influence cytokine production of mononuclear cells incubated with *C pneumoniae*. Taken the results of these studies into account, the role for *C pneumoniae* infection in clinical stroke seems not to be very important, although we cannot be 100% sure of this given the above mentioned considerations. Therefore, there might be a more aspecific effect of infections in general as opposed to a specific effect of *C pneumoniae*. As of yet, there seems to be no rationale for a large secondary prevention trial on the effect of antibiotics against *C pneumoniae*. The outcome of a small trial using surrogate endpoints may definitely forestall such a trial, or could surprisingly argue in its favour. The results of the ACSI trial (Azithromycin for the prevention of the progression of Cerebral ischaemic lesions in patients Surviving a non-cardioembolic cerebral Infarct), are therefore eagerly awaited.

References

1. Johnsen SP, Overvad K, Ostergaard L, Tjonneland A, Husted SE, Sorensen HT. Chlamydia pneumoniae seropositivity and risk of ischemic stroke: a nested case-control study. *Eur J Epidemiol.* 2005;20:59-65.
2. Ngeh J, Gupta S, Goodbourn C, Panayiotou B, McElligott G. Chlamydia pneumoniae in elderly patients with stroke (C-PEPS): a case-control study on the seroprevalence of Chlamydia pneumoniae in elderly patients with acute cerebrovascular disease. *Cerebrovasc Dis.* 2003;15:11-6.
3. Heuschmann PU, Neureiter D, Gesslein M, Craiovan B, Maass M, Faller G, Beck G, Neundoerfer B, Kolominsky Rabas PL. Association between infection with Helicobacter pylori and Chlamydia pneumoniae and risk of ischemic stroke subtypes: Results from a population-based case-control study. *Stroke.* 2001;32:2253-8.
4. Cook PJ, Honeybourne D, Lip GYH, Beevers DG, Wise R, Davies AH. Chlamydia pneumoniae antibody titers are significantly associated with acute stroke and transient cerebral ischemia: the West Birmingham Stroke Project. *Stroke.* 1998;29:404-10.
5. Elkind MS, Lin IF, Grayston JT, Sacco RL. Chlamydia pneumoniae and the risk of first ischemic stroke : The Northern Manhattan Stroke Study. *Stroke.* 2000;31:1521-5.
6. Fagerberg B, Gnarpe J, Gnarpe H, Agewall S, Wikstrand J. Chlamydia pneumoniae but not cytomegalovirus antibodies are associated with future risk of stroke and cardiovascular disease: a prospective study in middle-aged to elderly men with treated hypertension. *Stroke.* 1999;30:299-305.
7. Wimmer ML, Sandmann Strupp R, Saikku P, Haberl RL. Association of chlamydial infection with cerebrovascular disease. *Stroke.* 1996;27:2207-10.
8. Dowell SF, Peeling RW, Boman J, Carlone GM, Fields BS, Guarner J, Hammerschlag MR, Jackson LA, Kuo CC, Maass M, Messmer TO, Talkington DF, Tondella ML, Zaki SR. Standardizing Chlamydia pneumoniae Assays: Recommendations from the Centers for Disease Control and Prevention (USA) and the Laboratory Centre for Disease Control (Canada). *Clin Infect Dis.* 2001;33:492-503.
9. Einarsson S, Sigurdsson HK, Magnusdottir SD, Erlendsdottir H, Briem H, Gudmundsson S. Age specific prevalence of antibodies against Chlamydia pneumoniae in Iceland. *Scand J Infect Dis.* 1994;26:393-7.
10. Maass M, Gieffers J, Krause E, Engel PM, Bartels C, Solbach W. Poor correlation between microimmunofluorescence serology and polymerase chain reaction for detection of vascular Chlamydia pneumoniae infection in coronary artery disease patients. *Med Microbiol Immunol Berl.* 1998;187:103-6.
11. Wohlschlaeger J, Wimmer ML, Nagler DK, Haberl R, Weis S. Identification of Chlamydia pneumoniae in intracranial and extracranial arteries in patients with stroke and in controls: combined immunohistochemical and polymerase chain reaction analyses. *Hum Pathol.* 2005;36:395-402.
12. Vink A, Poppen M, Schoneveld AH, Roholl PJ, de Kleijn DP, Borst C, Pasterkamp G. Distribution of Chlamydia pneumoniae in the human arterial system and its relation to the local amount of atherosclerosis within the individual. *Circulation.* 2001;103:1613-7.
13. Virok D, Kis Z, Karai L, Intzedy L, Burian K, Szabo A, Ivanyi B, Gonczol E. Chlamydia pneumoniae in atherosclerotic middle cerebral artery. *Stroke.* 2001;32:1973-6.
14. Rassa M, Cazzavillan S, Scagnelli M, Peron A, Bevilacqua PA, Facco M, Bertoloni G, Lauro FM, Zambello R, Bonoldi E. Demonstration of Chlamydia pneumoniae in atherosclerotic arteries from various vascular regions. *Atherosclerosis.* 2001;158:73-9.
15. Cochrane M, Pospischil A, Walker P, Gibbs H, Timms P. Distribution of Chlamydia pneumoniae DNA in atherosclerotic carotid arteries: significance for sampling procedures. *J Clin Microbiol.* 2003;41:1454-7.
16. Ramirez JA. Isolation of Chlamydia pneumoniae from the coronary artery of a patient with coronary atherosclerosis. The Chlamydia pneumoniae/Atherosclerosis Study Group. *Ann Intern Med.* 1996;125:979-82.

17. Maass M, Bartels C, Engel PM, Mamat U, Sievers HH. Endovascular presence of viable *Chlamydia pneumoniae* is a common phenomenon in coronary artery disease. *J Am Coll Cardiol*. 1998;31:827-32.
18. Moazed TC, Campbell LA, Rosenfeld ME, Grayston JT, Kuo CC. *Chlamydia pneumoniae* infection accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *J Infect Dis*. 1999;180:238-41.
19. Fong IW, Chiu B, Viira E, Jang D, Mahony JB. De Novo induction of atherosclerosis by *Chlamydia pneumoniae* in a rabbit model. *Infect Immun*. 1999;67:6048-55.
20. Gonzales-Scarano F, Baltuch G. Microglia as mediators of inflammatory and degenerative diseases. *Annu. Rev. Neurosci*. 1999;219-240.
21. Bova IY, Bornstein NM, Korczyn AD. Acute infection as a risk factor for ischemic stroke. *Stroke*. 1996;27:2204-6.
22. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med*. 1997;336:973-9.
23. Ridker PM, Buring JE, Shih J, Matias M, Hennekens CH. Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation*. 1998;98:731-3.
24. Ford ES, Giles WH. Serum C-reactive protein and self-reported stroke: findings from the Third National Health and Nutrition Examination Survey. *Arterioscler Thromb Vasc Biol*. 2000;20:1052-6.
25. de Jong G, van Raak L, Kessels F, Lodder J. Stroke subtype and mortality. a follow-up study in 998 patients with a first cerebral infarct. *J Clin Epidemiol*. 2003;56:262-8.
26. Folsom AR, Pankow JS, Tracy RP, Arnett DK, Peacock JM, Hong Y, Djousse L, Eckfeldt JH. Association of C-reactive protein with markers of prevalent atherosclerotic disease. *Am J Cardiol*. 2001;88:112-7.
27. Sitzer M, Markus HS, Mendall MA, Liehr R, Knorr U, Steinmetz H. C-reactive protein and carotid intimal medial thickness in a community population. *J Cardiovasc Risk*. 2002;9:97-103.
28. Gurfinkel E. Inflammation, infection, or both in atherosclerosis: the ROXIS trial in perspective. *J Infect Dis*. 2000;181 Suppl 3:S566-8.
29. Gupta S. *Chlamydia pneumoniae*, monocyte activation, and azithromycin in coronary heart disease. *Am Heart J*. 1999;138:S539-41.
30. O'Connor CM, Dunne MW, Pfeffer MA, Muhlestein JB, Yao L, Gupta S, Benner RJ, Fisher MR, Cook TD. Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. *Jama*. 2003;290:1459-66.
31. Grayston JT, Kronmal RA, Jackson LA, Parisi AF, Muhlestein JB, Cohen JD, Rogers WJ, Crouse JR, Borrowdale SL, Schron E, Knirsch C. Azithromycin for the secondary prevention of coronary events. *N Engl J Med*. 2005;352:1637-45.
32. Vainas T, Stassen FR, Schurink GW, Tordoir JH, Welten RJ, van den Akker LH, Kurvers HA, Bruggeman CA, Kitslaar PJ. Secondary prevention of atherosclerosis through *chlamydia pneumoniae* eradication (SPACE Trial): a randomised clinical trial in patients with peripheral arterial disease. *Eur J Vasc Endovasc Surg*. 2005;29:403-11.
33. Gabriel AS, Ahnve S, Gnarpe H, Gnarpe J, Martinsson A. Azithromycin therapy in patients with chronic *Chlamydia pneumoniae* infection and coronary heart disease: immediate and long-term effects on inflammation, coagulation, and lipid status in a double-blind, placebo-controlled study. *Eur J Intern Med*. 2003;14:470-8.
34. Semaan HB, Gurbel PA, Anderson JL, Muhlestein JB, Carlquist JF, Horne BD, Serebruany VL. The effect of chronic azithromycin therapy on soluble endothelium-derived adhesion molecules in patients with coronary artery disease. *J Cardiovasc Pharmacol*. 2000;36:533-7.
35. Parchure N, Zouridakis EG, Kaski JC. Effect of Azithromycin treatment on endothelial function in patients with coronary artery disease and evidence of *Chlamydia pneumoniae* infection. *Circulation*. 2002;105:1298-303.
36. Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Reduced progression of early carotid atherosclerosis after antibiotic treatment and *Chlamydia pneumoniae* seropositivity. *Circulation*. 2002;106:2428-33.

37. Sander D, Winbeck K, Klingelhofer J, Etgen T, Conrad B. Progression of early carotid atherosclerosis is only temporarily reduced after antibiotic treatment of *Chlamydia pneumoniae* seropositivity. *Circulation*. 2004;109:1010-5.
38. Anderson JL, Muhlestein JB, Carlquist J, Allen A, Trehan S, Nielson C, Hall S, Brady J, Egger M, Horne B, Lim T. Randomized secondary prevention trial of azithromycin in patients with coronary artery disease and serological evidence for *Chlamydia pneumoniae* infection: The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with *Chlamydia* (ACADEMIC) study. *Circulation*. 1999;99:1540-7.
39. Kutlin A, Roblin PM, Hammerschlag MR. Effect of prolonged treatment with azithromycin, clarithromycin, or levofloxacin on *Chlamydia pneumoniae* in a continuous-infection Model. *Antimicrob Agents Chemother*. 2002;46:409-12.
40. Gieffers J, Fullgraf H, Jahn J, Klinger M, Dalhoff K, Katus HA, Solbach W, Maass M. *Chlamydia pneumoniae* infection in circulating human monocytes is refractory to antibiotic treatment. *Circulation*. 2001;103:351-6.
41. Tomimoto H, Akiguchi I. The role of immunologic reactions in the pathogenesis in Binswanger's disease; a clue to therapeutic approach. *Rinsho Shinkeigaku*. 1999;39:56-8.

Summary

Summary

Atherosclerosis is defined as an inflammatory, immune-mediated disorder, which develops following endothelial injury. Causes of endothelial injury include hypertension, smoking and elevated homocysteine. One of the factors implicated in the pathogenesis of atherosclerosis is infection, more specifically infection with *C pneumoniae*. Extensive research has been done in the area of infections and atherosclerosis of the coronary arteries; atherosclerosis of the (pre-) cerebral vessels has received less attention in this respect. In both areas results and opinions about the role of *C pneumoniae* in vascular events have been controversial. In this thesis we aim to answer questions on the association between *C pneumoniae* infection and (pre-) cerebral vascular disease.

In **chapter 2** we describe the presence of anti-Chlamydia antibodies in patients with an ischaemic stroke under the age of fifty. Immunoglobulin G (IgG) and IgA antibodies are produced after each infection, and the presence anti-Chlamydia IgG and IgA antibodies therefore is proof of a prior infection. This study included 41 patients, with atherosclerosis as the probable cause of the stroke, and 55 healthy controls. The presence of IgG and IgA antibodies was determined in the serum of these patients. We found that IgA antibodies against *C pneumoniae* were more prevalent in patients with ischaemic stroke than in the healthy controls (crude OR: 2.1; 90% CI 1.1-9.5, $p = 0.04$). After adjusting for hypertension, smoking and hypercholesterolaemia, positive Chlamydia antibodies remained associated with stroke (adjusted OR: 2.8, 90% CI 1.1-7.1, $p = 0.04$).

In **chapter 3** we describe a mouse experiment, issued to address the question whether *C pneumoniae* could enter the brain after an intraperitoneal injection, and whether the presence of *C pneumoniae* caused microglial activation. Microglial cells are the immune effector cells of the brain, and they respond to infection, trauma and ischaemia. Activation of microglial cells leads to the production of potentially neurotoxic agents such as tumour necrosis factor alpha (TNF α) and interleukin (IL)-1 beta (IL-1 β). The presence of activated microglial cells has been described in conditions such as Alzheimer's disease, and in areas of chronic cerebral ischaemia. Furthermore, we investigated whether *C pneumoniae* infection leads to a higher permeability of the blood brain barrier, the natural protection wall of the brain against micro-organisms and toxic agents. For this purpose we used three types of mice: mice with atherosclerosis, i.e. Apolipoprotein E (ApoE) knockout (KO) and ApoE/Low Density Lipoprotein receptor (ApoE/LDLr) KO mice both on a C57BL/6J background, and mice without atherosclerosis, i.e. wild type C57BL/6J mice.

Using a highly sensitive polymerase chain reaction, we could detect *C pneumoniae* DNA in the brain tissue of 30% of the infected mice. The number of infected mice was the highest shortly after infection (3 days) and declined in the three weeks after infection. The permeability of the blood brain barrier was not altered in the infected mice. Activated microglial cells were visualized using immunohistochemistry. We found a significant increase in the number of activated microglial cells in the brains of *C pneumoniae* PCR positive ApoE/LDLr KO mice, compared to mock-infected mice. There were no differences in microglial activation between the *C pneumoniae* infected *C pneumoniae* PCR negative mice and to the mock-infected mice. Furthermore we observed a higher number of activated microglia in atherosclerotic mice compared to non-atherosclerotic mice. We concluded that intraperitoneal injection of *C pneumoniae* leads to cerebral infection, and in the susceptible mouse, this leads to microglial activation.

Ischaemic cerebrovascular disease can be divided in two entities. Large vessel disease, which is due to atherosclerosis of the larger cerebral vessels, causes mainly larger, usually called territorial infarcts, which often involve the cortex. Small vessel disease causes small infarcts, less than 15 mm in diameter on cerebral imaging, which are located in the deep brain structures (basal ganglia and internal capsule) or the pons; these are often referred to as lacunar infarcts. Small vessel disease is also the cause of more diffuse damage to the white matter of the brain: leukoaraiosis. *C pneumoniae*, Cytomegalovirus (CMV) and Herpes Simplex virus type 1 (HSV-1) have been linked to large vessel atherosclerosis, and have been detected in brain tissue. The role of these micro-organisms in cerebral small vessel disease however, is still unknown. In **chapter 4** we describe the post-mortem presence of *C pneumoniae*, CMV and HSV-1 in the cerebral large vessels, small vessels and brain tissue of patients who died from various causes. In the majority of these cases, signs of large vessel disease and small vessel disease were present; in most cases this was asymptomatic. A PCR was performed on paraffin embedded tissue, to establish the presence of DNA of the above mentioned micro-organisms. Subsequently immunohistochemistry was used on adjacent paraffin slides to locate the micro-organisms within the brain tissue. Using the PCR method, we found no evidence of the presence of *C pneumoniae*, CMV, or HSV-1 in any of the cerebral large vessels. We found *C pneumoniae* in the brain tissue (periventricular white matter) of one case and CMV in the brain tissue (basal ganglia) of another case. By immunohistochemistry, the presence of CMV in the PCR positive sample could not be confirmed. *C pneumoniae* was present in ganglion cells and underneath the basal membrane of an arteriole. We concluded that, even though signs of large and small vessel disease were present in most cases, the presence of

the above mentioned micro-organisms was minimal. *C pneumoniae*, CMV and HSV-1, therefore do not seem to play an important role in (asymptomatic) cerebral large vessel and small vessel disease.

The chapters 5, 6 and 7 are clinical studies, using a patient population derived from the ACSI study (Azithromycin for the prevention of the progression of Cerebral ischaemic lesions in patients Surviving a non-cardio embolic cerebral Infarct), which investigates the effect of a three-month treatment schedule of azithromycin on ischaemic cerebral damage progression measured at two years.

In **chapter 5**, 149 patients are divided into two groups, patients with a cortical infarct or a single symptomatic lacunar infarct were assigned to the large vessel disease group, and patients with asymptomatic lacunar infarcts and leukoaraiosis were assigned to the small vessel disease group. *C pneumoniae* and inflammatory parameters such as high sensitive C-reactive protein (hsCRP) and cytokines such as IL-6 have been linked to cerebral large vessel disease, but it is unclear whether *C pneumoniae* infection and inflammation are related to small vessel disease, especially to the chronic, progressive type which results in silent lacunar infarcts and leukoaraiosis (silent brain lesions: SBL). For this purpose, we investigated the relation between the presence of *C pneumoniae* IgG antibodies, and inflammatory parameters (tumour necrosis factor alpha (TNF α), interleukin (IL)-6, IL-1 β , IL-10 and IFN γ) and the presence of SBL on magnetic resonance imaging of the brain. Inflammatory markers did not differ between patients with and without silent brain lesions, nor did the presence of *C pneumoniae* antibodies. Furthermore, inflammatory markers were not different in patients with and without *C pneumoniae* antibodies. Therefore, we concluded that *C pneumoniae* infection is not related to cerebral small vessel disease.

Common carotid intima-media thickness (CCA-IMT) can be assessed non-invasively by B-mode ultrasonography. Increases in IMT are associated with an increased risk of myocardial infarction and stroke, but this has only been investigated in large population based studies in healthy individuals. The same thing is true for the relation between *C pneumoniae* infection and inflammatory markers and CCA-IMT. It is unknown whether CCA-IMT is a predictive factor in a selected population of stroke patients, and how CCA-IMT is related to stroke subtype. In **chapter 6**, we sought to find out whether CCA-IMT is related to stroke subtype, and the presence of ipsilateral SBL on MRI. Furthermore, we investigated the relation between serum inflammatory parameters, *C pneumoniae* serology, and CCA-IMT. In our patient population (N=75), there were no differences in mean CCA-IMT with regard to the presence of ipsilateral

SBL. Furthermore, we found that, in a selected population of stroke patients, CCA-IMT was not related to *C pneumoniae* serology and inflammatory parameters. We concluded that CCA-IMT values are not different in the different stroke subtypes and are not related to the presence of SBL, in a population of patients with a symptomatic stroke. Furthermore, CCA-IMT is not related to *C pneumoniae* serology, or inflammatory parameters.

In **chapter 7**, we investigated whether treatment with azithromycin, an antimicrobial and anti-inflammatory agent, reduced *C pneumoniae* induced cytokine production by blood mononuclear cells. For this purpose levels of the cytokines TNF α , IL-6, IL-1 β , IL-10 and IFN γ were assessed in mononuclear cells. These cells were stimulated with *C pneumoniae* antigen, and subsequently, cytokine levels were assessed in the supernatant. The cytokine levels of 75 patients, which had been randomised in the ACSI trial, were assessed, before and after treatment. The patients were randomised to receive either azithromycin 500 mg or a matching placebo once daily for 3 days, then once weekly for twelve weeks. Blood was drawn at randomisation and at three months, after finishing the course of study medication. After three months of treatment, there were no significant differences in cytokine levels in the supernatant of *C pneumoniae* stimulated cells between the two treatment groups. Furthermore, when we measured the changes in the cytokine levels before and after treatment, we found no significant differences in changes in the cytokine levels, between the two patient groups. We concluded that azithromycin does not influence *C pneumoniae* induced cytokine production of mononuclear cells.

In this thesis the relation between *C pneumoniae* infection and ischaemic stroke is addressed from different directions. The animal experiment showed that *C pneumoniae* can infect the brain through haematogenic spread and, that cerebral infection with *C pneumoniae* causes microglial activation in susceptible animals. Furthermore, we found anti-Chlamydia IgA antibodies more often in young patients with ischaemic stroke than in controls. In spite of the above mentioned results, we conclude that there is no significant role for *C pneumoniae* infection in ischaemic stroke. The presence of *C pneumoniae* in cerebral vessels and brain tissue is scarce, and in several clinical studies we found no relation between *C pneumoniae* infection and SBL, nor was *C pneumoniae* infection or inflammation related to CCA-IMT. Furthermore, there was no effect of azithromycin on *C pneumoniae* induced cytokine production of mononuclear cells.

Samenvatting

Samenvatting

Atherosclerose is een chronisch progressief inflammatoir proces, waarbij algemeen aangenomen wordt dat endotheelschade aan de basis van dit proces ligt. Verschillende oorzaken voor deze endotheelschade zijn al bekend, zoals hypertensie, roken en een verhoogd homocysteïne gehalte. Infecties, vooral infecties met *C pneumoniae*, worden ook genoemd als mogelijke bijdragers aan het proces van atherosclerose. In het verleden is uitgebreid onderzoek verricht naar de relatie tussen *C pneumoniae* infectie en atherosclerose van de coronair vaten; de cerebropetale vaten zijn hierin onderbelicht gebleven. De resultaten van studies op dit terrein die werden beschreven door diverse onderzoekers waren zeer tegenstrijdig; sommigen vonden een duidelijke relatie tussen *C pneumoniae* infectie en atherosclerose, anderen vonden deze relatie juist niet. Dit proefschrift probeert de vraag of er een relatie is tussen *C pneumoniae* infectie en cerebrovasculaire ischemie te beantwoorden.

In **hoofdstuk 2** beschrijven we de relatie tussen de aanwezigheid van imuunglobuline (Ig) G en IgA antilichamen tegen *C pneumoniae* in patiënten met een herseninfarct op jonge leeftijd, in vergelijking met gezonde controles. IgG en IgA antilichamen worden geproduceerd na iedere infectie en de aanwezigheid van anti-Chlamydia IgG en IgA antilichamen wijst derhalve op een doorgemaakte *C pneumoniae* infectie. In deze studie werden 41 patiënten geïncludeerd die jonger waren dan 50 jaar en een herseninfarct hadden doorgemaakt, waarvan de meest waarschijnlijke oorzaak atherosclerose was. De aanwezigheid van IgG en IgA antistoffen werden bepaald in het serum van deze patiënten en dit werd vergeleken met het serum van 55 gezonde controles. We vonden dat IgA antistoffen tegen *C pneumoniae* vaker in de patiëntengroep voorkwamen dan in de controle groep (OR 2.1; 90% CI 1.1-9.5, $p=0.04$). Ook na corrigeren voor factoren als hypertensie, roken, hypercholesterolemie en diabetes mellitus, was er nog een significante relatie tussen de aanwezigheid van IgA antistoffen tegen *C pneumoniae* en een herseninfarct op jonge leeftijd (gecorrigeerde OR: 2.8, 90% CI 1.1-7.1, $p=0.04$).

In **hoofdstuk 3** wordt een muisexperiment beschreven. In dit experiment werd onderzocht of *C pneumoniae* de hersenen kan infecteren na intraperitoneale toediening en of deze infectie leidt tot een reactie, namelijk tot activatie van microglia cellen. Microglia cellen zijn de immuuncellen van de hersenen en deze cellen reageren op infectie, trauma of ischemie van de hersenen. De activatie van microglia cellen leidt tot de productie van verschillende cytokines zoals tumor necrosis factor alpha ($TNF\alpha$) en interleukine 1 bèta ($IL-1\beta$). Deze stoffen zijn potentieel neurotoxisch. De aanwezigheid van geactiveerde

microglia cellen is reeds beschreven bij de ziekte van Alzheimer en in gebieden van de hersenen die zijn blootgesteld aan chronische ischemie. Daarnaast werd gekeken of *C pneumoniae* infectie werd veroorzaakt door, of leidde tot een verhoogde doorlaatbaarheid van de bloed-hersen barrière. De bloed-hersen barrière is de natuurlijke beschermer van de hersenen tegen schadelijke stoffen in de circulatie. Een verhoogde doorlaatbaarheid van deze bloed-hersen barrière kan ertoe leiden dat de hersenen worden bloot gesteld aan deze schadelijke stoffen. Om deze vragen te beantwoorden werden drie typen muizen geïnfecteerd, namelijk muizen met atherosclerose: de Apolipoproteïne E (ApoE) knockout (KO) en ApoE/Low Density Lipoprotein receptor (ApoE/LDLr) KO muizen en controle muizen, de C57BL/6J muizen. Met een zeer gevoelige polymerase ketting reactie test werd het DNA van *C pneumoniae* gevonden in 30% van de geïnfecteerde muizen. Het aantal geïnfecteerde muizen was het hoogst drie dagen na de intraperitoneale injectie. Uit het onderzoek bleek ook dat de bloed-hersen barrière niet was aangetast na infectie. De activatie van microglia cellen werd in beeld gebracht door een immunohistochemische kleuring. Hierbij vonden we in de hersenen van de geïnfecteerde ApoE/LDLr KO muizen significant meer geactiveerde microglia cellen dan in de niet geïnfecteerde ApoE/LDLr KO muizen. Daarnaast bleken de ApoE/LDLr KO muizen meer geactiveerde microglia cellen te hebben dan de C57BL/6J muizen. We concludeerden dat intraperitoneale injectie van *C pneumoniae* leidt tot een cerebrale infectie, die in de muizen met atherosclerose leidde tot een verhoogd aantal geactiveerde microglia cellen.

Het herseninfarct kan worden ingedeeld in twee groepen: large vessel disease en small vessel disease. Large vessel disease staat voor de atherosclerose van de grotere hersenvaten; waardoor de grotere zogenaamde corticale herseninfarcten worden veroorzaakt. Small vessel disease staat voor vaatwandveranderingen in de kleinere hersenvaten, het type infarct wat hierbij wordt gezien is de zogenaamde lacunaire infarct. Lacunaire infarcten zijn niet groter dan 15 mm in doorsnede en worden vooral gezien in de diepere hersenstructuren zoals de capsula interna en de basale ganglia. Daarnaast wordt small vessel disease geassocieerd met chronische progressieve afwijkingen zoals stille lacunaire infarcten en leukoaraïosis ("silent brain lesions" = SBL). *C pneumoniae*, Cytomegalovirus (CMV) en Herpes Simplex virus type 1 (HSV-1), zijn allen in verband gebracht met atherosclerose en zijn allen eerder aangetoond in hersenweefsel. Echter, de rol van deze micro-organismen in de vaatwandveranderingen van de kleine hersenvaten is nog nooit eerder beschreven. In **hoofdstuk 4** beschrijven we de onderzoek naar aanwezigheid van *C pneumoniae*, CMV en HSV-1 in de grote hersenarteriën,

de kleine hersenvaten en hersenweefsel, dat is verkregen bij obductie van mensen die waren overleden aan diverse oorzaken.

De polymerase ketting reactie werd hier uitgevoerd op weefsel wat gefixeerd was in formaline en daarna ingebed in paraffine. Daarnaast werd op weefsel waar een van de micro-organismen werd gevonden een immuno-histochemische kleuring toegepast om de locatie van het micro-organisme aan te tonen. Met de polymerase ketting reactie werd in slechts twee casus DNA gevonden van *C pneumoniae* (in de periventriculaire witte stof) en CMV (in de basale kernen). Geen van de micro-organismen werd aangetoond in de grote hersenarteriën, hoewel in bijna alle hersenarteriën tekenen van atherosclerose werden gezien. Met immunohistochemie konden alleen *C pneumoniae* antigenen worden aangetoond; deze bevonden zich in de ganglion cellen van de hersenen en onder de basale membraan van een hersenarteriole. Hieruit concludeerden we dat ondanks dat er in het merendeel van de weefsels aanwijzingen werden gevonden voor atherosclerose, arteriolosclerose en ischemische hersenschade, de aanwezigheid van de bovengenoemde micro-organismen minimaal was. Deze micro-organismen lijken dus geen rol te spelen in asymptomatische ischemische hersenschade en vaatwandveranderingen.

In **hoofdstuk 5** werden 149 patiënten verdeeld in twee groepen: patiënten met corticale infarcten en één enkel symptomatisch lacunair infarct werden ingedeeld in de groep large vessel disease en patiënten met stille lacunaire infarcten en leukoaraiosis werden ingedeeld in de small vessel disease groep. *C pneumoniae* antistoftiters en inflammatoire parameters zoals high sensitive C-reactief proteïne (hsCRP) en cytokines als IL-6, worden in het algemeen geassocieerd met atherosclerose van de grote cerebrale vaten. Het is onduidelijk of *C pneumoniae* infectie en inflammatie ook een rol spelen in het ontstaan van small vessel disease en de of *C pneumoniae* infectie gerelateerd is aan de aanwezigheid van asymptomatische ischemische laesies zoals stille lacunaire infarcten en leukoaraiosis (SBL). Hiertoe onderzochten we de relatie tussen de aanwezigheid van *C pneumoniae* IgG antilichamen en inflammatoire parameters in het serum (hsCRP, IL-6, TNF α , IL-1 β , IL-10, en IFN γ) en de aanwezigheid van SBL op de magnetische resonantie scan (MRI) van de hersenen. Het resultaat van dit onderzoek was dat er geen verschil gevonden werd in inflammatoire parameters tussen de groep patiënten met en zonder SBL, ook de aanwezigheid van *C pneumoniae* IgG antilichamen verschilde niet tussen beide patiëntengroepen. *C pneumoniae* infectie is dus niet gerelateerd aan cerebrale small vessel disease.

Het meten van de intima-media dikte van de arteria carotis communis (CCA-IMT) is een veilig en non-invasief onderzoek. Het is alom bekend dat de dikte

van de CCA-IMT gerelateerd is aan het risico op het krijgen van een vasculair event. Dit is echter alleen beschreven in grote studies in gezonde patiënten populaties. Hetzelfde geldt voor de relatie tussen inflammatoire parameters en de dikte van de CCA-IMT. Het is niet bekend of de CCA-IMT in een selecteerde patiëntenpopulatie ook een voorspellende factor is. Tevens zijn data over de relatie tussen CCA-IMT en de verschillende subtypen van het herseninfarct spaarzaam. Zoals beschreven is in **hoofdstuk 6** onderzochten we of de CCA-IMT gerelateerd is aan de aanwezigheid van ipsilaterale SBL. Dit werd vervolgens gerelateerd aan de aanwezigheid van *C pneumoniae* IgG antistoffen en inflammatoire parameters in het bloed. In onze patiëntenpopulatie (N = 75) vonden we geen verschillen in de gemiddelde CCA-IMT waarden in de aan- en afwezigheid van ipsilaterale SBL. Verder vonden we in een populatie die exclusief bestond uit patiënten met een herseninfarct geen relatie tussen de dikte van de CCA-IMT, inflammatoire parameters en *C pneumoniae* serologie.

In **hoofdstuk 7** beschrijven we het effect van een drie maanden durende antibiotica kuur met azithromycine op de cytokine productie van mononucleaire cellen in het bloed. In dit experiment werden de mononucleaire cellen gestimuleerd met *C pneumoniae* antigeen, waarna de productie van de cytokines TNF α , IL-1 β , IL-6, IL-10 en IFN γ werd gemeten in het supernatant. Van 75 patiënten die deelnamen aan het ACSI onderzoek werd bloed afgenomen voor en na de azithromycine inname. De patiënten waren bij loting in twee groepen verdeeld en kregen azithromycine of een placebo: 500 mg per dag gedurende de eerste drie dagen, en 500 mg per week voor een periode van twaalf weken. Na drie maanden was er geen verschil in de bloedspiegel van de cytokines tussen beide patiëntengroepen. Daarnaast werd gekeken naar het verschil in bloedspiegels van de cytokines voor en na de behandeling, ook hier werd geen verschil gevonden. Uit dit onderzoek kan worden geconcludeerd dat azithromycine heeft geen effect op de cytokine productie van mononucleaire cellen die worden gestimuleerd met *C pneumoniae* antigeen.

In dit proefschrift werd op verschillende manieren gekeken naar een eventuele relatie tussen een infectie met *C pneumoniae* en herseninfarcten. Het diermodel liet zien dat *C pneumoniae* het brein kan infecteren en microglia activatie veroorzaakt, zoals ook wordt gezien bij chronische ischemie van de hersenen. Jonge patiënten met een herseninfarct hadden vaker anti-Chlamydia IgA antistoffen dan gezonde controles. Ondanks bovengenoemde resultaten lijkt er geen (grote) rol weggelegd voor *C pneumoniae* infectie in het ontstaan van cerebrale ischemie. De aanwezigheid van *C pneumoniae* in de hersenvaten en hersenweefsel bleek zeer minimaal te zijn en in de klinische

studies werd geen relatie gevonden tussen *C pneumoniae* serologie en de aanwezigheid van SBL op MRI en de dikte van de CCA-IMT. Daarnaast werd geen effect gevonden van behandeling met azithromycine op de cytokine productie van mononucleaire cellen.

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Dankwoord

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Mijn familie.

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Curriculum vitae

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Johanna Maria Voorend (roepnaam Manuela) werd geboren op 12 september 1971 te Breukelen. In 1989 deed ze eindexamen VWO aan de RSG Brokdele te Breukelen, waarna ze geneeskunde ging studeren aan de Universiteit van Amsterdam. In 1996 behaalde ze het artsendiploma en werkte vervolgens een jaar als AGNIO neurochirurgie in het Slotervaart ziekenhuis in Amsterdam. Na twee jaar AGNIO neurologie werd in 2000 gestart met een AGIKO constructie neurologie, waarin werd gestart met het onderzoek naar de relatie tussen *Chlamydia pneumoniae* en herseninfarcten, waarvan dit proefschrift het resultaat is. In februari 2003 startte zij met de opleiding tot neuroloog, die ze in augustus 2008 hoopt af te ronden.