On the inflammatory and infectious aspects of atherosclerosis: a serological, molecular biological & clinical treatise
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ISBN 90 5278 512 0

Layout: Tiny Wouters
Production: Datawyse | Universitaire Pers Maastricht

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Greek soldiers finally entered and conquered the city of Troy, after a siege of 10 years, hidden in the belly of a wooden horse. Similarly, many proatherogenic factors, such as Chlamydia pneumoniae may reach the vascular wall within the macrophages.
On the inflammatory and infectious aspects
of atherosclerosis:
a serological, molecular biological & clinical treatise

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. mr. G.P.M.F. Mois,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 17 februari 2005 om 16.00 uur

door

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geboren op 26 oktober 1969
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The studies described in this thesis were supported by a grant of the Netherlands Heart Foundation (NHF-98.149)

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.
In memory of Marcel van den Berg.

The soul of a man is immortal and imperishable.

Plato

Στους γονείς μου και στην Σάκη
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Chapter 1

General introduction & Aims of the thesis
1.1 Definition

The term *atherosclerosis* is derived from the Hellenic ἀθερή (atherē = gruel) and σκληρός (skēros = hard). It refers to the condition of large- and medium-sized arteries associated with thickening and hardening of the vessel wall due to accumulation of lipids and fibrous elements. This leads to narrowing of the vascular lumen, hence obstructing blood flow to the organs supplied by the affected artery. Acknowledging the consistent association of fatty degeneration and arterial stiffening, Dr. Felix Marchand of Leipzig introduced the term in 1904.¹

Atherosclerotic changes are a common feature of the vasculature of even asymptomatic individuals. Autopsies in infants, children and adolescents have shown that the first atherosclerotic lesions appear very early in life. Advanced lesions are a consistent finding in middle-aged men and women. Patients with atherosclerotic cardiovascular disease exhibit complicated atherosclerotic lesions, often at multiple sites.

1.2 Atherosclerotic lesion types and development

By convention, atherosclerotic lesion types are designated with the numerals I to VIII, signifying the order in which the distinct lesion types develop.² The earliest manifestation of atherosclerosis, the type I lesion consists of small isolated groups of macrophages that may contain lipid droplets (the macrophage foam cell) and extra-cellular accumulations of lipoproteins in the intima that do not disrupt the intercellular matrix structure. Type I lesions can be observed in atherosclerosis prone locations of the vascular tree of foetusses³ and young infants⁴,⁵ as well as in moderately susceptible locations of arteries of older children and adults.⁴,⁵

During childhood and puberty, type II lesions develop at atherosclerosis prone sites of the vascular tree. They consist of macrophage foam cells arranged in layers and indigenous smooth muscle cells. Occasionally, T-lymphocytes and mast cells can be found in these lesions. In addition to macrophages, intimal smooth muscle cells may contain lipid. Furthermore, minute quantities of lipid droplets and small lipoprotein particles can be found in the extra-cellular space.⁶ Some, but not all type II lesions are visible to the unaided eye as fatty streaks, i.e., yellow coloured streaks, patches, or spots on the intimal surface of arteries. Of all the type II lesions present in a person, only a small group will develop into type III and subsequently into the more advanced lesions. Mainly, those type II lesions developing at the predictable, atherosclerosis-prone sites will further proceed to more advanced lesions. Local hemodynamic conditions seem to play an important role herein.⁷ Type I and II lesions are frequently
referred to as early lesions, implying that they are found early in life and/or that they precede the development of advanced lesions. Type III lesions form the morphological and chemical bridge between type II and type IV lesions (i.e., atheromas) and are therefore also known as transitional lesions or preatheroma. Type III lesions have the same cellular constituents as type II lesions but are characterized by extra-cellular lipid droplets that coalesce to form lipid pools among the layers of intimal smooth muscle cells, without however forming a single massive lipid core, i.e., a confluent accumulation of extra-cellular lipid. Compared to type II lesions, preatheromas contain more free cholesterol, fatty acid, sphingomyelin, lyssolecithin and triglycerides.

Atherosclerotic lesions characterized by cellular and molecular changes resulting in disruption of intimal structure and luminal and vessel-wall architecture are considered advanced lesions. Histologically, advanced lesions are classified as types IV, V, VI, VII, and VII. During early adolescence, type IV advanced atherosclerotic lesions that potentially may have clinical sequelae develop at susceptible arterial sites. The characteristic feature of the type IV lesion or atheroma is the lipid core, i.e., a massive accumulation of extra-cellular lipid at the abluminal part of the intima. At the luminal site, the core is covered by a layer of tissue with the usual thickness of the intima at that site. Type IV lesions do not necessarily narrow the vessel lumen but may become symptomatic when the tissue overlying the lipid core is disrupted leading to acute thrombotic obstruction. Type V, fibroatheromatous lesions develop from atheroma through deposition of fibrous connective tissue either by organization of a luminal thrombus, extension of adjacent fibroatheroma, or resorption of the lipid core. Extensive fibrous degeneration may lead to the development of a lipid core free lesion designated type VIII (fibrotic) lesion. In contrast, excessive deposition of calcium crystals in the lipid core may lead to the development of type VII (calcified) lesions when more than 50% of the cross sectional area of the lesion consists of mineral. When (fibro)atheromatous (type IV or V) lesions develop surface disruptions, haematomas, thrombi or a combination of the above, they are designated type VI or unstable plaques. In case the above episodes are not fatal, fissures, haematomas and thrombi are colonized by intimal smooth muscle cells that will produce collagen, resulting in development of a lesion with type V morphology and with a greater luminal obstruction than before the complicating event.

It has generally been accepted that clinically overt atherosclerotic lesions develop from early, type I and II lesions. An increasing amount of experimental and clinical evidence suggests that atherosclerotic plaques may regress or even disappear. It has been suggested that plaque senescence with plaque volume reduction combined with continuous outward remodelling, may result in aneurysm formation. Thus, formation of aneurysm
may represent the latest stage of atherosclerotic degeneration. Figure 1.1 shows the atherosclerotic lesion types and their suggested pathways of progression and regression.

```
Adaptive intimal thickening
   ↓
Type I lesion
   ↓
Type II lesion (fatty streak)
   ↓
Type III lesion (preatheroma)
   ↓
Type IV lesion (atheroma)
   ↓
Type V lesion (fibroatheroma)
   ↓
Type VI lesion (unstable plaque)
   ↓
Type VII (calcified) lesion
   ↓
Type VIII (fibrotic) lesion
   ↓
Aneurysm formation
```

Figure 1.1 Schematic representation of the sequence in the development of atherosclerotic lesions. A black arrow signifies a pathway of progressions and a grey arrow represents a regression pathway. Adapted from Stary et al.
1.3 Clinical manifestations of atherosclerosis and their treatment in historical perspective

Obstructed blood flow resulting from luminal stenosis or occlusion is the common denominator of the clinical conditions caused by atherosclerosis. Luminal stenosis or complete occlusion at the site of an atherosclerotic plaque may result from plaque growth or thrombosis triggered by plaque rupture or ulceration. Alternatively, atherothromboembolism may result in arterial occlusions remote of the culprit atherosclerotic lesion when plaque matter or thrombus dislodges from the unstable plaque and occludes a smaller artery further downstream. This is the case in strokes that are related to carotid artery disease. Since it might affect any artery, atherosclerosis may virtually afflict all organs. However, mostly, the heart, the brain, the (lower) limbs, the kidneys or the intestines are affected. Regardless the affected organ, ischemic pain, tissue necrosis and/or loss of organ function are the clinical manifestations of decreased blood flow.

1.3a Coronary artery disease.

Atherosclerotic lesions in coronary arteries give rise to ischemic heart disease. The clinical presentation varies from chest pain of increasing severity (i.e., exertional chest pain (angina pectoris) or chest pain at rest (unstable angina)) to myocardial infarction. The ischemic damage to the heart may cause rhythm abnormalities, valvular dysfunction, cardiac failure, and may ultimately result in the death of the patient.

The first description of angina pectoris was given by William Heberdeen when he addressed the Royal College of Physicians in 1788.\(^\text{18}\) Shortly thereafter, in 1799, it had been suggested that ‘...the principal cause of angina pectoris may be looked for in the disordered coronary arteries.’\(^\text{19}\) In 1928, Keefor and Resnik confirmed that myocardial anoxemia was the underlying cause of angina, and that it could be caused by such diverse conditions as coronary artery disease, vasospasm, and decreased oxygen saturation of the blood. Although James B. Herrick had already in 1912 speculated that ‘...hope for the damaged myocardium lies in the direction of securing a supply of blood ... so as to restore as far as possible its functional integrity,’\(^\text{20}\) medical and surgical reperfusion of obstructed coronary artery in order to relief ischemic symptoms became only available in the 1960’s. Despite the fact that the first report of successful thrombolytic therapy was published in 1958,\(^\text{21}\) it took another quarter of a century before the life-saving potential of fibrinolytic therapy in acute myocardial infarction was unequivocally demonstrated.\(^\text{22}\) The first direct surgical myocardial revascularization was performed in 1962 when Effler and his team alleviated a severe obstruction of the left coronary artery using the technique of endarterectomy combined with patch grafting.\(^\text{23}\) DeBakey and
Garrett have probably performed the first successful coronary artery vein bypass graft in response to an intra-operative misadventure. The first elective coronary artery bypass graft using a venous conduit was performed by Rene Favaloro in 1967, who subsequently developed the technique for use in multivessel coronary artery disease. In 1977, Andreas Gruentzig performed the world's first percutaneous transluminal angioplasty of an obstructed coronary artery, offering the medical community a minimally invasive technique for the desobstruction of coronary arteries. This technique has been taken a step further with the introduction of coronary stents to increase the patency of angioplastied arteries. Thanks to these technological advances and to the implementation of more effective primary and secondary preventive measurements, mortality from myocardial infarction alone has decreased more than 30% in the last 30 years.

1.3b Cerebrovascular disease

Atherothrombotic occlusion of the arteries supplying the brain may lead to temporary or permanent ischemic brain damage resulting in temporary (transient ischemic attack) or permanent (stroke) focal neurological deficit. Approximately 30% of cerebrovascular accidents result from atherothrombotic disease of the aortic arch or extracranial arteries. Another 20-25% of ischemic cerebral events result from embolism of cardiac origin. Lacunar strokes resulting from occlusion of intracranial small vessels account for 15-20% of cerebral infarctions. The remaining 30% of ischemic strokes are due to less common conditions, such as vasculitis and paradoxical embolism through a patent foramen ovale or are of unknown origin.

The ancient Greeks were familiar with the concept of paralysis following injuries to the contralateral site of the brain, a phenomenon Hippocrates called apoplexy. Hippocrates furthermore gave the first account of a transient ischemic attack, but ischemia or haemorrhage as cause of apoplexy eluded him. In 1658 the Swiss physician Wepfer was the first to describe carotid thrombosis. In his Apoplexia he described the clinical pictures of completed stroke, progressing stroke and transient ischemic attack and noted that apoplexy may result from occlusion of the extra-cranial cerebral arteries. Subsequently Virchow and Penzoldt reported cases of carotid thrombosis associated with blindness and hemiplegia. Nevertheless, until fairly recently, the prevailing notion of the medical community was that strokes were caused by intracranial vascular disease. Sir William Osler attributed apoplectic stroke largely to cerebral haemorrhage or to thromboembolic occlusion of intracranial arteries. In 1914, Hunt emphasized the importance of occlusions of extracranial arteries in cerebrovascular disease and he was later joined by Fisher who stated: 'It is even conceivable that some day vascular surgery will find a way to bypass the occluded portion of the artery during the period of
ominous fleeting symptoms. Indeed, in the same year DeBakey\textsuperscript{35} performed the first successful carotid endarterectomy followed a few months later by Eastcott and Rob.\textsuperscript{36} Following these landmark cases, carotid endarterectomy has proven to be a valuable prophylactic treatment option for patients with (potential) cerebrovascular disease due to (asymptomatic extracranial carotid artery plaques.\textsuperscript{37,38} Another recent development, i.e., the introduction of thrombolytic therapy in acute ischemic cerebral events, has contributed significantly to the prevention of the devastating sequela related to ischemic cerebrovascular disease.\textsuperscript{39}

1.3c Peripheral arterial disease

Peripheral arterial disease refers to the clinical manifestations of stenosis or occlusion of arteries supplying the lower extremities. Atherosclerosis is nowadays the most frequent cause of peripheral arterial obstructive disease. Depending on the degree of arterial stenosis and presence of collateral circulation, patients may be symptom-free or suffer from ischemic symptoms of increasing severity. Moderate obstructions in the presence of collateral circulation may give rise to exercise induced leg pain that subsides after resting (intermittent claudication). With increasing severity of arterial obstruction, ischemic pain may already be present at rest and/or tissue viability may be compromised leading to non-healing arterial ulcers.

The association between ischemic leg symptoms (claudication, arterial ulcers) and occlusions of supplying arteries had only been laid in the 19th century. In fact, intermittent claudication due to arterial occlusion was first diagnosed by the French veterinary surgeon Jean-Francois Bouley in a horse drawing a carriage in 1831. The animal started to limp with the hind legs after standard exercise. At autopsy, a partially thrombosed aneurysm of the abdominal aorta with occlusions of both femoral arteries were seen and the femoral occlusions were correctly identified as the cause of symptoms.\textsuperscript{40} Jean-Martin Charcot, who was aware of Bouley's work, first described intermittent claudication, in 1858, in a patient who developed claudication secondary to a thrombosed traumatic iliac aneurysm.\textsuperscript{40} The combination of exercise induced leg pain, tension or weakness that disappears after a period of rest, due to arterial occlusions, has also been called Charcot's syndrome or angina cruris.

For the longest part of medical history, amputation of an ischemic gangrenous limb was the only therapeutic option available for severe peripheral arterial disease. Direct arterial reconstruction for atherothrombotic disease had to await the development of vascular suture techniques and the introduction of heparin. In 1947, Jao Cid Dos Santos of Lissabon performed the first successful endarterectomy of an obstructed iliac artery.\textsuperscript{41} This major breakthrough, was soon followed by another important milestone of vascular surgery when Jean Kunlin performed the first femoropopliteal bypass for atherosclerotic obstructive
disease using the patients own greater saphenous vein. It should be mentioned however that the first successful vascular reconstruction using a saphenous vein graft in a patient with a traumatic lesion of the femoral artery and vein after sustaining a gunshot wound to the groin was performed by Murphy in Chicago in 1897. Aortic occlusions represented a surgical challenge not suitable for saphenous vein bypass grafting. The use of arterial homografts had been introduced in 1948 for the management of coarctation, and in 1950 Jacque Oudot performed the first reconstruction of an occluded aorta in a patient with non-healing ulcers of the left leg using an arterial homograft. In an attempt to overcome the drawbacks of arterial homografts, such as, limited availability and preservation, and graft degeneration, several surgeons have worked towards the development of fabric arterial grafts. In 1952, Voorhees reported on the first use of a Vinyon N tube as arterial graft. Soon thereafter Nylon, Teflon and Dacron grafts were tested. The knitted Dacron prosthesis described by DeBakey and colleagues was the first consistently successful arterial substitute. The expanded polytetrafluorethylene (PTFE) conduit took graft technology a step further. In addition to the advancement of surgical therapies, minimally invasive, percutaneous techniques were developed for the treatment of peripheral arterial disease. Dotter was the first to relief arterial stenoses using a rigid teflon sound introduced through percutaneous arterial punctures. Gruentzig improved Dotter’s technique by performing the dilatation with the distension of a balloon mounted onto the tip of an intravascular catheter. Finally, the use of stents has improved long term patency of percutaneous interventions.

1.3d Visceral ischemia

Atherothrombotic disease of renal and mesenteric arteries may be associated with ischemic pain, tissue necrosis and organ dysfunction. Goldblatt et al. have shown that atherosclerotic obstruction of the renal arteries results in reno-vascular hypertension, renal atrophy and loss of renal function. Remarkably, the association between hypertension, albuminuria and granular shrunken kidneys had been described by Bright a century earlier. Renal-vascular hypertension has been treated initially with simple nephrectomy. In 1954, while performing an endarterectomy for occlusive aortic disease, Freeman performed the first documented reconstructive renal artery procedure for hypertension. Many other surgeons have followed revascularising the kidney either by means of endarterectomy or bypass procedures. Chronic mesenteric atherothrombotic disease may be associated with post-prandial abdominal pain (angina abdominale) suggestive of intestinal arterial insufficiency but remains asymptomatic in the majority of cases. In an autopsy study, approximately 10% of unselected specimens demonstrated a significant
stenosis of at least one of the main intestinal aortic branches.\textsuperscript{57} Despite the high prevalence of asymptomatic mesenteric atherosclerotic disease, chronic mesenteric ischemia manifested by post-prandial abdominal pain is a rare disorder, thereby illustrating the adequacy of the mesenteric collateral circulation. Acute mesenteric ischemia, often on the background of chronic asymptomatic mesenteric disease, characterized by abrupt onset pain, represents an acute abdominal catastrophe with a high mortality. Frequently the severity of pain is out of proportion to the findings at clinical examination.

The first historical description of mesenteric vascular occlusion is attributed to Antonio Benveniste from Florence, Italy in the 15\textsuperscript{th} century.\textsuperscript{58} Although Hodgson described a case of acute intestinal ischemia from Guy's Hospital in 1815,\textsuperscript{59} the interest of the medical community for this pathology had awakened only after Litten published his classic experimental work on the effect of occlusions of the mesenteric vessels in 1875.\textsuperscript{60} The most important progress in the treatment of this invariably lethal condition was achieved when Elliot reported the first patient who survived an acute mesenteric ischemic event, by resection of the ischemic bowel and creation of two stomas which were re-anastomosed two weeks later.\textsuperscript{61} Thus, the modern concept of diagnosing an acute ischemic bowel by laparotomy and treating it by resection with primary or second stage intestinal re-anastomosis has essentially been unchanged for more than 100 years. The association between abdominal pain and chronic mesenteric occlusion was first laid by Councilman who described three cases of chronic occlusion of the superior mesenteric artery associated with postprandial abdominal pain in 1894.\textsuperscript{62} In 1958, Shaw and Maynard described the first thrombendarterectomy of the superior mesenteric artery for the treatment of acute as well as chronic intestinal ischemia.\textsuperscript{63} Mesenteric revascularization using bypass grafts from the infrarenal aorta to the superior mesenteric artery was first described in 1962 by Morris.\textsuperscript{64} Recently, balloon angioplasties of stenoses of the celiac trunk and superior mesenteric artery have been performed successfully.\textsuperscript{65}

1.3e Abdominal aortic aneurysms

Aneurysms are localised pathologic dilations of arteries, mostly located at the infrarenal part of the abdominal aorta. Other arteries such as the thoracic aorta, the femoral, popliteal and carotid artery may occasionally be affected as well. In their majority, aneurysms remain asymptomatic and are accidentally discovered when screening patients for other purposes. Occasionally, patients with aneurysms may present with acute, thrombo-embolic ischemia. Thrombus depositions form in the dilated arterial segment and small thrombus particles may break off and occlude more distal arteries leading to acute ischemic symptoms. Alternatively the weakened wall of aneurysmal arteries may rupture leading to fatal exsanguination of the patient.
Probably the first account of successful treatment of aneurysms came from Antyllus, a 3rd century Greek surgeon, who had already recognized the distinction between true (degenerative) and false (traumatic) aneurysms. Antyllus advocated opening the (popliteal) aneurysmal sac after applying ligatures directly proximal and distal to the aneurysm. This technique for the treatment of popliteal aneurysms remained basically unchanged for more than a millennium until John Hunter advocated ligation of the nondilated femoral artery at the level of the subsartorial canal. This technical improvement led to a significant decrease of post-operative exsanguinating haemorrhage associated with Antyllus’ technique. Soon, ligation of other peripheral arteries for aneurysms was reported, such as the brachial artery by Rudolph Matas and the external iliac artery by John Abermethy. Sir Astley Cooper was the first to ligate the lower part of the abdominal aorta in an attempt to treat a patient with iliac artery aneurysm. The specimen of this ligated aorta has been preserved and can be seen to date in the museum at St Thomas’ Hospital, London, UK. In order to obliterate aneurysms, unsuccessful attempts were made to induce thrombosis using wires or electrical currents. Cellophane wrapping of aneurysms has also been attempted without any long-term success. Exclusion of a popliteal pseudoaneurysm with direct arterial reconstruction using saphenous vein graft was first performed by James Hogarth Pringle from Glasgow. Rudolph Matas of New Orleans developed the technique of endoaneurysmorrhaphy for the direct reconstruction of aneurysmal arteries in order to maintain arterial continuity and blood flow. In 1951, Charles Dubost performed the first successful resection of an infrarenal aortic aneurysm using an arterial homograft to reconstruct the aorta. Cooley and DeBakey introduced the use of artificial (Dacron) conduits for aortic replacement after resection of an aneurysm. The latest improvement in the treatment of aneurysms is the development of endovascular stentgrafts. With this technique aneurysms can be excluded from the circulation minimally invasively by introducing the stentgraft percutaneously through a puncture of the femoral artery and placing it in the dilated aorta. This technique has already proven to dramatically reduce the mortality of elective and emergency aneurysm repair.

1.4 Epidemiology of atherosclerotic disease
Cardiovascular disease, mainly resulting from atherosclerosis, is the leading cause of death and disability worldwide. According to the World Health Report 2003 (available online at http://www.who.int/whr/2003/en/), an estimated 16,700,000 deaths, i.e., 29.2% of total global mortality resulted from cardiovascular diseases in 2002. Additionally, at least 20,000,000 patients worldwide survived a myocardial infarction or stroke. Cardiovascular morbidity
and mortality is no longer a prerogative of developed countries. Approximately 80% of cardiovascular deaths in 2002 took place in low and middle-income countries. The World Health Organization has estimated that by 2010 cardiovascular disease will be the leading cause of death in developing countries as well. The increase in cardiovascular disease prevalence in developing countries reflects a change in dietary habits, physical activity levels and tobacco consumption worldwide as a result of industrialization, economic development and food market globalization.

Considering the fact that cardiovascular diseases mainly affect people in their productive mid-life years, and taking into account the number of patients surviving myocardial infarctions and strokes that require costly clinical care, the extent of the socio-economic burden of cardiovascular disease becomes evident. More than 50% of the deaths and disability from cardiovascular disease could be prevented by simple efforts to reduce cardiovascular risk factors. As a matter of fact, modest reductions in major risk factors in England and Wales during the last 20 years resulted in a significant gain in life-years. Therefore, in light of the upcoming global cardiovascular epidemic, identification and elimination of (novel) cardiovascular risk factors seems of enormous importance, both, in global health and socio-economic terms.

The prevalence of cardiovascular diseases in the Netherlands followed the trend of other developed countries, reaching epidemic proportions in the second half of the 20th century but showing a decrease towards the end of the 20th century. Table 1.1 shows the estimated prevalences of cardiovascular diseases for the total population in developed countries. For all types of cardiovascular diseases, prevalence is age-dependent and increases almost exponentially with advancing age. Although the number of deaths due to cardiovascular diseases did not change significantly between 1972 and 2002 in the Netherlands (i.e., 50,143 deaths in 1972 vs. 48,799 deaths in 2002), age-adjusted death rates decreased significantly during this period. Cardiovascular diseases continue to impose a serious burden on national health care budget. The direct and indirect costs of the treatment of coronary artery disease and stroke alone, in 2005 in the USA has been estimated at an excess of $200 billion. Of 1.5 million hospital admissions in 2002 in the Netherlands, 18%, i.e., 272,275 were related to cardiovascular diseases. Although adjusted cardiovascular related hospital admissions have declined in the period 1972-2002, this has been mainly due to the increased percentage of cardiovascular patients treated in day-care setting so that the cost of treatment of cardiovascular diseases in the Netherlands remains high and parallels the US figures.
Table 1.1. Population based prevalence of cardiovascular diseases in developed countries.

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<th>Prevalence</th>
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<tr>
<td>CVD</td>
<td>2.6%</td>
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<tr>
<td>VLD</td>
<td>0.1% - 20%</td>
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<tr>
<td>PAD</td>
<td>0.6% - 8.8%</td>
</tr>
<tr>
<td>AAA</td>
<td>4.7%-7.7%</td>
</tr>
</tbody>
</table>

CAD: coronary artery disease; CVD: cerebrovascular disease; VLD: visceral ischemic disease (i.e., renal artery stenosis and mesenteric ischemia); PAD: peripheral arterial disease; AAA: abdominal aortic aneurysms.

1.5 Risk factors for atherosclerotic disease

Probably one of the most important medical developments of the 20th century has been the identification of risk factors for atherosclerotic disease through large prospective studies such as the Framingham Heart study and the Seven countries study. Obviously, certain risk factors (i.e., age, sex) are not amenable to treatment but most of them are very well modifiable. It has been proven that simple measures achieving modest reductions in major risk factors result in significant decrease in atherosclerotic cardiovascular events. Cigarette smoking, hypercholesterolemia, low serum HDL cholesterol, hypertension, diabetes mellitus, and advancing age are considered major risk factors and are used in several models to determine cardiovascular risk. Other factors such as obesity, physical inactivity, high serum triglyceride level, increased serum lipoprotein (a), hyperhomocysteinemia, family history of premature atherosclerotic manifestations, and several coagulation factors increase the likelihood for developing atherosclerotic vascular disease but have not been incorporated in current risk prediction equations. Some pathological studies have suggested that only about half of the variation in size of atherosclerotic lesions can be attributed to known risk factors. Similarly, it has been suggested that approximately 50% of patients with atherosclerotic vascular disease lack any of the major risk factors. However, recently published meta-analyses involving 122,458 patients from 14 randomised clinical trials and 386,915 patients from three observational studies have shown that 80-90% of patients who develop clinically significant coronary heart disease and as much as 95% of patients who experienced a fatal coronary event had at least one major cardiovascular risk factor. In view of these statistics, the need to develop "primordial prevention" strategies, i.e., preventing the risk factor to develop, is increasingly being appreciated.

The identification of atherosclerotic risk factors through large epidemiological studies has contributed to the understanding of the causes of atherosclerosis, leading to the formulation of hypotheses regarding its development. The recent
realisation that atherosclerosis is an inflammatory disease and that thrombosis is related to acute cardiovascular events has prompted the search for and identification of novel atherothrombotic risk factors. Table 1.2 lists some of the proposed novel risk factors for atherosclerotic vascular disease. It remains to be seen whether they have an additive value over conventional risk factors for risk estimation.

<table>
<thead>
<tr>
<th>Inflammatory markers</th>
<th>Thrombotic/haemostatic factors</th>
<th>Other factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein</td>
<td>Fibrinogen</td>
<td>Infectious agents:</td>
</tr>
<tr>
<td>Serum amyloid A</td>
<td>Von Willebrand factor antigen</td>
<td>C. pneumoniae,</td>
</tr>
<tr>
<td>Interleukins</td>
<td>Plasminogen activator inhibitor</td>
<td>Cytomegalovirus,</td>
</tr>
<tr>
<td>Vascular and cellular adhesion molecules</td>
<td>Tissue-plasminogen activator</td>
<td>H. pylori,</td>
</tr>
<tr>
<td>Soluble CD40 ligand</td>
<td>D-dimer</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>Leucocyte count</td>
<td>Platelet aggregation</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate</td>
<td>Platelet activity</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td></td>
<td>Platelet size and volume</td>
<td>Lipoprotein (a)</td>
</tr>
</tbody>
</table>

* Adapted from Hackam & Anand.

1.6 Pathogenesis of atherosclerosis – the inflammatory paradigm

In his landmark 1999 review, Russel Ross pointed out that atherosclerosis develops as an inflammatory response to endothelial dysfunction. From the initial atherosclerotic lesion, which is a pure inflammatory lesion as it consists only of monocyte-derived macrophages, to complex plaques, all atherosclerotic lesions represent different stages of a chronic inflammatory process in the artery.

The initial step in atherogenesis is endothelial dysfunction caused by several proatherogenic factors such as oscillating shear stress and turbulent flow, increased and/or modified LDL, hypertension, free radicals caused by smoking, elevated plasma homocysteine levels, certain bacteria and viruses, e.g. Chlamydia pneumoniae and Cytomegalovirus, and combinations of these and other factors. These initiating stimuli result in increased endothelial permeability, increased endothelial adhesiveness for leucocytes or platelets, development of endothelial procoagulant instead of anticoagulant properties and production of vasoactive molecules, cytokines and growth factors. One of the earliest events in atherosclerosis is the retention of LDL in the subendothelial matrix where it undergoes modification. Modified LDL stimulates the overlying endothelium to produce a number of pro-inflammatory molecules, such as growth factors and adhesion molecules. Being a potent chemoattractant for monocytes and a mitogen for macrophages and
smooth muscle cells,\textsuperscript{118} modified LDL contributes to inflammation and foam cell formation. Under its influence, monocytes and specific subsets of T-cells are attracted to the subintimal space.\textsuperscript{119} Macrophages rapidly take up modified LDL becoming foam-cells and leading to fatty streak formation.\textsuperscript{120} The ongoing inflammatory response stimulates migration and proliferation of smooth muscle cells, leading to intermediate, preatheromatous type III lesions. If unabated, the inflammatory reaction results in further influx of macrophages and T-cells, proliferation of smooth muscle cells and apposition of extra-cellular matrix. Through the action of an array of inflammatory molecules such as hydrolytic enzymes, chemokines and cytokines the lesions evolve into advanced (fibro)atheromatous and/or unstable plaques.\textsuperscript{113,121}

1.7 Atherosclerosis and C-reactive protein

In view of the inflammatory nature of atherosclerosis, several investigators have studied inflammatory molecules as markers of atherosclerosis (see Table 1.2). Among the plethora of analysed inflammatory markers, C-reactive protein (CRP) has been studied most extensively and has shown the most consistent association with atherosclerotic events in diverse clinical settings. CRP is an acute phase reactant present in trace concentrations in healthy subjects (<1 mg/l). Its concentration can increase up to a 1,000-fold in response to infection, ischemia, trauma, burns and (acute) inflammatory conditions.\textsuperscript{122,123} The development of high sensitivity CRP (hsCRP) assays has facilitated the analysis of the role of CRP in atherosclerotic vascular disease.\textsuperscript{124,125} as hsCRP levels below 10 mg/l have demonstrated specificity for vascular events.\textsuperscript{128} It has been shown that serum hsCRP is elevated in patients with coronary artery disease\textsuperscript{127-133} and that in these patients, as well as in patients with peripheral arterial disease, serum hsCRP is related to (cardiovascular) mortality\textsuperscript{134-136} and future coronary events.\textsuperscript{104,137-145} Furthermore, several prospective and nested case control studies have shown that a single, non-fasting measure of serum hsCRP is strongly associated with the development of coronary,\textsuperscript{146-157} cerebral,\textsuperscript{147,158,159} and peripheral\textsuperscript{160} vascular events, in apparently healthy, middle-aged and elderly individuals. These studies have established CRP as an independent predictor of future cardiovascular events that adds prognostic information to lipid screening, and to Framingham Risk Score.\textsuperscript{181} Recently, evidence has emerged to show that primary and secondary preventive strategies are most effective in patients with elevated serum hsCRP levels suggesting that lowering CRP may reduce cardiovascular event rates. The risk reduction for aspirin was higher in patients with elevated baseline serum hsCRP.\textsuperscript{147} This may suggest that aspirin has clinically important anti-inflammatory as well as antiplatelet effects. Nevertheless, the CRP-lowering properties of aspirin remain controversial.\textsuperscript{133,162} In contrast, statins
(hydroxymethyl glutaryl coenzyme A reductase inhibitors) have shown to be able to reduce serum hsCRP concentrations as well as plasma cholesterol levels.\textsuperscript{163-166} and both in the setting of primary (AFCAPS/TexCAPS primary prevention trial)\textsuperscript{167} and secondary prevention (CARE).\textsuperscript{162} the effect of statins on cardiovascular risk reduction was most pronounced in patients with high hsCRP levels. Recently published data of a randomised clinical trial has shown that among patients with acute coronary syndromes, statin treatment achieving low level of hsCRP (<2 mg/l) was associated with a significant improvement in event-free survival. This effect was present at all levels of LDL cholesterol achieved and suggested that strategies designed to reduce inflammation may improve cardiovascular outcome.\textsuperscript{168}

Assuming that CRP is produced by hepatocytes after stimulation with interleukin-6 and interleukin 1\(\beta\).\textsuperscript{169} the association between serum hsCRP and cardiovascular disease may reflect (i) the amount of circulating proinflammatory (pro-atherosclerotic) cytokines, (ii) vascular inflammation related to the extent and severity of atherosclerosis, (iii) (vascular) inflammation resulting from chronic (vascular) infections, and (iv) inflammation related to tissue (heart, brain, skeletal muscle) ischemia. However, CRP is an ancient host defence protein whose phylogenetic origins can be traced as far back as the horseshoe crab (Limulus polyphemus).\textsuperscript{170} Therefore it would be anticipated that many tissues of the body would preserve their ability to generate this protein as part of their innate immune defences. Intriguingly, it has been shown that various types of damaged tissues, such as Alzheimer's disease brains,\textsuperscript{171,172} infarced hearts,\textsuperscript{173} and coronary atherosclerotic plaques\textsuperscript{174} produce CRP. Since CRP can be found in atherosclerotic tissue\textsuperscript{175} and lowering hsCRP results in atherosclerotic event-reduction\textsuperscript{168} it may well be suggested that CRP could actually participate in the development of atherothrombosis. In support of this hypothesis, in vitro experiments have shown that CRP exerts pro-atherogenic effects on all cellular constituents of the atherosclerotic lesion. CRP colocalizes with the membrane attack complex in atherosclerotic plaques and activates complement.\textsuperscript{174,176} It is chemotactic for circulating monocytes and CRP plaque deposits actually precede the appearance of monocytes, suggesting that it may play a major role in the recruitment of monocytes during atherogenesis.\textsuperscript{177} Furthermore, CRP stimulates monocyte tissue factor production\textsuperscript{178} and up-regulates some macrophage pro-inflammatory cytokines.\textsuperscript{179} CRP causes a sustained increase in native LDL-uptake by macrophages\textsuperscript{180}, possibly through uptake of CRP-opsonized native LDL via the CRP-receptor CD32.\textsuperscript{181} Through activation of nuclear factor kappa B of endothelial and smooth muscle cells, CRP may induce pro-inflammatory and pro-atherosclerotic phenomena. CRP may thus induce endothelial production of adhesion molecules,\textsuperscript{182} stimulate endothelial monocyte chemoattractant protein-1 release,\textsuperscript{183} and may inhibit basal and stimulated endothelial NO
release.\textsuperscript{104} Similarly, CRP activates monocyte chemoattractant protein-1, interleukin-6 and inducible nitric oxide synthetase expression in vascular smooth muscle cells.\textsuperscript{105}

1.8 Atherosclerosis and infections

The notion that infections play an important role in the development of atherosclerosis is surprisingly old. At the beginning of the previous century, it had already been suggested that infections may contribute to the development of cardiovascular disease.\textsuperscript{186-188} During the interbellum, Collins observed that every influenza epidemic was followed by an increase in cardiac deaths.\textsuperscript{189} Nevertheless, experimental evidence supporting this hypothesis only came available when half a century later Fabricant showed that infection of chickens with Marek's disease virus, an avian herpes virus, produces typical atherosclerotic lesions.\textsuperscript{190} Subsequently, and in accordance with the early observations of Selwyn Collins, clinicians had observed that acute (respiratory tract) infections were associated with coronary events.\textsuperscript{191-194} Several pathogens have been related to the development of cardiovascular disease, such as the herpesviridae cytomegalovirus,\textsuperscript{195} Epstein-Barr virus and herpes simplex virus,\textsuperscript{196} Helicobacter pylori,\textsuperscript{197,198} the periodontal pathogens Streptococcus sanguis,\textsuperscript{199} Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Bacteroides forsythus, and Prevotella intermedia,\textsuperscript{200} hepatitis A virus,\textsuperscript{201} influenza virus,\textsuperscript{202-204} Coxsackie B virus,\textsuperscript{205} and Chlamydia pneumoniae.\textsuperscript{193,206} The latter has been studied most extensively in relation to atherosclerosis, not in the last place because two early small antibiotic intervention trials had suggested that antichlamyoidal treatment may reduce cardiovascular risk in patients with coronary artery disease.\textsuperscript{207,208}

1.8a Chlamydia pneumoniae biology & epidemiology

Chlamydia pneumoniae was initially isolated in 1965 from a child's conjunctiva as an atypical strain that could not be identified as Chlamydia trachomatis.\textsuperscript{209} A similar atypical chlamydial strain was isolated from the pharynx of a patient with an upper respiratory tract infection in 1983.\textsuperscript{210} In 1989, these atypical isolates were recognized as a new chlamydial species that was named Chlamydia pneumoniae.\textsuperscript{211} It is an obligate intracellular, Gram-negative microbe with a typical chlamydial developmental life cycle of two alternating functional and morphological forms (Figure 1.2).\textsuperscript{212} The small, dense elementary body (EB) is the metabolically inactive, highly infectious form of the microbe, responsible for attaching to the target host cell. After attachment to the host cell, the EB transforms, within an endosome, into the larger, metabolically active reticulate body (RB) that divides by binary fission within the endosome. After a period of growth and division, the RBs reorganize into EBs and cause lysis of the host.
cell thereby completing the developmental cycle. The released EBs may initiate new infectious cycles. Under certain conditions Chlamydiae may follow an altered intracellular development. Nutrient deficiency, several antimicrobial agents and cytokines may delay RB maturation and inhibit differentiation into EBs, giving rise to a morphologically altered RB form which occasionally is referred to as persistent body. Re-establishment of a favourable milieu will allow persistent bodies to re-enter and complete the normal chlamydial developmental cycle (Figure 1.2).

*Chlamydia pneumoniae* causes community acquired respiratory tract infections. Among adults with community acquired pneumonia, *Chlamydia pneumoniae* was the causative agent in approximately 3-50%. Considering the high prevalence of *Chlamydia pneumoniae* IgG antibody in the general population, which may be as high as 80% in octogenarians, it is not surprising that up to 90% of *Chlamydia pneumoniae* respiratory tract infections may remain asymptomatic. This high antibody prevalence may be the result of frequent reinfections and suggests that virtually everyone will at least once get infected with *Chlamydia pneumoniae* during his/her lifetime.

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**Figure 1.2** The developmental cycle of *Chlamydia pneumoniae*. 

- Elementary body (EB)
- Reticulate body (RB)
- Persistent body (PB)
1.8b  *Chlamydia pneumoniae* and atherosclerosis

In the early 1940's several investigators showed that patients with cardiovascular disease and without any history of lymphogranuloma venereum often demonstrated a positive intradermal Frei test, which measures hypersensitivity to all chlamydial species, thereby providing the first hint that chlamydial infections may be related to arterial disease.\(^{223-225}\) The last 20 years ample evidence has gathered to suggest that *Chlamydia pneumoniae* infections play a (modulatory) role in the development of atherosclerosis.\(^{226}\)

1.8b.i  Sero-epidemiological evidence

Since Sakku and colleagues have shown that *Chlamydia pneumoniae* antibodies were elevated in patients with acute myocardial infarction or stable coronary artery disease,\(^{193}\) a large number of studies have explored the relation between *Chlamydia pneumoniae* serology and atherosclerotic disease, determining *Chlamydia pneumoniae* antibodies or immune-complexes in patients with coronary, carotid, aortic, and peripheral arterial disease and healthy controls. Although a few studies failed to verify the association between infection and atherosclerosis,\(^{227}\) the majority of these studies supported an association between *Chlamydia pneumoniae* serology and atherosclerosis.\(^{228-231}\) Despite the numerous sero-epidemiological reports, no consensus has been reached regarding the serological detection of chronic active or persistent vascular *Chlamydia pneumoniae* infection.\(^{232}\) Hence, various serological assays and different serological criteria for chronic or persistent *Chlamydia pneumoniae* infection have been used insofar leading to the conflicting results of hitherto published studies. Standardization of *Chlamydia pneumoniae* detection assays in patients with cardiovascular disease has therefore become mandatory.

1.8b.ii  Histological evidence

Traces of *Chlamydia pneumoniae* have been found abundantly in human atherosclerotic tissue.\(^{233}\) *Chlamydia pneumoniae* proteins and/or DNA have been detected in more than 50% of atherosclerotic specimens using immunohistochemical techniques or PCR, respectively. Although *Chlamydia pneumoniae* seems ubiquitously present in the atherosclerotic vascular tree, as it has been detected in atheroma derived from any vascular territory, i.e., coronary, carotid, aortic, femoral and popliteal, no traces of the bacterium could be found in normal arteries.\(^{234,235}\) The prevalence of chlamydial presence in atherosclerotic tissue varies considerably.\(^{236}\) Detection rates ranging from 0 to 100% have been reported.\(^{237}\) Despite this variation, the frequent finding of *Chlamydia pneumoniae* in atherosclerotic plaques and not in normal tissue strongly suggests that the organism is undeniably present in atherosclerotic lesions and supports an association with atherosclerosis, although the
presence of the microbe in atheroma has not invariably been associated with severity or extent of the disease.\textsuperscript{236,239}

1.8b.3 Experimental evidence

\textit{Chlamydia pneumoniae} shows a strong tropism for macrophages, but has proven to infect and survive in endothelial cells and smooth muscle cells as well.\textsuperscript{240} This suggests that local infection of the vasculature may provide a focus for in-situ vascular damage promoting a pro-atherogenic inflammatory response.

The microbe may gain access to the vasculature after respiratory tract infections through infected monocytes.\textsuperscript{241} Indeed, \textit{in vitro} experiments have shown that monocytes infected with \textit{Chlamydia pneumoniae} exhibit enhanced plasma membrane fluidity\textsuperscript{242} and adherence to human endothelial cells,\textsuperscript{243} resulting in transmission of the pathogen to endothelial cells.\textsuperscript{242,244} Infection of macrophages with \textit{Chlamydia pneumoniae} induces excessive LDH uptake leading to foam cell formation.\textsuperscript{245} Lipopolysaccharide (LPS) has been shown to be the chlamydial component that induces foam cell formation.\textsuperscript{246} By means of its Heat shock protein 60 (HSP-60), \textit{Chlamydia pneumoniae} induces monocytes to oxidize lipoproteins.\textsuperscript{247,248} Furthermore, infected macrophages secrete increased levels of inflammatory cytokines (interleukin (IL)-1β, IL-6, tumor necrosis factor-α, interferon-γ),\textsuperscript{249} monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α)),\textsuperscript{250} and matrix metalloproteinase-9 (MMP-9).\textsuperscript{251,252}

\textit{Chlamydia pneumoniae} upregulates atherosclerosis-related gene expression in human umbilical vein endothelial cells.\textsuperscript{253,254} Infection of endothelial cells augments endothelial adhesion molecule expression providing additional means for the promotion of monocyte adherence to the endothelial surface.\textsuperscript{255} Furthermore, \textit{Chlamydia pneumoniae} infection causes endothelial dysfunction,\textsuperscript{256} stimulates transendothelial migration of inflammatory cells,\textsuperscript{257} induces endothelial hypercoagulability through increased expression of tissue factor,\textsuperscript{258,259} and promotes endothelial secretion of inflammatory mediators.\textsuperscript{260} Smooth muscle cells respond to endothelial \textit{Chlamydia pneumoniae} infection by proliferating.\textsuperscript{261} Direct infection of smooth muscle cells also has a mitogenic effect, possibly through induction of endogenous heat shock protein,\textsuperscript{262} inhibits apoptosis,\textsuperscript{263} and induces secretion of cytokines\textsuperscript{258,259} and matrix metalloproteinases.\textsuperscript{264}

Studies using cholesterol fed New Zealand white rabbits,\textsuperscript{267,268} several mouse strains,\textsuperscript{269} rats\textsuperscript{270} and pigs\textsuperscript{271} have supported the atherogenic properties of \textit{Chlamydia pneumoniae} infection. Although two investigators reported a lack of association between experimental \textit{Chlamydia pneumoniae} infection and development of atherosclerosis,\textsuperscript{272,273} the majority of animal studies has shown that \textit{Chlamydia pneumoniae} has a tropism for the vasculature and the capacity
to initiate or promote atherosclerotic lesion development. Repeated
inoculations and a hyperlipidemic background were prerequisites for the
demonstration of the atherogenic properties of *Chlamydia pneumonias*. After
intranasal inoculation, *Chlamydia pneumonias* reaches distant organs,
including the arteries, by infected macrophages. In hyperlipidemic
mice and cholesterol fed New Zealand white rabbits, *Chlamydia pneumonias*
infection accelerated atherosclerotic lesion development. In contrast, in normallipidemic animals, infection did not induce plaque formation
although in these circumstances arterial inflammatory reactions were
observed. Furthermore, infected animals showed more advanced
atherosclerotic lesions and unstable plaque phenotypes. Experimental
*Chlamydia pneumonias* infection has been associated with endothelial
dysfunction, increased fibrinogen activity, and increased gelatinolytic
activity. Antibiotic treatment of experimental animals infected with *Chlamydia pneumonias* ameliorated the progression of atherosclerotic lesions, but only
when administered shortly after inoculation.

1.8b.IV Antibiotic trials

The sero-epidemiological and experimental data showing an association
between *Chlamydia pneumonias* infection and atherosclerosis triggered the
interest of (cardio)vascular clinicians for this pathogen based on the premises
that anti-chlamydial antibiotics may offer a new treatment modality for patients
with atherosclerotic vascular disease. Indeed, two early small randomised
clinical trials suggested that antibiotics may reduce cardiovascular risk in
patients with coronary artery disease.

Several investigators have tried to evaluate the effect of antibiotics on
cardiovascular risk by observational case-control studies. A number of these
studies showed a survival benefit from exposure to anti-chlamydial antibiotics,
although others refuted it. Considering the methodological shortcomings of these studies, randomised clinical trials were
needed to determine the effectiveness of antibiotics in atherosclerotic disease
prevention. Following the two initial positive pilot studies a number of antibiotic
trials have been designed and carried out worldwide with contradictory
results. In general, favourable results were shown in studies with small
sample size and/or limited follow-up studies that have included
patients with abdominal aortic aneurysms or peripheral arterial disease,
or studied surrogate endpoints. Three larger studies, involving
patients with coronary artery disease, demonstrated no beneficial effect of
antibiotics in the prevention of acute coronary events and/or death.
1.8b.V Mechanism of disease

The available data suggest that *Chlamydia pneumoniae* may play a (modulatory) role (i) in the initiation of atherosclerosis through stimulation of inflammatory cell recruitment and foam cell formation, (ii) in the progression of atherosclerosis through stimulation of smooth muscle cell proliferation and inhibition of apoptosis, and (iii) in the development of an unstable plaque through stimulation of collagenase activity and induction of hypercoagulability, mainly by virtue of its *in situ* effects. Next to its presumed local effects, *Chlamydia pneumoniae* may promote atherosclerosis through systemic effects. Chronic *Chlamydia pneumoniae* infection may induce a chronic inflammatory state characterized by elevated levels of pro-inflammatory and pro-atherogenic molecules, and/or a hypercoagulable state characterized by raised fibrinogen levels.\textsuperscript{310,311} Furthermore, infection may trigger an autoimmune reaction to human HSP-60 by antigenic mimicry resulting in endothelial cytotoxicity\textsuperscript{312} which has been associated with enhanced development of atherosclerosis.\textsuperscript{313}

Finally, infection may be associated with atherosclerosis through interaction with atherosclerotic risk factors such as smoking,\textsuperscript{314} lipid metabolism,\textsuperscript{315,316} and hypertension.\textsuperscript{317} Considering the high prevalence of exposure to *Chlamydia pneumoniae* among patients with atherosclerotic vascular disease and age matched healthy controls, it is conceivable that host factors, such as variations in genes involved in inflammatory processes, may render individuals susceptible for the pro-atherogenic effects of infection.
1.9 Aims of the study and outline of the thesis

Atherosclerosis has emerged as a multi-factorial inflammatory process of the vascular wall. It is unlikely that one inflammatory molecule (C-reactive protein) and one pathogen (Chlamydia pneumoniae) alone can initiate or propagate the development of atherosclerosis. Keeping this in mind, the primary goal of this study was to assess the role of C-reactive protein and Chlamydia pneumoniae infection in patients with (non-coronary) atherosclerotic disease.

The relation between CRP and peripheral arterial disease or abdominal aortic aneurysms is dealt with in section 2 (chapters 2, 3). Chapter 2 describes the association between serum concentration of CRP and severity of peripheral arterial disease and future cardiovascular events in patients with PAD. In chapter 3 the relation between serum CRP and extent of abdominal aortic aneurysms is studied. Furthermore, the production of CRP by diseased vascular tissue was explored in these chapters.

In section 3 (chapters 4, 5, 6) the role of Chlamydia pneumoniae infection in peripheral vascular disease was studied. In chapter 4, the use of a commercially available enzyme immunoassay for the detection of Chlamydia pneumoniae IgA- and IgG-antibodies in patients with cardiovascular disease is validated against the gold standard serological assay. The association between Chlamydia pneumoniae antibodies and presence of the pathogen in atherosclerotic tissue is assessed in chapter 5. Furthermore the relation between the extent of atherosclerotic disease and antibody titers or presence of the pathogen in atherosclerotic tissue is analysed. Considering the reported association between Chlamydia pneumoniae antibodies and cardiovascular events, and bearing in mind that plaque destabilization (i.e., plaque rupture or ulceration) and thrombus formation underly acute cardiovascular events, we explored whether Chlamydia pneumoniae is more likely to induce plaque instability or a hypercoagulable status. This question is answered in chapter 6 using an in vivo model that allows to differentiate between plaque instability and hypercoagulability, i.e. the patient undergoing carotid endarterectomy for symptomatic carotid artery disease.

In section 4 (chapter 7), host factors potentially accounting for individual susceptibility to the pro-atherogenic properties of infection are studied. In chapter 7, the association between common polymorphisms of two pattern-recognition receptor genes (Toll-like Receptor 4 and CD14) and extent of atherosclerotic disease is analysed in patients with peripheral arterial disease. The infectious and inflammatory paradigm for atherosclerosis development offers new potential therapeutic targets. In section 5 the effect of a short course of antibiotics in the progression of atherosclerosis in patients with PAD was studied in a randomised clinical trial (chapter 8). Finally, the data presented in this thesis are discussed and put in perspective in section 6 (chapter 9).
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Section 2

Inflammation & atherosclerosis
Chapter 2

C-reactive protein in peripheral arterial disease: relation to severity of the disease and to future cardiovascular events

Vainas T, Stassen FR, de Graaf R, Twiss EL, Herngreen SB, Welten RJ, van den Akker LH, van Dieijen-Visser MP, Bruggeman CA, Kitslaar PJ

2.1. Abstract

Background
Serum CRP has proven to be an independent marker of the extent of atherosclerosis in patients with coronary, cerebrovascular and peripheral arterial disease. In this prospective observational study we wanted to assess the relationship between serum CRP and extent of disease transversely and longitudinally in time, as well as future cardiovascular complications in patients with peripheral arterial disease (PAD). Hypothesizing that CRP not only is a marker of but also actively participates in atherogenesis, we explored the possibility of CRP production by femoral atherosclerotic plaques.

Patients & methods
Serum CRP was measured highly sensitive (hsCRP) in 387 patients with PAD attending the vascular clinic of a university and two affiliated teaching hospitals. Serum hsCRP was related to ankle-brachial pressure index (ABPI), as an indication of severity of disease at inclusion and at 12 months’ follow-up and to future events (death, coronary, cerebral, and peripheral arterial events). In femoral plaques, the production of CRP was analyzed with reverse transcription polymerase chain reaction (RT-PCR), and CRP plaque localization was assessed with immunostaining on serial tissue sections with antibodies toward CRP, smooth muscle cells, T-cells and macrophages.

Results
The hsCRP (average ± SD) was 3.26±2.41 mg/l. Serum hsCRP showed a correlation with baseline and 12-month follow-up ABPI (Spearman rank correlation, P<0.05 for both correlations). When the patients were divided into three equally sized groups according to baseline serum hsCRP, the ABPI at baseline and at 12 months decreased significantly from the low to the high hsCRP group (baseline ABPI: 0.70, 0.65 and 0.57, P<0.01; and 12-month follow-up ABPI: 0.76, 0.70 and 0.65, P<0.01). These associations persisted after correction for conventional risk factors. Furthermore, serum hsCRP was related to the combined endpoint ‘death and/or any cardiovascular event’ (log rank test, P<0.04) during a median 24-month follow-up period. RT-PCR analysis showed CRP production in 4 of 14 femoral plaques. CRP was detected in all femoral plaques, but not in healthy brachial arteries. Immunoreactivity for CRP was observed in smooth muscle cells, macrophages and T-cells.

Conclusion
Serum hsCRP was related to severity of PAD, showing a relation to future hemodynamic function and cardiovascular events in PAD patients. In addition to coronary plaques, aneurysmal aortas and failed venous coronary bypasses, femoral plaques as well produce CRP, illustrating that the production of CRP may represent a universal response to vascular injury and suggesting that vascular CRP may contribute to plaque development.
2.2 Introduction

In concord with the inflammatory nature of atherosclerosis, several inflammatory markers, such as, white blood cell count,\(^1\) erythrocyte sedimentation rate\(^2\) and C-reactive protein (CRP) have been associated with coronary artery disease. Of these, CRP is the most extensively studied in this regard. It has been shown that serum CRP is increased in patients with coronary artery disease,\(^3\) whereas in healthy middle-aged and elderly individuals, it is associated with the development of coronary,\(^4,5\) cerebral\(^6,7\) and peripheral vascular events.\(^7\)

Peripheral arterial disease (PAD) is a common manifestation of atherosclerosis. Its estimated prevalence varies between 0.6% and 8.8%.\(^8\) Compared with healthy controls, patients with PAD have increased serum CRP levels.\(^9,10\) Furthermore, serum CRP seems to be associated with a low ankle-brachial pressure index (ABPI<0.9)\(^10\), walking performance\(^11\) and endothelial function in these patients.\(^9\) Nevertheless, it remains to be seen whether serum CRP is longitudinally related in time to severity of PAD, and whether it is related to future cardiovascular complications in patients with PAD.

The relationship between CRP and the extent of atherosclerosis does not necessarily reflect the systemic nature of a low-grade inflammatory reaction associated with atherosclerosis that leads to stimulated hepatic CRP production. In fact, it has been shown that coronary plaques, aneurysmal aortas and failed venous bypass grafts produce CRP.\(^12,13\) It remains to be established whether local production of CRP is a generalized phenomenon of all vascular territories afflicted by atherosclerosis.

In the present study we studied the relation between serum CRP and the extent and progression of peripheral atherosclerotic disease, as well as the development of cardiovascular complications, in patients with PAD. We further assessed the possible production of CRP by atherosclerotic plaques of lower limb arteries.

2.3 Materials and methods

2.3.1 Patients

Patients with symptomatic PAD (ABPI<0.9) were recruited at the vascular clinic of a university medical centre and two affiliated teaching hospitals. Patients with a suspected acute phase reaction (CRP>10mg/l),\(^15\) inflammatory co-morbidity, malignancy, recent (<3 months) antibiotic use, recent (<6 months) vascular events or interventions, renal or liver failure, or limited life expectancy (<2 years) were not included. The study was approved by the medical ethics
committee of all participating centres and conformed with the principles outlined in the declaration of Helsinki. All patients gave written informed consent.

2.3.2 Assessment of ABPI

The ABPI was measured in the supine position after a 15-minute rest period. At inclusion the ankle pressure was measured in, both, the posterior tibial and dorsal pedal artery of the (most) symptomatic leg. The highest ankle-pressure was used to determine the ABPI. At follow-up visits, the blood pressure in the same crural artery was used to determine the follow-up ABPI.

2.3.3 Risk factor profile

At inclusion, the presence of classic atherosclerotic risk factors was assessed, i.e., smoking (currently smoking or stopped <10 years ago), diabetes (currently using antihyperglycemic medication or insulin or fasting blood glucose level >7 mmol/l), hypertension (currently using antihypertensive medication or systolic blood pressure >160 mmHg), dyslipidemia (currently using antilipidemic medication or fasting cholesterol >5 mmol/l), and family history (1st degree relative with ischemic cardiovascular disease before the age of 70).

2.3.4 Follow-up and cardiovascular events

All patients were followed up semi-annually for a variable time period, but minimally for one year. The median follow-up was 24 months and ranged from 12 to 48 months.

The primary endpoint was the combined variable 'all cause mortality and/or any cardiovascular event'. Coronary events included myocardial infarction, de novo unstable angina pectoris, and any coronary revascularization procedure. A cerebral event was any stroke or transient ischemic attack. A peripheral arterial event was defined as any increase in peripheral ischemic symptoms (i.e., ischemic pain) that was accompanied either by a significant decrease in ABPI (i.e., Δ[ABPI] >0.1) or by any peripheral revascularization procedure (percutaneous transluminal angioplasty or operation). To minimize the risk of scoring a revascularization as an event soon after inclusion in the study even though no actual progression in atherosclerotic disease had occurred, patients were admitted to the study only after a definitive inclusion vascular treatment plan had been designed for each patient (conservative vs. angioplasty vs. surgery). Only revascularization procedures that developed beyond this inclusion treatment plan were scored as events.
2.3.5 Serum CRP level

Venous blood was obtained during baseline assessment. To obtain serum, blood was immediately centrifuged at 1200 rpm at 4°C for 10 minutes, and the serum was stored at -20°C until analysis. CRP was determined highly sensitive (hsCRP) with the IMMULITE assay (Diagnostic Products Corporation Nederland BV, Breda, The Netherlands). It provides a detection limit of 0.10 mg/l (zero calibrator + 2 SD) and a measurable range of 0.1–500 mg/l (manufacturer's claim). The coefficient of variation of this assay depends on the average hsCRP values, and at our laboratory it varied from 2.1% to 6.7%. This assay thus provides adequate precision for cardiovascular risk stratification, and it has been approved by the Food and Drug Administration for clinical use in the United States of America.

2.3.6 Atherosclerotic vascular tissue

Femoral endarterectomy specimens from 34 patients who underwent operation consecutively at our clinic were collected. Of these, 12 patients participated in the prospective, serological part of the study. Femoral plaques used for RT-PCR (n=14) were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Plaques for immunohistochemical analysis (n=20) were formalin-fixed and paraffin-embedded.

2.3.7 CRP RT-PCR and Immunohistochemical analysis

The presence of messenger RNA points to active transcription of a gene implicating synthesis of the gene-product. To assess whether CRP was produced in femoral atherosclerotic plaques, the presence of CRP mRNA was determined in this tissue by RT-PCR as previously described. The presence and localization of CRP in atherosclerotic plaques of femoral arteries was further assessed immunohistochemically. In order to identify CRP-positive cells, smooth muscle cells, T-cells, and macrophages were stained immunohistochemically on serial tissue sections. Femoral atherosclerotic plaques (n=20) were formalin fixed and embedded in paraffin. Serial 4 μm sections were deparaffined and incubated with a monoclonal antibody directed against CRP, an α-actin smooth muscle cell antibody, a CD-3 (T-cell) antibody and a CD-68 (macrophage) antibody (all from DAKO, Denmark). Subsequently, incubation with a rabbit anti mouse antibody (DAKO) and streptavidine biotine complex was performed in a multistep immunostaining procedure. The color reaction was created with diaminobenzidine. Two healthy brachial artery specimens obtained at post-mortem examination from fatal trauma victims were used as negative tissue control in each run.
2.3.8 Statistics

SPSS 10.0 for Windows (SPSS Inc, Chicago, Ill) was used for the statistics. The \( \chi^2 \)-test was used for comparison of dichotomous/categorical variables. Spearman rank correlation was used to explore the association between serum \( h\text{sCRP} \) and ABPI at inclusion and at 12 months. The paired samples \( t \)-test was used for comparison of baseline and 12-month follow-up ABPI. After the patient population was divided into three equally sized groups according to baseline serum \( h\text{sCRP} \), analysis of variance (ANOVA) with the Bonferroni correction was used to compare mean baseline and 12-month follow-up ABPI among the three groups. Parameters that were significantly associated with ABPI in the univariate analyses were entered as potential confounders in a multivariate ANOVA to study the relationship between ABPI and serum \( h\text{sCRP} \). Kaplan-Meier models with log rank statistics were constructed to compare freedom from clinical endpoints between patients in the three CRP groups. Cox proportional hazard models were used to correct for relevant co-variables.

2.4 Results

Patient characteristics and vascular status are shown in Table 2.1. The patients analyzed had severe PAD as revealed by an average (SD) baseline ABPI at inclusion of 0.64 (0.18) (Table 2.2). After 12 months the average (SD) ABPI increased significantly to 0.72 (0.20) (paired samples \( t \)-test, \( P<0.001 \)). This increase resulted mainly from interventions (Table 2.2).

The extent of systemic atherosclerotic disease in these patients was reflected in the high prevalence of coronary and cerebrovascular co-morbidity (Table 2.1) and in the significant number of cardiovascular and cerebrovascular complications and deaths during follow-up. Approximately one third of the PAD patients had coexisting coronary artery disease, and 18% had cerebrovascular comorbidities. During a 24-month follow-up period, 136 (35.1%) PAD patients developed 184 vascular events (coronary, cerebral, peripheral) or died. Thirty-two patients died (all-cause mortality), 29 developed 30 coronary events, 25 patients suffered 26 transient ischemic attacks or strokes and 76 had, in total, 96 peripheral arterial events (Table 2.3).

The median (inter-quartile range, IQR) serum \( h\text{sCRP} \) in PAD patients was 2.65 (1.35-4.41) mg/l. Because the mean \( h\text{sCRP} \) value (3.26 mg/l) was greater than 3 mg/l, these patients should be considered as being at high risk for future complications according to guidelines from the American Heart Association and the Centres for Disease Control and Prevention.\(^\text{13} \)
### Table 2.1 Patient characteristics.

<table>
<thead>
<tr>
<th>N</th>
<th>All patients</th>
<th>Low CRP</th>
<th>Intermediate CRP</th>
<th>High CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>387</td>
<td>129</td>
<td>129</td>
<td>129</td>
</tr>
<tr>
<td>Age, y, mean (SD)</td>
<td>65 (9.5)</td>
<td>64 (8.9)</td>
<td>65 (10.4)</td>
<td>66 (8.7)</td>
</tr>
<tr>
<td>Male / female</td>
<td>264 / 123</td>
<td>85 / 44</td>
<td>88 / 41</td>
<td>91 / 38</td>
</tr>
<tr>
<td>Smoking*, n (%)</td>
<td>297 (77)</td>
<td>95 (74)</td>
<td>101 (78)</td>
<td>101 (78)</td>
</tr>
<tr>
<td>Dislipidemia*, n (%)</td>
<td>321 (83)</td>
<td>106 (84)</td>
<td>108 (84)</td>
<td>105 (81)</td>
</tr>
<tr>
<td>Hypertension*, n (%)</td>
<td>221 (57)</td>
<td>76 (59)</td>
<td>70 (55)</td>
<td>75 (58)</td>
</tr>
<tr>
<td>Diabetes*, n (%)</td>
<td>105 (27)</td>
<td>38 (29)</td>
<td>33 (26)</td>
<td>34 (26)</td>
</tr>
<tr>
<td>Positive Family History*, n (%)</td>
<td>239 (62)</td>
<td>79 (61)</td>
<td>81 (63)</td>
<td>79 (61)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Claudication, n (%)</td>
<td>351 (91)</td>
<td>119 (92)</td>
<td>120 (93)</td>
<td>112 (87)</td>
</tr>
<tr>
<td>Critical limb ischemia, n (%)</td>
<td>36 (9)</td>
<td>10 (8)</td>
<td>9 (7)</td>
<td>17 (13)</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conservative, n (%)</td>
<td>273 (70)</td>
<td>90 (70)</td>
<td>90 (70)</td>
<td>93 (72)</td>
</tr>
<tr>
<td>Angioplasty, n (%)</td>
<td>73 (19)</td>
<td>29 (22)</td>
<td>22 (17)</td>
<td>22 (17)</td>
</tr>
<tr>
<td>Surgery, n (%)</td>
<td>41 (11)</td>
<td>10 (8)</td>
<td>17 (13)</td>
<td>14 (11)</td>
</tr>
<tr>
<td>Vascular co-morbidity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD</td>
<td>126 (32)</td>
<td>42 (33)</td>
<td>41 (32)</td>
<td>42 (33)</td>
</tr>
<tr>
<td>CVD</td>
<td>62 (16)</td>
<td>13 (10)</td>
<td>28 (22)</td>
<td>21 (16)</td>
</tr>
<tr>
<td>AAA</td>
<td>37 (10)</td>
<td>15 (12)</td>
<td>9 (7)</td>
<td>13 (10)</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylsalicylic acid, n (%)</td>
<td>362 (93)</td>
<td>120 (93)</td>
<td>123 (95)</td>
<td>119 (92)</td>
</tr>
<tr>
<td>Coumarin, n (%)</td>
<td>42 (11)</td>
<td>12 (9)</td>
<td>15 (12)</td>
<td>15 (12)</td>
</tr>
<tr>
<td>Bi-blocker, n (%)</td>
<td>121 (31)</td>
<td>43 (33)</td>
<td>39 (30)</td>
<td>39 (30)</td>
</tr>
<tr>
<td>RAS inhibitor, n (%)</td>
<td>129 (33)</td>
<td>46 (36)</td>
<td>40 (31)</td>
<td>43 (33)</td>
</tr>
<tr>
<td>Calcium channel blocker, n (%)</td>
<td>110 (28)</td>
<td>32 (25)</td>
<td>40 (31)</td>
<td>38 (29)</td>
</tr>
<tr>
<td>Statin, n (%)</td>
<td>321 (83)</td>
<td>108 (84)</td>
<td>108 (84)</td>
<td>105 (81)</td>
</tr>
</tbody>
</table>

CRP: C-reactive protein; CAD: coronary artery disease; CVD: cerebrovascular disease; AAA: abdominal aortic aneurysm; RAS: renin-angiotensin system.

* currently smoking or stopped less than 10 years ago; ** fasting cholesterol level > 5.1 mmol/l or the use of antilipolemic medication; * systolic blood pressure > 160 mmHg, diastolic blood pressure > 95 mmHg, or the use of antihypertensive medication; * fasting glucose level > 7 mmol/l or the use of antidiabetic medication or insulin; * first-degree relative with first ischemic cardiovascular event before age of 70 years.

Serum hsCRP was related to severity of PAD. Baseline serum hsCRP was significantly correlated with the ABI at inclusion (Spearman's \( r = 0.306 \), \( P < 0.01 \)), and with the ABI at 12 months (Spearman's \( r = 0.256 \), \( P < 0.01 \)). When patients were divided into three equally sized groups according to baseline hsCRP level, low hsCRP group (CRP ≤ 1.72), intermediate hsCRP group (1.72 < hsCRP ≤ 3.56), and high hsCRP group (hsCRP > 3.56), ABI decreased from lowest to upper tertile (average baseline ABI: 0.70, 0.65, and 0.57 in the low, intermediate and high hsCRP group, respectively; ANOVA, \( P = 0.001 \) for the trend; Figure 2.1a). This association persisted after correcting for relevant risk factors, i.e., diabetes, hypertension, smoking, age, sex and critical limb ischemia (multivariate ANOVA, \( P = 0.001 \) for the trend). Intriguingly, the ABI at 12 month follow-up showed a similar significant
decrease with baseline serum hsCRP (average 12 month follow-up ABPI: 0.78, 0.70 and 0.66 in the low, intermediate and high hsCRP group, respectively, ANOVA, \( P=0.001 \); Figure 2.1b). Again, the association between baseline hsCRP and ABPI at 12 months persisted after correction for the risk factors mentioned above (multivariate ANOVA, \( P=0.001 \) for the trend).

Table 2.2  ABPI at inclusion and after 12 months’ follow-up in the entire PAD patient population and in the subgroups of patients with or without an intervention within one year after inclusion.

<table>
<thead>
<tr>
<th></th>
<th>Entire population (N=387)</th>
<th>Patients without intervention (N=259)</th>
<th>Patients undergoing intervention (N=128)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPI at inclusion</td>
<td>0.64 (0.18)</td>
<td>0.67 (0.18)</td>
<td>0.59 (0.17)</td>
</tr>
<tr>
<td>ABPI at 12 months</td>
<td>0.72 (0.20)</td>
<td>0.69 (0.18)</td>
<td>0.76 (0.23)</td>
</tr>
<tr>
<td>( P ) (paired sample T test)</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

ABPI: ankle-brachial pressure index; PAD: peripheral arterial disease. Data are mean (SD) ABPI.

Serum hsCRP exhibited a relation with the occurrence of complications during follow-up. The incidence of all vascular events increased from the low hsCRP group to the high hsCRP group (Table 2.3). However, only for the combined endpoint did this association reach a statistically significant level (\( \chi^2 \) for trend, \( P=0.020 \)). Accordingly, the freedom from any clinical endpoint seemed shorter with higher baseline serum hsCRP. Again, only for the combined endpoint of all cause mortality and/or any cardiovascular complication did this association reach statistical significance (log rank statistics, \( P=0.036 \), Figure 2.2). The significance of this relation survived correction for conventional cardiovascular risk factors and clinical severity of PAD in a Cox proportional hazards model (\( P=0.003 \)).

Table 2.3  Incidence of cardiovascular events in PAD patients with low, intermediate or high baseline serum hsCRP level.

<table>
<thead>
<tr>
<th></th>
<th>Total group (N=387)</th>
<th>Low CRP group(^a) (N=129)</th>
<th>Intermediate CRP group(^b) (N=129)</th>
<th>High CRP group(^c) (N=129)</th>
<th>( P^d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined endpoint</td>
<td>136 (35.1)</td>
<td>33</td>
<td>51</td>
<td>52</td>
<td>0.020</td>
</tr>
<tr>
<td>Death</td>
<td>32 (8.2)</td>
<td>6</td>
<td>12</td>
<td>14</td>
<td>0.170</td>
</tr>
<tr>
<td>Coronary endpoint</td>
<td>29 (7.4)</td>
<td>7</td>
<td>12</td>
<td>10</td>
<td>0.493</td>
</tr>
<tr>
<td>Cerebral endpoint</td>
<td>25 (6.5)</td>
<td>6</td>
<td>7</td>
<td>12</td>
<td>0.266</td>
</tr>
<tr>
<td>Peripheral endpoint</td>
<td>76 (19.6)</td>
<td>23</td>
<td>28</td>
<td>25</td>
<td>0.733</td>
</tr>
</tbody>
</table>

PAD: peripheral arterial disease; hsCRP: highly sensitive C-reactive protein. Data are numbers (percentages) of patients with an event. \(^a\) \( \chi^2 \)-test for trend; \(^b\) low CRP group: CRP≤1.72; \(^c\) intermediate CRP group: 1.72<hsCRP≤3.56; \(^d\) high CRP group: hsCRP>3.56
Figure 2.1 Relationship between ankle-brachial pressure index (ABPI) at inclusion and at 12 months and serum hsCRP at inclusion.

A: The average ABPI at inclusion decreased from the low CRP group to the high CRP group (analysis of variance [ANOVA]; F_{trend}=0.001). B: The average ABPI at 12 months follow-up also decreased from high to low baseline serum hsCRP (ANOVA, F_{trend}=0.001). CI: confidence interval.

Fourteen atherosclerotic femoral plaques from 14 patients undergoing vascular surgery of the lower limb were analysed by RT-PCR to assess the local production of CRP in femoral atherosclerotic plaques. Four (29%) of the 14 plaques showed evidence of CRP messenger RNA production. The housekeeping gene cyclophosphin was detected in all samples (Figure 2.3).

Figure 2.2 Difference in freedom from the combined endpoint death and/or any vascular event' (coronary, cerebral, peripheral) among patients with low vs. intermediate vs. high C-reactive protein (CRP; log rank statistics; P=0.036). This association persisted after correction for conventional cardiovascular risk factors and clinical extent of peripheral arterial disease (Cox proportional hazards model, P=0.003).
Furthermore, immunohistochemical analysis of 20 femoral atherosclerotic plaques from 20 consecutive patients and two healthy brachial arteries showed omnipresence of CRP in all plaques and almost complete absence of CRP in normal arteries (Figure 2.4). CRP-immunoreactive cells were also positive for CD-3 (T-cells), α-actin (smooth muscle cells) or CD-68 (macrophages) (Figure 2.4).

![Diagram of gel showing CRP and cyclophilin](image)

**Figure 2.3** Representative gel showing C-reactive protein (CRP) messenger RNA in femoral atherosclerotic plaque from a patient with peripheral arterial disease. Lane 1 contains a marker for size (SmartLadder, Eurogentec Seraing, Belgium). Lane 2 has been loaded with CRP amplification product from femoral plaque. Lane 3 has been loaded with femoral plaque CRP amplification product without prior transcription of RNA into complementary DNA (-RT), demonstrating the specific amplification of RNA instead of genomic DNA. Lane 4 was loaded with cyclophilin polymerase chain reaction product from femoral plaque and lane 5 was loaded with water.
Figure 2.4  Immunohistochemical detection of C-reactive protein (CRP) in femoral atherosclerotic plaques and apparently healthy brachial artery. CRP was abundantly present in femoral atherosclerotic plaque (A) and almost completely absent in healthy tissue (B). CRP immunostaining (C) co-localized with T-cells (D), smooth muscle cells (E) and macrophages (F) in serial tissue sections of femoral atherosclerotic plaques.
2.5 Discussion

In this study, we assessed the association between *hs*CRP and the extent of atherosclerotic disease in PAD patients. Our data showed that PAD patients have increased serum *hs*CRP. Furthermore, *hs*CRP was inversely related to ABPI. This is in agreement with previous studies that showed that *hs*CRP is associated with hemodynamic (eg, ABPI)\(^{10}\) and functional (eg, outcome 6-minute walk)\(^{11}\) outcomes, and with clinical severity of PAD.\(^{19}\) Previously published data have shown that preprocedural serum CRP is independently related to early and late clinical cardiovascular events and to restenosis after coronary percutaneous intervention.\(^{20,21}\) Accordingly, our data showed that baseline serum *hs*CRP was inversely related to ABPI measured 12 months later. This may suggest that high baseline serum *hs*CRP may identify patients that are most likely to develop restenosis or experience accelerated atherosclerosis of the native lower limb arteries. Serum *hs*CRP has been associated with the development of complications of the coronary, cerebral and peripheral arterial circulation in apparently healthy individuals.\(^{4,7}\) With this prospective study we were able to demonstrate that serum *hs*CRP was also related to the development of atherosclerosis-related events or death in PAD patients. To the best of our knowledge, this is the first study to demonstrate that serum *hs*CRP is related to future hemodynamic function and future cardiovascular events in patients with PAD. Hence, serum *hs*CRP may be used to estimate the extent of atherosclerotic disease, and may facilitate cardiovascular risk estimation in PAD patients and healthy individuals. Additionally, recently published data have demonstrated that decreasing serum CRP by statins resulted in a significant reduction of cardiovascular event rate regardless of the resultant low-density lipoprotein cholesterol level. Consequently, cardiovascular risk-reduction strategies involving statins should include serum CRP monitoring.\(^{22}\)

It is believed that cytokine-stimulated production of CRP by hepatocytes is responsible for the elevated CRP levels in atherosclerosis. Localization of CRP extracellularly and in macrophages shown in human atherosclerotic aortic lesions was thought to result from uptake of CRP-lipid complexes by macrophages.\(^{23}\) However, recent evidence suggests that diseased vascular tissue can produce CRP as well. Coronary plaques,\(^{12}\) aneurysmal aortas\(^{13}\) and failed venous coronary bypasses\(^{14}\) have shown to produce CRP. In this study, we demonstrated that femoral atherosclerotic plaques produce CRP as well. Using immunohistochemical staining on serial tissue sections we identified smooth muscle cells, macrophages and T-cells as the putative cellular production sites of CRP. Yasojima *et al.* have suggested that smooth muscle-like cells and macrophages produce CRP in coronary atherosclerotic plaque,\(^{12}\) whereas Kuta *et al.*\(^{24}\) and Ikuta *et al.*\(^{25}\) independently showed that lymphocytes
produce CRP when stimulated with LPS or 10-O-tetradecanoyl-phorbol-13-acetate, respectively.

Considering the presence and local production of CRP in atherosclerotic tissue it is logical to assume that CRP actively participates in the atherosclerotic process. Recent evidence suggests that CRP has proatherogenic effects on all cellular constituents of the vascular wall. CRP co-localizes with the membrane attack complex in atherosclerotic plaques and activates complement.\textsuperscript{12,26} CRP is chemotactic for circulating monocytes,\textsuperscript{27} stimulates monocyte tissue factor production,\textsuperscript{28} up-regulates some macrophage pro-inflammatory cytokines,\textsuperscript{28} and causes a sustained increase in native low-density lipoprotein uptake by macrophages.\textsuperscript{30} Through activation of nuclear factor \kappaB, CRP may induce endothelial adhesion molecules,\textsuperscript{31} stimulate endothelial monocyte chemoattractant protein-1 release,\textsuperscript{32} and inhibit basal and stimulated endothelial nitric oxide release.\textsuperscript{33} Similarly, CRP activates monocyte chemoattractant protein-1, interleukin 6 and inducible nitric oxide synthetase expression in vascular smooth muscle cells.\textsuperscript{34} The majority of the above described pro-inflammatory effects have been demonstrated \textit{in vitro} using CRP concentrations ranging between 5 and 900mg/l. Therefore, it has recently been questioned whether plasma concentration of CRP used to denote a high risk for future cardiovascular complications (>3mg/l) are able to elicit these pro-inflammatory effects in the vascular wall.\textsuperscript{35} Local production of CRP in the vascular wall as demonstrated in the present paper may overcome this problem by yielding high CRP concentrations in the microenvironment of the atherosclerotic vessel wall.

The associations described here reflect the inflammatory nature of atherosclerosis. Because chronic infections have also been associated with atherosclerosis development as well,\textsuperscript{36} it is very reasonable to suggest that micro-organisms may be related to the increased serum CRP levels in atherosclerotic patients. In our study population, \textit{Chlamydia pneumoniae} serology was not related to the serum CRP concentration. Furthermore, although both \textit{Chlamydia pneumoniae} seropositivity (see chapter 8) and serum CRP had an independent effect on cardiovascular events, we were not able to demonstrate a synergistic effect of these variables on cardiovascular risk. This may be related to the fact that only patients with no recent infections and vascular interventions were analyzed. Additionally, it is more likely that total pathogen burden rather than infection with one micro-organism may be related to CRP levels and cardiovascular risk.

In conclusion, our data demonstrate that in addition to being a marker of atherosclerosis, CRP is produced within the atherosclerotic plaque and may locally participate in plaque development. Unravelling the parameters governing vascular CRP production may offer new ways of interfering with atherogenesis.
2.6. References


Chapter 3

Serum C-reactive protein level is associated with abdominal aortic aneurysm size and the protein may be produced by aneurysmal tissue

Vainas T, Lubbers T, Stassen FR, Herngreen SB, van Dieijen-Visser MP, Brugeman CA, Kitslaar PJ, Schurink GW

_Circulation_ 2003;107:1103-07
3.1 Abstract

Background
Abdominal aortic aneurysms (AAA) are characterized by extensive transmural inflammation and C-reactive protein (CRP) has emerged as independent risk factor for the development of cardiovascular disease. Therefore, we evaluated a possible association between serum CRP and aneurysm dimension in patients with asymptomatic AAA. Furthermore the possibility of CRP production by aneurysmal tissue has been examined.

Patients & methods
Serum CRP was determined highly sensitive (hsCRP) and aneurysmal size was measured in 39 patients with AAA. The presence of CRP messenger RNA was assessed in aneurysmal tissue of 16 patients.

Results
Mean (SD) hsCRP was 3.23 (2.96) mg/l. After log-transformation, hsCRP correlated significantly with aneurysm size \( r=0.477, p=0.002 \). When the patients were divided into 3 equally sized groups according to hsCRP level, aortic diameter increased from 54mm to 61mm and 67mm respectively, \( P<0.05 \) for 3rd vs. 1st tertile. This association persisted after correction for risk factors. CRP messenger RNA was found in 25% of aneurysmal aortic tissues.

Conclusion
This is the first report showing that serum hsCRP is associated with aneurysm size and that at least some patients' CRP may be produced by aneurysmal tissue. These data underscore the inflammatory nature of AAA formation, suggesting that serum hsCRP may serve as marker of AAA disease and that CRP produced in vascular tissue might contribute to aneurysm formation.
3.2 Introduction

The degenerative form of the abdominal aortic aneurysm (AAA) is a medial disease characterized by dilatational remodeling with degradation of extracellular matrix components and medial thinning. Inflammation appears to be an important component of aneurysm formation as illustrated by the extensive medial/adventitial inflammatory cell infiltrations. Accordingly, increased expression of pro-inflammatory cytokines can be found in aneurysmal tissue and circulating levels of inflammatory cytokines are elevated in patients with AAA. The serum concentration of several cytokines is associated with aneurysm diameter (interleukin-8), AAA symptomatology (tumour necrosis factor-α), and increased AAA expansion rate (interferon-γ). C-reactive protein (CRP) has recently emerged as a strong independent risk factor for atherosclerosis and atherosclerosis-related complications in apparently healthy individuals and patients with cardiovascular disease. In 1987, Powell and colleagues showed that, among patients undergoing elective aortic reconstruction, serum CRP was elevated in AAA-patients compared with patients with obstructive disease. Although subsequently elevated serum CRP has been reported in patients with symptomatic or ruptured AAA, this early observation of elevated CRP level in patients with asymptomatic AAA has not been verified. Therefore, in the present study, serum CRP was, for the first time, measured highly sensitive in patients with asymptomatic AAA. The association between serum CRP and AAA dimension, and the possibility of local CRP-production by aneurysmal tissue were also explored.

3.3 Materials and methods

Patients with AAA (n=39), admitted to the surgical clinic of the University Hospital of Maastricht for vascular reconstruction were included in this study. Patients with ruptured/asymptomatic AAAs, recent infections, active inflammatory disorders and/or serum CRP>10 mg/l were excluded. The study was approved by the institutional medical ethical committee, and all patients gave written informed consent.

3.3.1 Determination of serum CRP

Venous blood samples drawn on admission 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). 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 USA.
3.3.2 AAA dimension

Computer assisted tomography was used to visualize the aorta and to determine the maximal aneurysm diameter. Because of logistical reasons, echodoppler ultrasonography was used to determine aneurysm dimensions in 5 of the 39 patients. hsCRP-level and aneurysm size did not differ between patients who had tomography or ultrasonography (data not shown).

3.3.3 CRP RT-PCR

Aneurysm tissue was snap frozen in liquid nitrogen after explantation and stored at -80°C until analysis. Total RNA from approximately 0.1 g of each tissue sample (n=16) was isolated according to the manufacturer's instructions using TRIZOL® Reagent (Life Technologies). After treatment with DNase I in the presence of RNA guard (both Amersham Pharmacia Biotech), the RNA concentration was determined measuring the optical density at 260nm. One microtitre of RNA was reverse transcribed into cDNA using oligo dT (RACE-1, Amersham Pharmacia Biotech) and Superscript II RNase H- (Invitrogen). For every RNA isolate, a RT-PCR reaction was also performed in the absence of reverse transcriptase to demonstrate the specific amplification of messenger RNA instead of genomic DNA. For amplification of CRP the following primer pair (amplifying a 441 bp sequence) was used: forward: 5'-TCGTATGGCCACCAAGACAGACA-3', reverse: 5'-AACACTTGCTTGCACCTTC AATGT-3'. Two microlitres cDNA were transferred to an amplification mixture containing HotStar Taq DNA polymerase (Qiagen) and amplified for 40 cycles. Amplification products were separated on a 1% ethidium bromide stained agarose gel. Resulting bands were imaged using a FluorChem™ 8000 analyzer (Alpha Innotech Corporation). To control for the identity of the bands, PCR product was (i) sequenced and (ii) treated with the endonuclease Apal splitting the amplified CRP sequence in 2 fragments of 381bp and 60bp respectively. The cDNA of the housekeeping gene cyclophilin (primer pair: forward: 5'- AATGCTGAACCACGCCGTTCTTCG-3'; reverse: 5'-CGTGGAAGTCACCA CCCTGACACA-3') was amplified in parallel in every run to control for RNA integrity.

3.3.4 Statistical analysis.

Pearson's r was computed to explore the correlation between aneurysm size and log-transformed serum hsCRP. ANOVA was used to further study the univariate association between serum hsCRP and aneurysm dimension and to assess the independent effect of sex, age, smoking, hypertension, dislipidemia, diabetes and extent of vascular disease on aneurysm size. Parameters that associated significantly with aneurysm size in the univariate analyses were
entered as potential confounders in a multivariate ANOVA to study the relation between aneurysm size and serum hsCRP.

3.4 Results

A total of 39 patients (5 women) aged 71 (±5) years with an atherosclerotic risk profile (hypertension, 74%; diabetes, 15%; dislipidemia, 74%; smoking, 77%) were included in this study. Seventy-seven percent of the patients had extensive vascular disease affecting at least 2 vascular territories (coronary, cerebral and/or aortofemoral).

3.4.1 CRP in aneurysmal disease.

The average (SD), 25th, 50th, and 75th percentile of serum hsCRP was 3.23 (2.96), 1.03, 1.71, and 4.99 mg/l, respectively. CRP mRNA was detected in 4 of 16 tissue samples (25%), whereas cyclophilin was detected in all samples (Figure 3.1).

![Figure 3.1](image)

Figure 3.1 Representative gel showing CRP messenger RNA in a aneurysmal sample from a patient with AAA. Lane 1 contains a marker for size and quantity (SmartLadder™, Eurogentec). CRP amplification product from human liver (lane 2) and aneurysmal tissue (lane 3) are seen. Endonuclease treatment of aneurysmal CRP PCR-product with Apal yielded fragments of the expected size (lane 4), whereas CRP amplification without prior transcription of RNA into cDNA (-RT) demonstrated the specific amplification of RNA instead of genomic DNA (lane 5). Lane 6 was loaded with cyclophilin PCR-product from aneurysmal tissue.
3.4.2 Aneurysm dimension and serum CRP

The average (SD) maximal aneurysm dimension was 59 (15) mm. Aneurysm size correlated with (log-transformed) serum hsCRP (r=0.477, P<0.002). When patients were divided into 3 equally sized groups according to hsCRP level ([i] hsCRP<1.13, [ii] 1.13≤hsCRP<4.15, and [iii] hsCRP≥4.15), aortic diameter increased from lowest to upper tertile (49mm, 61mm, and 67mm respectively; P<0.05 for 3rd vs 1st tertile; Table 3.1). From the risk factors for cardiovascular disease, only sex and diabetes showed an (trend towards) association with aneurysm size. When these confounders were entered in a multivariate model, the association between hsCRP and aneurysm size persisted. The corrected (for sex and diabetes) aneurysm diameter (mean [95%-CI], mm) of patients in the upper hsCRP tertile (73 [63-83], P=0.003) was significantly elevated, and that of the patients in the middle tertile (60 [51-68], P=0.083) tended to be elevated compared to corrected aneurysm size of patients in the lowest hsCRP tertile (49 [40-58], Table 3.1).

<table>
<thead>
<tr>
<th>hsCRP tertile</th>
<th>N</th>
<th>Mean (95%-CI) maximal diameter (in mm)</th>
<th>P*</th>
<th>Mean (95%-CI) corrected max. diameterb (in mm)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lowest (hsCRP&lt;1.09mg/l)</td>
<td>13</td>
<td>49 (41-56)</td>
<td>-</td>
<td>49 (40-58)</td>
<td>-</td>
</tr>
<tr>
<td>Middle (1.09mg/l≤hsCRP&lt;4.15mg/l)</td>
<td>13</td>
<td>61 (54-67)</td>
<td>0.098</td>
<td>60 (51-68)</td>
<td>0.083</td>
</tr>
<tr>
<td>Upper (hsCRP≥4.15mg/l)</td>
<td>13</td>
<td>67 (57-78)</td>
<td>0.004</td>
<td>73 (63-83)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* for comparison with lowest tertile; b correction for sex and diabetes.

3.5 Discussion

Moderately elevated serum hsCRP has been reported in patients with stenotic atherosclerotic disease, and is associated with an increased risk of developing cardiovascular events. However, scarce information exists about serum hsCRP in patients with aneurysmal disease. Powell et al. showed that patients with asymptomatic AAA had increased serum CRP compared to patients with obstructive disease. The high mean serum CRP level (56±10 mg/l) reported, may reflect the suboptimal performance of the CRP-method used compared to modern highly sensitive assays or may suggest that patients with acute inflammatory conditions were included in that analysis. A recent study showed that patients with symptomatic and ruptured aneurysms had elevated serum CRP compared with patients with asymptomatic AAAs, but failed to verify the elevated serum CRP in asymptomatic AAA. The CRP assay used by these investigators lacked the sensitivity (detection limit=5 mg/l) to be used in the assessment of CRP in cardiovascular disease. In the present study, for the first time, serum CRP has been measured highly sensitive in patients with
asymptomatic AAAs. Even though patients with symptomatic/ruptured AAA, active inflammatory/infectious disorders, or hsCRP>10mg/l were excluded, the mean serum hsCRP was above the range for supposedly healthy individuals\(^7\) and was elevated compared to serum hsCRP of a healthy population measured in our lab.\(^{12}\)

Intriguingly, serum hsCRP of asymptomatic AAA-patients showed in this study a strong association with aneurysm dimension. Despite the fact that hsCRP has proven to be an independent risk factor for cardiovascular complications it remains unclear whether serum hsCRP would be a useful marker for the prediction of aneurysm growth and rupture in patients with AAA.

It is believed that moderately elevated serum hsCRP (<10 mg/l) results from chronic hepatic stimulation. However, it has been shown that CRP is produced in coronary plaques,\(^{13}\) Alzheimer’s disease brain tissue,\(^{14}\) and myocardial infarcts.\(^{15}\) In the present study we were able to show that, in some patients, CRP was produced in aneurysmal tissue as well. These findings corroborate the notion that CRP upregulation is a generalized reaction to several types of tissue injury. Macrophages and smooth muscle cells might be the producers of ‘vascular’ CRP.\(^{13}\) Quantitative analysis of CRP mRNA and protein in normal and AAA tissue should give more insight into the up-regulation of CRP production during aneurysm formation.

In conclusion our data suggest that serum hsCRP is associated with aneurysm size in patients with asymptomatic AAA and that, in some cases, aneurysmal tissue is capable of producing CRP. Future studies should evaluate the usefulness of serum hsCRP as marker of disease progression and elucidate the role of locally produced CRP in AAA formation.
3.6 References

Section 3

Infection & atherosclerosis
Chapter 4

Chlamydia pneumoniae serology: comparing a commercial enzyme immunoassay and micro-immunofluorescence test in patients with cardiovascular disease

Vainas T, de Graaf R, Stassen FR, Kurvers HA, Grauls GE; Kitslaar PJ, Bruggeman CA

APMIS 2003;111:363-9
4.1 Abstract

Background
Chlamydia pneumoniae has been associated with cardiovascular disease and the detection of Chlamydia pneumoniae antibodies has subsequently challenged many cardiovascular investigators. The micro-immunofluorescence (MIF) test is considered the gold standard for detection of Chlamydia pneumoniae antibodies, but requires a high-level of expertise for adequate interpretation. Therefore, we compared the technically less demanding enzyme immunoassay (EIA) with the MIF test for the detection of Chlamydia pneumoniae antibodies in patients with cardiovascular disease.

Patients & methods
Chlamydia pneumoniae IgG- and IgA-antibodies were measured in sera of patients with cardiovascular disease (n=141) using an EIA and a MIF test. The first 44 sera were tested in duplicate by EIA in order to assess its intratest agreement. To define interobserver variability for the MIF test, MIF test results were read by two independent observers. Regarding the MIF test as gold standard, the sensitivity, specificity, positive and negative predictive value of the EIA for the detection of Chlamydia pneumoniae IgG- or IgA-seropositivity using various cut-off levels for seropositivity, as well as the agreement (kappa) between EIA and MIF, were calculated.

Results
The interobserver agreement of the MIF test for detection of seropositivity at various cut-off levels was good for IgG and for IgA. The intratest agreement of the EIA was excellent for IgG and IgA. The agreement between EIA and MIF in detection of IgG- and IgA-antibodies was adequate at low but not at high titre levels. At low titre levels, the sensitivity, specificity, positive and negative predictive value of EIA compared to the MIF-test was sufficient. The sensitivity of the EIA increased, improving the agreement with the MIF at high titre levels by retesting sera with elevated titres at higher pre-dilutions.

Conclusion
The EIA shows sufficient agreement with the MIF test in the detection of Chlamydia pneumoniae seropositivity. Therefore, the EIA is a practical alternative to the MIF in the detection of Chlamydia pneumoniae antibodies in patients with cardiovascular disease, bearing in mind that the sensitivity of the EIA depends on the antibody titre.
4.2 Introduction

*Chlamydia pneumoniae* infections have been associated with the development of atherosclerotic disease of the coronary, cerebral and peripheral arterial system,\(^1\) opening the field of *Chlamydia pneumoniae*-related research to a vast number of non-microbiologically trained (cardiovascular) investigators and laboratories. As a result, various serological assays, immunoglobulin subtypes and different cut-off points have been employed in the sero-epidemiological studies analysing the association between *Chlamydia pneumoniae* infection and atherosclerotic disease, which may explain the conflicting results of hitherto published reports.\(^2\) Hence, the development of a simple and reliable detection assay for *Chlamydia pneumoniae* antibodies has become mandatory. Nowadays, the micro-immunofluorescence (MIF) test is considered the gold standard for the detection of *Chlamydia pneumoniae* antibodies during (acute) infections.\(^3\) Although this is a specific and sensitive test, it is also technically demanding, time-consuming and it is hampered by the subjective reading of the results. Several enzyme immunoassays (EIA) characterised by high throughput, objective endpoints, technical accessibility and cost effectiveness are commercially available. However, the performance of these assays compared to the MIF has yet to be properly evaluated.

In this study we evaluated the performance of an EIA compared with the MIF in the detection of *Chlamydia pneumoniae* IgA- and IgG-antibodies in patients with cardiovascular disease.

4.3 Materials and methods

Sera of patients admitted to the Vascular Surgical Clinic of Maastricht University Hospital were used for this analysis. All patients undergoing vascular reconstruction surgery for atherosclerotic disease whose serum was available for analysis were included. The study subjects (n=141) represented a random subpopulation of vascular surgical patients treated between January 1, 1997 and December 31, 1998, at our vascular surgical clinic. The included patients underwent arterial reconstruction of the carotid artery (n=33), aorta (n=22) or lower limb arteries (n=86). The study was approved by the local medical ethical committee and all patients gave their informed consent.

4.3.1 Micro-immunofluorescence test (MIF)

The commercial MIF-test is an indirect fluorescent antibody technique for measurement of IgG- and IgA-antibodies to *Chlamydia pneumoniae*. The test utilises purified, formalinized whole elementary bodies (EB) of *Chlamydia*
pneumoniae, fixed onto microscope slides as distinct dots of antigen, leading to detection of surface exposed protein reactive antibodies. The MIF test was performed according to the instructions of the manufacturer (Labsystems; Helsinki, Finland). Briefly, serial serum dilutions were incubated with antigen on a microscope slide for 30 minutes. After slides were rinsed and dried, fluorescein isothiocyanate (FITC)-labelled anti-IgG or -IgA conjugates were applied and incubated for 30 minutes. A coverslip was mounted with appropriate mounting fluid over the antigen spot after the microscope slides were again rinsed and dried. The microscopic examination was done with a Zeiss Axioskop (Zeiss, Jena, Germany) with epillumination at 100x and 400x magnification. According to the recommendations of a consensus committee, only evenly distributed, typical fluorescence of Chlamydia pneumoniae EBs was considered a positive reaction.3 Endpoints were read as the last serum dilution that exhibited specific fluorescence of the EBs throughout the antigen dot. Two experienced investigators evaluated the slides independently. Sera were tested at serial dilutions from 1/32 to 1/512 for IgG-, and from 1/8 to 1/128 for IgA-antibody titre determination.

4.3.2 Enzyme immunoassay (EIA)

The EIA used in this study was a commercially available test (Labsystems; Helsinki, Finland) detecting species-specific IgG- and IgA-antibodies to surface expressed proteins of Chlamydia pneumoniae. Sera were tested according to the instructions of the manufacturer. In short, serum diluted at 1/100 was incubated with Chlamydia pneumoniae antigens coated onto a 96-well plate. After washing, horseradish peroxidase conjugated anti-immunoglobulin (IgG or IgA) was added to the wells and incubated for 60 min at room temperature. After further washing, the chromogen containing tetramethylbenzidine was added. The reaction was stopped with sulphuric acid after 30 min and optical density was immediately read at 450 nm. Antibody titres were calculated from optical density readings in enzyme immuno-units (EIU) according to manufacturer’s instructions. In the first 44 patients, IgG- and IgA-antibodies were determined in double serum samples. Due to the excellent agreement of the measured antibody titre in duplicate serum samples (see results), Chlamydia pneumoniae IgG and IgA were determined in single serum specimens in the remaining 97 patients. According to the manufacturer the EIA has been developed so that the calculated EIUs correspond to the reciprocal of the titre determined with the MIF-test. Therefore, the EIU values calculated with the EIA were transformed into MIF titre categories (Table 4.1) in order to assess the agreement between the EIA and the MIF.
Due to the weak agreement between the EIA and MIF at the high titre range (see results), sera with elevated titres (IgG ≥ 64 EIU or IgA ≥ 16 EIU) were retested at a higher pre-dilution (1/400 in stead of 1/100).

<table>
<thead>
<tr>
<th>EIA titre (EIU)</th>
<th>Corresponding MIF-titre</th>
<th>IgG</th>
<th>Corresponding MIF-titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG &lt; 32</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>32 ≤ IgG &lt; 64</td>
<td>1 / 32</td>
<td>8 ≤ IgA &lt; 16</td>
<td>1 / 8</td>
</tr>
<tr>
<td>64 ≤ IgG &lt; 128</td>
<td>1 / 64</td>
<td>16 ≤ IgA &lt; 32</td>
<td>1 / 16</td>
</tr>
<tr>
<td>128 ≤ IgG &lt; 256</td>
<td>1 / 128</td>
<td>32 ≤ IgA &lt; 64</td>
<td>1 / 32</td>
</tr>
<tr>
<td>256 ≤ IgG &lt; 512</td>
<td>1 / 256</td>
<td>64 ≤ IgA &lt; 128</td>
<td>1 / 64</td>
</tr>
<tr>
<td>IgG ≥ 512</td>
<td>1 / 512</td>
<td>IgA ≥ 128</td>
<td>1 / 128</td>
</tr>
</tbody>
</table>

4.3.3 Statistics

SPSS 10.0 for windows (SPSS Inc. Chicago, Illinois, USA) was used for the statistical analysis. Pearson’s correlation coefficient of the (log-transformed) EIA titre measurements in duplicate samples was computed. Spearman rank correlation between the two independent readings of the MIF test, and between the MIF and EIA titres (after transformation into MIF titre categories) was calculated. The intra-test and inter-test variability was assayed by calculating the coefficient of agreement kappa (κ). The sensitivity, specificity and positive- (PPV) and negative predictive value (NPV) was calculated for detection of Chlamydia pneumoniae seropositivity using various cut-off points for IgG and IgA, regarding the MIF test as the gold standard.

4.4 Results

4.4.1 MIF – Inter-observer agreement

The MIF test results were read by two independent observers. The two independent readings of both IgG- (Spearman’s $P=0.873$, $P<0.001$) and IgA-antibodies (Spearman’s $P=0.860$, $P<0.001$) correlated adequately. The prevalence of seropositivity varied from approximately 85% to 12% for IgG, and from approximately 70% to 4% for IgA, using different cut-off levels for IgG- and IgA-seropositivity (Table 4.2). There were no structural differences in outcome between the two observers; i.e., observer 1 assigned higher titres to some sera, whereas observer 2 did so to some other samples. The degree of agreement (inter-observer agreement) when categories of serodiagnosis (seropositive or seronegative) using 1/32, 1/64, 1/128, 1/256 or 1/512 as cut-off
point for IgG-seropositivity, and using 1/8, 1/16, 1/32, 1/64 or 1/128 as cut-off for IgA-seropositivity, varied from $\kappa = 0.67$ to $\kappa = 0.80$ for IgG, and from $\kappa = 0.62$ to $\kappa = 0.85$ for IgA (Table 4.2).

Table 4.2: MIF: Interobserver agreement at various cut-off levels for *Chlamydia pneumoniae* seropositivity (n=141)

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>% seropositivity</th>
<th>kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observer 1</td>
<td>Observer 2</td>
</tr>
<tr>
<td>IgG ≥ 1/32</td>
<td>84</td>
<td>67</td>
</tr>
<tr>
<td>IgG ≥ 1/64</td>
<td>73</td>
<td>77</td>
</tr>
<tr>
<td>IgG ≥ 1/128</td>
<td>55</td>
<td>61</td>
</tr>
<tr>
<td>IgG ≥ 1/256</td>
<td>32</td>
<td>38</td>
</tr>
<tr>
<td>IgG ≥ 1/512</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>IgA ≥ 1/8</td>
<td>76</td>
<td>67</td>
</tr>
<tr>
<td>IgA ≥ 1/16</td>
<td>62</td>
<td>49</td>
</tr>
<tr>
<td>IgA ≥ 1/32</td>
<td>40</td>
<td>51</td>
</tr>
<tr>
<td>IgA ≥ 1/64</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>IgA ≥ 1/128</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

4.4.2 EIA – Intra-test agreement

The IgG- and IgA-antibody titres of the first 44 patients were assayed by EIA in duplicate serum samples. Duplicate measurements showed (after log-transformation) an excellent correlation for both IgG- (Pearson’s correlation coefficient: 0.989, $P<0.001$) and IgA-antibodies (Pearson’s correlation coefficient: 0.973, $P<0.001$). Accordingly, the intra-test agreement of duplicate measurements comparing diagnostic categories (seropositive vs seronegative) at various cut-off levels was excellent for IgG and IgA (Table 4.3).

Table 4.3: EIA: Intra-test agreement at various cut-off levels for *Chlamydia pneumoniae* seropositivity (n=44)

<table>
<thead>
<tr>
<th>Cut-off</th>
<th>% seropositivity</th>
<th>kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td>IgG ≥ 1/32</td>
<td>82</td>
<td>77</td>
</tr>
<tr>
<td>IgG ≥ 1/64</td>
<td>52</td>
<td>46</td>
</tr>
<tr>
<td>IgG ≥ 1/128</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>IgG ≥ 1/256</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgG ≥ 1/512</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgA ≥ 1/8</td>
<td>58</td>
<td>58</td>
</tr>
<tr>
<td>IgA ≥ 1/16</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>IgA ≥ 1/32</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>IgA ≥ 1/64</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>IgA ≥ 1/128</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Therefore, in the remaining 97 patients IgG- and IgA-antibody levels were determined in single serum samples. The *Chlamydia pneumoniae* seroprevalence varied from 79% to 0% for IgG, and from 69% to 0% for IgA, using increasing antibody levels as cut-off points for IgG- and IgA-seropositivity (Tables 4.4 & 4.5). Considering the lower sensitivity of the EIA, especially at the high titre range, sera with an IgG≥64 EU or IgA≥16 EU were retested at a higher pre-dilution (1/400). This resulted in higher seropositivity prevalences when elevated titres were used as cut-off (Tables 4.4 & 4.5).

<table>
<thead>
<tr>
<th>Table 4.4</th>
<th>Comparing EIA to MIF test (gold standard). Inter-test agreement (kappa), sensitivity, specificity, positive and negative predictive value of EIA for the detection of <em>Chlamydia pneumoniae</em> IgG seropositivity at various cut-off points (n=141). Numbers in parentheses represent values calculated after retesting sera with IgG≥64 EU at higher pre-dilution.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cut-off for IgG-seropositivity at</td>
</tr>
<tr>
<td></td>
<td>1/32</td>
</tr>
<tr>
<td>% seropositive</td>
<td>EIA</td>
</tr>
<tr>
<td>Kappa</td>
<td>0.70</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>92</td>
</tr>
<tr>
<td>Specificity</td>
<td>87</td>
</tr>
<tr>
<td>PPV</td>
<td>97</td>
</tr>
<tr>
<td>NPV</td>
<td>67</td>
</tr>
</tbody>
</table>

PPV: positive predictive value; NPV: negative predictive value

4.4.3 EIA vs MIF

In the comparison of EIA vs. MIF, the EIA outcomes were weighted against the MIF readings of the same observer throughout all analyses. Of the titres determined by EIA in double serum samples in the first 44 patients, the first measurement was compared to the MIF. The titres of the remaining 97 were analysed in single serum samples by EIA and compared to the MIF results. When titres (in EU) calculated from the optical density readings obtained by the EIA were transformed into categorical variables corresponding to the MIF titre categories according to the transformation scheme given in Table 4.1, the correlation between EIA and MIF for both IgG and IgA was good (Spearman’s p=0.706, P<0.001, and Spearman’s p=0.745, P<0.001, for IgG and IgA respectively). Compared to the MIF, the EIA was less sensitive yielding lower prevalence of high antibody titres. Accordingly, the inter-test agreements between EIA and MIF in the detection of *Chlamydia pneumoniae* IgG- and IgA-seropositivity, as well as, the sensitivity, specificity, PPV and NPV of EIA compared to MIF were dependent on the cut-off point used to determine seropositivity. As shown in Tables 4.4 and 4.5, the EIA compared favourably to
the MIF when seropositivity was defined at low cut-off points. However, the kappa values and sensitivity of the EIA decreased with increasing titre levels. For this reason, sera with IgG≥64 EIU or IgA≥16 EIU were retested at a higher pre-dilution. This manoeuvre resulted in a slight improvement of the correlation between MIF and EIA for both IgG (Spearman’s ρ=0.730, P<0.001) and IgA (Spearman’s ρ=0.762, P<0.001), and in increased sensitivity of the EIA, yielding adequate intertest agreements when high titres were used to define *Chlamydia pneumoniae* seropositivity (Tables 4.4 and 4.5).

### Table 4.5
Comparing EIA to MIF test (gold standard). Intertest agreement (kappa), sensitivity, specificity, positive and negative predictive value of EIA for the detection of *Chlamydia pneumoniae* IgA seropositivity at various cut-off points (n=141). Numbers in parentheses represent values calculated after retesting sera with IgA≥16 EIU at higher pre-dilution.

<table>
<thead>
<tr>
<th>% seropositive</th>
<th>MIF</th>
<th>EIA</th>
<th>Cut-off for IgA-seropositivity at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/8</td>
<td>1/16</td>
<td>1/32</td>
</tr>
<tr>
<td>Kappa</td>
<td>0.71</td>
<td>0.62</td>
<td>0.51 (0.56)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>93</td>
<td>78</td>
<td>60 (67)</td>
</tr>
<tr>
<td>Specificity</td>
<td>79</td>
<td>87</td>
<td>89 (88)</td>
</tr>
<tr>
<td>PPV</td>
<td>93</td>
<td>81</td>
<td>79 (79)</td>
</tr>
<tr>
<td>NPV</td>
<td>76</td>
<td>71</td>
<td>77 (80)</td>
</tr>
</tbody>
</table>

PPV: positive predictive value; NPV: negative predictive value.

### 4.5 Discussion
Since Saikku and his co-workers proposed an association between *Chlamydia pneumoniae* infection and coronary artery disease, the serological detection of *Chlamydia pneumoniae* infection has been of interest to a number of cardiovascular investigators. Various serological assays and different serological criteria for persistent or chronic active *Chlamydia pneumoniae* infection have been used so far in sero-epidemiological studies. This might partly explain the conflicting results of some of these reports. The development of a simple and technically accessible, objective test for the detection of *Chlamydia pneumoniae* antibodies is therefore mandatory. The MIF test is commonly used to measure IgG-, IgA-, and IgM-antibodies to *Chlamydia pneumoniae*. It is considered both a sensitive and specific technique for the detection of *Chlamydia pneumoniae* infection and is nowadays regarded the gold standard for the detection of *Chlamydia pneumoniae* antibodies. However, the MIF test is labour intensive, requires an experienced reader and is hampered by the subjectivity of the outcome measure. In the present study, the MIF test was compared to the EIA, a technically less demanding alternative to the MIF, characterised by high
throughput and an objective outcome measure. According to our results, in patients with cardiovascular disease the EIA test showed sufficient agreement with the MIF in the detection of *Chlamydia pneumoniae* antibodies, keeping in mind that the sensitivity of the EIA depends on the titre level. The seropositivity rate detected by the EIA was comparable to that obtained by the MIF when low cut-off levels for IgG- and IgA-seropositivity were used, but was lower compared to the MIF when high cut-off levels for IgG- and IgA-seropositivity were applied. In accordance, Numazaki et al reported an excellent correlation between MIF and ELISA in Japanese children although high *Chlamydia pneumoniae* antibody levels could only be detected with the MIF test. We were able to prevent this loss of sensitivity by retesting sera with elevated IgG- and IgA-titres at higher pre-dilutions. By this manoeuvre results could be obtained in the linear portion of the calibration curve, which resulted in increased agreement and sensitivity of EIA compared to the MIF at the high titre range.

Schumacher et al compared the Labsystems' MIF with the Labsystems' EIA and with Medac’s rELISA (Hamburg, Germany) using sera of 197 patients with coronary heart disease and 197 age- and sex-matched controls. The rELISA, which detects antibodies against chlamydial LPS, did not correlate with MIF and EIA, both detecting mainly the surface exposed proteins of chlamydial elementary bodies. Although the authors concluded that the results of the EIA were significantly different from those of the MIF test, the reported agreement coefficient between the protein-immunoreactive MIF and EIA for detection of IgG- and IgA-seropositivity (cut-offs IgG≥1/64 and IgA≥1/32) was $\kappa=0.628$ and $\kappa=0.426$, respectively, which is satisfactory. Likewise, in a randomly selected group of 20 to 29 year old blood donors, the seroprevalence of IgA (IgA≥1/8) was comparable when analysed by Labsystem's MIF and EIA. Although the agreement between EIA and MIF was satisfactory, the two tests did not yield the same outcome for all sera. Specimens that had been classified as seropositive by one test were seronegative by the other. Ideally the two serological assays should define the same sera as positive or negative according to the actual infectious status of the patient. Therefore, sera of true positive and true negative patients should have been included in the analysis. In this study we failed to comply with this requirement, as in case of patients with atherosclerotic disease, it is not known how to determine the (chronic) infectious status on the basis of serology. No correlation has been found between identification of *Chlamydia pneumoniae* in atherosclerotic tissue and *Chlamydia pneumoniae* antibodies. Furthermore, no consensus has as yet been reached regarding the serological criteria for chronic or persistent *Chlamydia pneumoniae* infection. Various cut-off points for IgG- and IgA- titres or even IgG and IgA titre-combinations have been proposed. It remains, however, unclear whether the presence or titre of *Chlamydia pneumoniae* IgG-
or IgA-antibody by MIF reflects persistent, chronic active infection or reinfection or may simply represent the immunological scar of past infection. For this reason, we limited our study to the evaluation of the agreement between the two serological assays using several antibody levels as cut-off for seropositivity.

Recent evidence suggests that the interlaboratory variation of the MIF is problematic. Taking into account that only minor antigenic differences have been found among the _Chlamydia pneumoniae_ strains used in the various (mostly in-house developed) MIF tests, this suggests that variation in reading of titres is probably the important factor for the variability in the MIF test results. In line herewith, the interobserver agreement of the MIF test in our study was good (Table 4.2) although the correlation between the independent readings was sub-optimal. In contrast, the intratest agreement of the EIA (Table 4.3) presented in this paper was excellent, which might reflect the advantages of the objective outcome measures.

The MIF is a species-specific assay for the detection of antibodies directed against surface expressed proteins of _Chlamydia pneumoniae_. The EIA has also been designed to detect _Chlamydia pneumoniae_ surface expressed protein reactive antibodies, but is frustrated by cross-reacting antibodies in populations with a high background level of _Chlamydia trachomatis_ antibodies. Nevertheless, in older populations with low incidence of _Chlamydia trachomatis_ infection, for example individuals from western countries with cardiovascular disease such as the patients analyzed in the present study, no significant cross-reactivity is observed.

In conclusion, our data suggest that, when measures are taken to obtain results in the linear portion of the detection curve, the EIA yields comparable results to the MIF. Considering the practical advantages of EIA testing and the fact that cardiovascular investigators with limited microbiological background and experience are involved in _Chlamydia pneumoniae_-related cardiovascular research, the use of EIA to detect _Chlamydia pneumoniae_ antibodies might increase the external validity of future sero-epidemiological studies.
4.6 References

Chapter 5

*Chlamydia pneumoniae* in atherosclerotic disease: evaluating the association between serum antibodies and presence of antigens or DNA in vascular tissue

Vainas T, Stassen FR, Kurvers HA, Grauls GE, Bruggeman CA, Kitslaar PJ

*Submitted*
5.1 Abstract

Background
Despite the large number of studies assessing the role of *Chlamydia pneumonia* in atherosclerotic disease, no consensus about the serological detection of clinically relevant (vascular) *Chlamydia pneumoniae* infection has been reached yet. The aim of this study was to identify serological criteria for the detection of *Chlamydia pneumoniae* in the vascular wall, and to study the relationship between signs of *Chlamydia pneumoniae* infection (i.e., serum antibodies or presence of *Chlamydia pneumoniae* particles in the vascular wall) and atherosclerotic plaque morphology.

Patients & methods
*Chlamydia pneumoniae* IgG- and IgA-antibodies were measured in the serum of 127 patients undergoing reconstruction surgery for severe atherosclerotic disease. *Chlamydia pneumoniae* DNA or antigens were detected in vascular tissue by PCR or immunohistochemistry, respectively. Receiver Operating Characteristic (ROC) curves were computed to study the association between *Chlamydia pneumoniae* serology and vascular presence of *Chlamydia pneumoniae*. Furthermore, the relation between plaque morphology and signs of *Chlamydia pneumoniae* infection (antibodies or vascular *Chlamydia pneumoniae* presence) was analysed.

Results
*Chlamydia pneumoniae* serum antibodies were frequently found. *Chlamydia pneumoniae* antigens were detected in approximately 80% of plaques although *Chlamydia pneumoniae* DNA was seen in only 9% of them. No association between serology and vascular presence of *Chlamydia pneumoniae* was found. The sensitivity and specificity of serology for detection of vascular *Chlamydia pneumoniae* was poor. No association could be found between signs of infection (serological or histological) and plaque morphology.

Conclusion
Although antibodies are frequently found in patients with cardiovascular disease, and *Chlamydia pneumoniae* particles can be detected in a significant number of plaques, only a poor association between *Chlamydia pneumoniae* serology and its vascular presence was found, rendering *Chlamydia pneumoniae* serology not suitable for the detection of patients with relevant vascular infection. Additionally, plaque morphology is not related to *Chlamydia pneumoniae* serology or vascular presence.
5.2 Introduction

In recent years it has been shown that *Chlamydia pneumoniae* seropositivity is associated with acute and chronic atherosclerotic complications.\(^1\) Moreover, it has become evident that atherosclerotic lesions harbour chlamydial DNA and antigens.\(^1,2\) Hence, it is believed that vascular *Chlamydia pneumoniae* infections, especially chronic active or persistent rather than acute ones, are associated with the development of atherosclerosis.\(^3\) However, the serological criteria for chronic active or persistent vascular *Chlamydia pneumoniae* infections have not yet been established conclusively.\(^4\) Therefore, in the present study we assessed the presence of either chlamydial antigens (i.e., major outer membrane proteins (MOMP) and heat shock protein 60 (HSP60)) by immunohistochemistry (IHC), or chlamydial DNA by polymerase chain reaction (PCR) in vascular tissue of patients undergoing reconstructive surgery of the carotid artery, aorta or lower limb arteries for severe atherosclerotic disease. In the same patients the *Chlamydia pneumoniae* antibodies were measured to reveal a possible association between the prevalence of either chlamydial antigens or DNA in vascular tissue and *Chlamydia pneumoniae* antibody titres, thereby determining the serological criteria that would most securely identify patients potentially harbouring a vascular *Chlamydia pneumoniae* infection. *Chlamydia pneumoniae* infection has been linked to atherosclerotic disease mainly on the basis of sero-epidemiological studies, showing on numerous occasions an association between elevated levels of *Chlamydia pneumoniae* antibodies and cardiovascular disease.\(^1\) However, it is not clear whether the presence of *Chlamydia pneumoniae* in vascular tissue is associated with atherosclerotic plaque morphology, or even with clinical manifestation of atherosclerotic disease. For this reason, we also studied the relation between plaque morphology, assessed according to the American Heart Association classification criteria, and (i) presence of *Chlamydia pneumoniae* antigens in the vascular tissue or (ii) *Chlamydia pneumoniae* serology.

5.3 Materials and methods

Vascular tissue and preoperatively drawn blood of 127 patients undergoing vascular reconstructions of the aorta, carotid, or lower limb arteries were collected. The study subjects represented a random subpopulation of vascular surgical patients treated at the vascular surgical clinic of Maastricht University Hospital. All patients undergoing vascular reconstruction surgery for atherosclerotic disease, whose serum and atherosclerotic tissue was available
for analysis, qualified for entry to the study, provided that they gave written informed consent.

Vascular tissue of the first 70 patients was fixed in formalin and embedded in paraffin for immunohistochemical detection of *Chlamydia pneumoniae* antigens. Arterial samples of the following 57 patients were snap frozen in liquid nitrogen for detection of *Chlamydia pneumoniae* DNA by PCR. *Chlamydia pneumoniae* IgA and IgG antibody titres were determined in all 127 patients. The study was approved by the local medical ethical committee.

### 5.3.1 *Chlamydia pneumoniae* antibody detection

Preoperatively collected blood was centrifuged at 1200 rpm for 10 minutes to obtain serum that was stored at -20°C until analysis. *Chlamydia pneumoniae* IgG- and IgA-antibodies were measured by means of a *Chlamydia pneumoniae* specific enzyme immunoassay (Labsystems, Helsinki, Finland) according to the manufacturer's instructions and as described earlier. In short, serum diluted at 1/100 was incubated with *Chlamydia pneumoniae* antigens coated onto a 96-wells plate. Anti-immunoglobulin (IgG or IgA) conjugated with horseradish peroxidase was added to the wells after washing, and incubated for 60 min at room temperature. Subsequently the chromogen containing tetramethylbenzidine was added to the wells after washing. The reaction was stopped with sulphuric acid after 30 min and optical density was immediately read at 450nm. Antibody titres were calculated from optical density readings according to manufacturer's instructions. Results are given in mean Enzyme ImmunoUnits (EIU) ± Standard deviation (SD). According to the manufacturer, the enzyme immunoassay is titrated in such a way that the EIU-value calculated from the optical density readings corresponded to the reciprocal value of the titre determined by the micro-immunofluorescence of the same manufacturer (Table 5.1). Although this method may not be the "gold standard" we previously demonstrated that the EIA yields comparable results to the MIF in patients with cardiovascular disease.

<table>
<thead>
<tr>
<th>Table 5.1</th>
<th>Transforming <em>Chlamydia pneumoniae</em> antibody titre calculated with the EIA (in EIUs) into MIF-titre categories.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIA titre (EIU)</td>
<td>Corresponding MIF-titre</td>
</tr>
<tr>
<td>IgG</td>
<td>IgA &lt; 8</td>
</tr>
<tr>
<td>32 ≤ IgG &lt; 64</td>
<td>1 / 32</td>
</tr>
<tr>
<td>64 ≤ IgG &lt; 128</td>
<td>1 / 64</td>
</tr>
<tr>
<td>128 ≤ IgG &lt; 256</td>
<td>1 / 128</td>
</tr>
<tr>
<td>256 ≤ IgG &lt; 512</td>
<td>1 / 256</td>
</tr>
<tr>
<td>IgG ≥ 512</td>
<td>1 / 512</td>
</tr>
</tbody>
</table>
5.3.2 Vascular tissue and plaque morphology

Atherosclerotic plaques obtained during operation of the first 70 patients were fixed in formal and embedded in paraffin for determination of plaque morphology and for immunohistochemical detection of Chlamydia pneumoniae proteins. Vascular tissue from the following 57 patients was snap frozen in liquid nitrogen for detection of Chlamydia pneumoniae DNA. Plaque morphology was assessed in one hematoxylin-eosine stained section per patient, according to the Stary classification system of atherosclerotic tissue.7,8 A plaque was characterized unstable in the presence of intra-plaque hemorrhage, fibrous cap rupture or presence of organized luminal thrombus.

5.3.3 Immunohistochemistry

For this purpose, deparaffined serial sections were incubated with two monoclonal Chlamydia pneumoniae-antibodies, i.e., (i) an antibody directed against Chlamydia pneumoniae MOMP (RR-402, DakoCytomation, Glostrup, Denmark) and (ii) an monoclonal antibody directed against Chlamydial HSP60 (ABR-Affinity BioReagents Inc, Golden, Colorado, USA). Normal mouse ascitic fluid was used as negative antibody control. Subsequently, incubation with a rabbit anti mouse antibody (DakoCytomation) and streptavidine biotine complex (DakoCytomation) was performed in a multi-step immunostaining procedure. The coloric reaction was created with diaminobenzidine. Chlamydia pneumoniae-infected and non infected human epithelial cells were used as positive and negative tissue controls in each run. According to the recommendations of a consensus committee, a cell was classified as Chlamydia pneumoniae-positive if it showed smooth or granular cytoplasmic staining whereas the same cell on the subsequent section incubated with mouse ascitic fluid stained negative.4

5.3.4 Chlamydia pneumoniae PCR

Chlamydia pneumoniae DNA fragments were detected by means of a ‘touchdown’ PCR.9 DNA was extracted from atheroma by conventional proteinase K digestion (Qiagen Genomics Inc, Bothell, Washington, USA). The primer pair used amplified a 207 bp species-specific target sequence from the major outer membrane protein gene of Chlamydia pneumoniae.10 DNA extraction, PCR amplification and electrophoresis were performed in separate rooms in order to minimize the risk of contamination.
5.3.5 Statistical analysis

Receiver-operating characteristic (ROC) curves were computed in order to evaluate the ability of IgG/IgA antibody titres to detect presence of *Chlamydia pneumoniae* antigens or DNA in vascular tissue. Mean *Chlamydia pneumoniae* antibody titres between patients with stable vs. unstable plaques were compared with the Mann-Whitney *U* test. The association between the presence of *Chlamydia pneumoniae* in the plaque and plaque morphology was analysed with the $\chi^2$-test. Pearson's *r* was computed to study the correlation between MOMP and HSP60 immunoreactivity. All analyses were done on a SPSS 10.0 software package for windows (SPSS Inc., Chicago, Illinois).

5.4 Results

5.4.1 Patients

Data of 127 patients operated in a single teaching hospital were analysed. Entry criteria were the existence of atherosclerotic occlusive or aneurysmal disease and the availability of, both, serum and vascular tissue for analysis. Patient characteristics are given in Table 5.2.

<table>
<thead>
<tr>
<th>Table 5.2 Patient characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>----------------------------------</td>
</tr>
<tr>
<td>N 127</td>
</tr>
<tr>
<td>Mean (SD) age in years            66.9 (8.5)</td>
</tr>
<tr>
<td>Male/female                       100/27</td>
</tr>
<tr>
<td>Diabetes Mellitus                 24 (19)</td>
</tr>
<tr>
<td>Dyslipidemia                      89 (70)</td>
</tr>
<tr>
<td>Hypertension                      89 (70)</td>
</tr>
<tr>
<td>Smoking                           88 (69)</td>
</tr>
<tr>
<td>Carotid surgery                   40 (31)</td>
</tr>
<tr>
<td>Aortic surgery                    24 (19)</td>
</tr>
<tr>
<td>Femorodistal surgery              63 (50)</td>
</tr>
</tbody>
</table>

Values represent number (percentage) of patients.

$^a$ Fasting glucose level $>$7 mmol/l or the use of antihyperglycemic medication or insulin; $^b$ Fasting cholesterol level $>$6.5 mmol/l and/or triglyceride level $>$1.55 mmol/l and/or use of antihyperlipidemic medication; $^c$ Systolic blood pressure $>$160 mmHg and/or diastolic blood pressure $>$90 mmHg and/or use of antihypertensive medication; $^d$ Currently smoking or stopped less than 10 years ago.

5.4.2 *Chlamydia pneumoniae* serology

Table 5.3 gives the mean (SD) IgG- and IgA-titre in EIUAs as well as the percentage IgG- and IgA-seropositive patients using various cut-off levels for seropositivity. Evidently, *Chlamydia pneumoniae* IgG- and IgA-antibodies are
frequently found in patients with atherosclerotic disease of the carotid, aortic or femorodistal arterial segment.

Table 5.3  Mean *Chlamydia pneumoniae* IgA- and IgG antibody titres (in EIU) and *Chlamydia pneumoniae* IgA- and IgG-seropositivity at various cut-off levels.

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Cut-off</th>
<th>Seropositive*</th>
<th>Mean (SD) in EIU</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td></td>
<td></td>
<td>26 (22)</td>
</tr>
<tr>
<td></td>
<td>IgA ≥ 5</td>
<td>97 (75)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgA ≥ 16</td>
<td>69 (54)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgA ≥ 32</td>
<td>40 (31)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgA ≥ 64</td>
<td>13 (10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgA ≥ 128</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td></td>
<td>108 (34)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG ≥ 16</td>
<td>116 (91)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG ≥ 32</td>
<td>104 (82)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG ≥ 64</td>
<td>78 (61)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG ≥ 128</td>
<td>44 (35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG ≥ 256</td>
<td>5 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG ≥ 512</td>
<td>2 (2)</td>
<td></td>
</tr>
</tbody>
</table>

EIU: Enzyme Immuno-unit  
* values represent number (percentage) of seropositive patients at various cut-off levels

5.4.3 Evidence of vascular *Chlamydia pneumoniae* infection

The presence of *Chlamydia pneumoniae* antigens in atheromatous tissue of 70 patients was assessed by means of immunohistochemical detection using two monoclonal antibodies, directed against (i) MOMP and (ii) HSP60. Thirty-one plaques (44%) stained positive for both antigens, 9 plaques (13%) stained positive for MOMP only, and 11 plaques (16%) were *Chlamydia pneumoniae*-HSP60 positive only. The 2 stainings correlated significantly with each other (r=0.412, p<0.0001).

The presence of *Chlamydia pneumoniae* DNA was assessed in 57 patients. Of these 57 plaques, 5 (9%) were *Chlamydia pneumoniae* DNA-positive.

5.4.4 Detecting vascular *Chlamydia pneumoniae* infection by means of *Chlamydia pneumoniae* serology

We further examined the correlation between *Chlamydia pneumoniae* serology and vascular presence of *Chlamydia pneumoniae*-antigens or *Chlamydia pneumoniae* DNA. Figure 5.1 shows the receiver operating characteristics of *Chlamydia pneumoniae* IgG- and IgA-titres for detection of vascular *Chlamydia pneumoniae* antigens (MOMP+ and/or HSP60+) or *Chlamydia pneumoniae* DNA. As shown, the sensitivity and specificity of *Chlamydia pneumoniae* serology for the detection of vascular *Chlamydia pneumoniae* infection was
poor, as the area under the curve did not exceed 0.565 in any case. Therefore, regardless of the cut-off point for positive serology, no significant association existed between *Chlamydia pneumoniae* seropositivity and vascular presence of either *Chlamydia pneumoniae* antigens or DNA, indicating that the presence of serum *Chlamydia pneumoniae* Ig's is in neither way indicative for a chronic or persistent vascular *Chlamydia pneumoniae* infection.

![Graphs](image)

**Figure 5.1** Sensitivity and specificity of *Chlamydia pneumoniae* serology for detection of vascular *Chlamydia pneumoniae* antigens/DNA. ROC curves demonstrating the sensitivity and specificity of *Chlamydia pneumoniae* IgG- and IgA-antibodies for the detection of vascular presence of (A) *Chlamydia pneumoniae* MOMP, (B) *Chlamydia pneumoniae* HSP60, (C) *Chlamydia pneumoniae* antigens (MOMP and/or HSP60), (D) *Chlamydia pneumoniae* DNA. AUC = Area Under Curve.

5.4.5 Plaque (in)stability and *Chlamydia pneumoniae* infection

Plaque instability defined as the presence of organised thrombus, fibrous cap rupture and/or intraplaque haemorrhage was present in 37/70 (53%) plaques. Plaque instability was not related to presence of *Chlamydia pneumoniae*
antigens (MOMP and/or HSP60) in vascular tissue ($P=\text{ns}$, $\chi^2$-test). Neither was the IgG- and IgA-titre (in EIU) significantly different between patients with stable versus patients with unstable plaques ($P=\text{ns}$, Mann-Whitney U-test).

### 5.5 Discussion

Ever since Saikku et al.\textsuperscript{11} showed that coronary artery disease is associated with elevated \textit{Chlamydia pneumoniae} antibody titres, numerous studies have explored the association between \textit{Chlamydia pneumoniae} infection and the development of atherosclerosis determining \textit{Chlamydia pneumoniae} antibodies in patients with coronary, carotid, aortic and peripheral arterial disease.\textsuperscript{1} Despite a mixture of studies confirming this original observation, the significance of these data has been questioned, as the pooled risk estimate for the relation between \textit{Chlamydia pneumoniae} and atherosclerosis did not support the suggested association between infection and atherosclerotic disease.\textsuperscript{12-14} Nevertheless, a recent meta-analysis including studies published between January 1997 and December 2000 showed that seropositivity is related to atherosclerosis with a pooled odds ratio of 1.6 (95% confidence interval: 1.3–2.0). The authors suggested that study design (cross sectional vs. nested case control studies) as well as duration of follow-up in the prospective studies was a source of variability.\textsuperscript{15}

Many pro-atherogenic effects of \textit{Chlamydia pneumoniae} on various components of the vascular wall have been reported.\textsuperscript{16} This implies that the presence of the pathogen in the vessel wall might be a prerequisite for exerting atherogenic effects and therefore depends on a chronic active or persistent \textit{Chlamydia pneumoniae} infection of the artery. Indeed, both \textit{Chlamydia pneumoniae} antigens or DNA have been demonstrated in vascular specimens, obtained from patients undergoing vascular reconstruction.\textsuperscript{17} Also, in their extensive meta-analysis Gutierrez and colleagues\textsuperscript{14} demonstrated the strongest association between \textit{Chlamydia pneumoniae} and atherosclerosis in immunohistochemical (OR=15.4) and (nested) PCR studies of arterial and non-arterial material (OR=4.3 and 16.7, respectively), suggesting that it would be most useful to analyse the presence of the pathogen in arterial as well as non-vascular biopsies to identify patients with \textit{Chlamydia pneumoniae} as potential risk factor. Due to ethical and practical constrains it might virtually be impossible to obtain vascular tissue for diagnostic purposes. In contrast, serum samples may readily be available for determination of \textit{Chlamydia pneumoniae} antibody titres. Assuming that the presence of \textit{Chlamydia pneumoniae} in the vascular wall is indicative for future cardiovascular complications, it might be of diagnostic use if serum antibody titres would closely correlate to the presence of \textit{Chlamydia pneumoniae} in vascular tissue. In the present study we explored the association between \textit{Chlamydia pneumoniae} antibodies and the presence
of *Chlamydia pneumoniae* antigens or DNA in the vessel wall attempting to identify an appropriate serological marker of potential vascular *Chlamydia pneumoniae* infection. Our results indicate that *Chlamydia pneumoniae* IgG- and IgA-antibodies are poorly associated with the presence of *Chlamydia pneumoniae* antigens or DNA in atherosclerotic vessel wall, detected by immunohistochemistry (IHC) or by means of PCR, suggesting that measurement of serum antibody titres is inadequate to detect patients with vascular *Chlamydia pneumoniae* infections. Despite the fact that Blasi et al. had shown that *Chlamydia pneumoniae* seropositivity (defined as 18≤IgG<512 and/or 32≤IgA<256) was significantly higher in subject with *Chlamydia pneumoniae* PCR-positive plaques than in subjects with PCR-negative plaques, several investigators found, in agreement with our data, no association between *Chlamydia pneumoniae* serology and detection of *Chlamydia pneumoniae* in vascular tissue by PCR and/or culture. However, in these studies, the relation between *Chlamydia pneumoniae* serology and presence of *Chlamydia pneumoniae* in vascular tissue, was analysed using a single cut-off point for seropositivity (IgG≥1/8 or IgG≥1/16), despite the fact that no uniform criteria for the serological detection of chronic active or persistent *Chlamydia pneumoniae* infection have been formulated yet. Measuring *Chlamydia pneumoniae* titres as a continuous variable with an EIA and applying ROC analysis, we essentially tested for an association between seropositivity and vascular presence of *Chlamydia pneumoniae* using virtually every single titre value as cut-off point for seropositivity bypassing the limitation of previous studies exploring the association between vascular *Chlamydia pneumoniae* and seropositivity defined by a single (not standardised) cut-off point. Nevertheless, even this more extensive approach did not reveal any correlation between *Chlamydia pneumoniae* serology and the presence of *Chlamydia pneumoniae* in vascular tissue. Both IHC and PCR techniques have been applied to demonstrate the presence of *Chlamydia pneumoniae* in vascular tissue and both *Chlamydia pneumoniae* antigens as well as *Chlamydia pneumoniae* DNA could be demonstrated. However, results were not always consistent and discrepancies have previously been reported. A similar discordance was found in the present study. While almost 50% of all samples examined stained positive for both antigens (MOMP and HSP60), *Chlamydia pneumoniae* DNA could only be detected in 9% of all samples examined. Meijer et al. suggested that this might be related to differences in kinetics of degradation of the various *Chlamydia pneumoniae* components, with rapid degradation of *Chlamydia pneumoniae* DNA and HSP60 and persistence of the membrane proteins. However, this is not confirmed by the present data as the number of MOMP or HSP60 positive specimens was not different. Alternatively, Hoynans and colleagues recently
demonstrated that the high percentage of immunohistochemical (IHC) positive samples might be due to cross-reaction of the antibodies used with non-Chlamydia plaque constituents like autofluorescent ceroid deposits. This may explain the repeatedly demonstrated discrepancies between PCR data and IHC assays. Unfortunately, due to logistic reasons we were not able to cross-test specimens by both methods. The disagreement between IHC and PCR may be related to the fact that the overall performance of IHC and PCR to detect vascular *Chlamydia pneumoniae* infection is poor. The sensitivity and specificity of these assays is far from optimal keeping in mind the reported differences in *Chlamydia pneumoniae* detection rate, varying between 0% and 100%.\textsuperscript{24,25} It may also represent a sampling error as in most cases only a limited amount of vascular tissue has been assayed. Cochrane *et al.* showed that the detection rate of *Chlamydia pneumoniae* DNA in carotid atheroma increased from 39% to 100% when 1, 2, 5, 10, 15 and 20 sections per carotid atheroma were analysed.\textsuperscript{26} Assuming that the presence of *Chlamydia pneumoniae* in vascular tissue is indeed 100% if tested thoroughly, the sensitivity and specificity of *Chlamydia pneumoniae* serology for the detection of presence of vascular *Chlamydia pneumoniae* would still remain very poor (data not shown). Hence, detection of *Chlamydia pneumoniae* antigens or DNA in vascular tissue is hampered by methodological pitfalls and is probably by the currently available techniques not pathognomonic for the presence of *Chlamydia pneumoniae* in vascular tissue.

Earlier sero-epidemiological studies suggested that *Chlamydia pneumoniae* infections have pro-atherogenic properties, since elevated *Chlamydia pneumoniae* antibodies are associated with the development of acute and chronic atherosclerosis-related cardiovascular events.\textsuperscript{1} Animal studies support the notion that *Chlamydia pneumoniae* infections stimulate atherogenesis\textsuperscript{27,28} and *in vitro* evidence suggests that this might be due to locally induced pro-atherogenic changes in the vessel wall. Yet, the data presented here and elsewhere\textsuperscript{29,30} failed to show any association between vascular presence of *Chlamydia pneumoniae* antigens or DNA and plaque morphology. This suggests that in humans, vascular *Chlamydia pneumoniae* infection may not stimulate advanced atherosclerotic lesion progression or transformation of an advanced stable plaque into an unstable one through *in situ* processes. In contrast, the association between *Chlamydia pneumoniae* and atherosclerosis might be mediated by the systemic effects of (non)vascular *Chlamydia pneumoniae* infection and/or by the host response to *Chlamydia pneumoniae* infection. We have shown in this respect that *Chlamydia pneumoniae* infection is associated with hypercoagulability but not with plaque morphology in patients with carotid artery disease.\textsuperscript{31} In addition, plasma fibrinogen levels are elevated in patients with *Chlamydia pneumoniae* infections\textsuperscript{32} and decrease upon antimicrobial treatment.\textsuperscript{33} *Chlamydia pneumoniae* infection might also contribute to
the development of atherosclerosis through stimulation of cytokine production and through alteration of lipid metabolism. Furthermore, it has been shown that antibodies elicited against chlamydial HSP60 cross-react with human autologous HSP60 and can cause lysis of stressed endothelial cells expressing on their surface human HSP60. In other words, although in vitro studies suggest that infection of vascular cells with *Chlamydia pneumoniae* is associated with atherosclerosis progression, the lack of an association between the presence of *Chlamydia pneumoniae* antigens and DNA in vessel wall and atherosclerotic plaque morphology, suggests that in humans systemic rather than local effects of *Chlamydia pneumoniae* infection such as stimulation of hypercoagulability and cytokine production or induction of an auto-immune response might have clinically significant pro-atherogenic properties.

In conclusion, the present data suggest that *Chlamydia pneumoniae* serology is not suitable to detect vascular presence of *Chlamydia pneumoniae* antigens or DNA. Moreover, *Chlamydia pneumoniae* infection does not seem to be associated with plaque morphology. The lack of association between *Chlamydia pneumoniae* infection (determined by serology or the detection of antigens/DNA in vascular tissue) and plaque morphology, suggests in the light of the suggested association between *Chlamydia pneumoniae* serology and atherosclerotic events, that in humans, *Chlamydia pneumoniae* infection might stimulate atherogenesis not via direct effects on components of the vascular wall but more likely through the induction of systemic atherothrombotic phenomena.
5.6 References


Chapter 6

*Chlamydia pneumoniae* serology is associated with thrombosis-related but not with plaque-related microembolization during carotid endarterectomy


*Stroke* 2002;33:1249-54
6.1 Abstract

Background
Chlamydia pneumoniae has repeatedly been associated with atherosclerotic disease. Our study was designed to clarify whether this association is based on Chlamydia pneumoniae-induced transformation of a stable into an unstable atherosclerotic plaque or on stimulation of hypercoagulability leading to increased thrombotic arterial occlusions by Chlamydia pneumoniae infection. Transcranial Doppler ultrasonographic monitoring of the middle cerebral artery during carotid endarterectomy offers the opportunity to study, before removal of the plaque, atherothrombotic emboli dislodging from an unstable carotid plaque (plaque-related emboli) and emboli related to (excessive) thrombus formation at the endarterectomy site after removal of the plaque and restoration of flow (thrombosis-related emboli).

Methods
Chlamydia pneumoniae IgA- (≥1/16) and IgG (≥1/64) seropositivity was assessed in 53 patients with symptomatic carotid artery disease undergoing carotid endarterectomy. The removed carotid plaques were studied histologically to assess plaque instability.

Results
Plaque- and thrombosis-related emboli were registered in 43 patients with an adequate transcranial window. IgA seropositivity (58%) was associated significantly with thrombosis-related embolization (P=0.03), but not with plaque-related embolization or with histological plaque instability.

Conclusion
Chlamydia pneumoniae serology is associated with micro-embolization after endarterectomy and restoration of flow. Since these micro-emboli represent platelet aggregations and are related to cerebrovascular complications, our data suggest that Chlamydia pneumoniae infection contributes to cerebrovascular events in patients with carotid artery disease through stimulation of thrombosis.
6.2 Introduction

An increasing body of evidence suggests that infections play a role in the initiation and progression of atherosclerotic disease. Particularly, chronic infections with the gram-negative, intracellular bacterium *Chlamydia pneumoniae* have been linked to the development of vascular disease. *Chlamydia pneumoniae* seropositivity has been associated with acute and chronic coronary artery disease, early and advanced asymptomatic carotid lesions, and stroke. In addition, it has been shown that *Chlamydia pneumoniae* can infect all cellular components of the vascular wall inducing proatherogenic changes such as foam cell formation, endothelial expression of adhesion molecules and chemokines, stimulation of transendothelial leucocyte migration, smooth muscle cell proliferation, endothelial production of tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1), and macrophage production of matrix metalloproteinase 9 (MMP-9). These observations suggest that *Chlamydia pneumoniae* infections could contribute to the development of atherosclerosis, leading to atherosclerotic plaque growth and increased arterial stenosis, and that *Chlamydia pneumoniae* infection may also play a role in the formation of an unstable atherosclerotic plaque, leading to acute cardiovascular and/or cerebrovascular events. Plaque rupture and thrombosis are the main mechanisms of acute arterial occlusion leading to an atherosclerotic event such as myocardial infarction or stroke. Although *Chlamydia pneumoniae* infection could theoretically contribute to both processes, it is unclear whether the earlier reported association between *Chlamydia pneumoniae* serology and acute cardiovascular and cerebrovascular events is based on stimulation of plaque- and/or thrombosis related mechanisms by *Chlamydia pneumoniae* infection.

Cerebrovascular events related to carotid artery disease are caused in the majority of cases by atherothrombotic emboli dislodging from the carotid plaque. Fibrous cap rupture and luminal thrombosis are the histological manifestations of carotid plaque instability and are the main source of cerebral micro-embolic signals (MES), detected by transcranial Doppler ultrasonographic (TCD) monitoring of the ipsilateral middle cerebral artery, in patients with high-grade carotid artery stenosis. MES not only correlate well with histological determinants of plaque instability but are also associated with clinical manifestations of plaque instability such as strokes and transient ischemic attacks (TIAs). Thrombus formation at the endarterectomy and clamping sites after carotid endarterectomy (CEA) resulting in either thrombotic occlusion of the carotid artery or downstream embolization of intracranial arteries is the main cause of postoperative stroke and TIA. Hence, MES occurring during the dissection phase of CEA are associated with carotid plaque instability, whereas MES observed after endarterectomy and
restoration of flow, as well as in the early postoperative period, are related to excessive platelet aggregation and thrombus formation at the endarterectomy and clamping sites.\textsuperscript{26} TCD monitoring during CEA therefore offers the unique opportunity to study, \textit{in vivo} plaque instability and thrombosis separately by distinguishing between plaque-related MES (during dissection, pMES) and thrombosis-related MES (after endarterectomy and restoration of flow, tMES).

In order to investigate the relation between \textit{Chlamydia pneumoniae} infection and atherothrombotic disease, we studied the association between \textit{Chlamydia pneumoniae} serology and carotid plaque histology as well as perioperative micro-embolization in patients undergoing CEA for symptomatic carotid artery disease, with special attention to both pMES and tMES.

### 6.3 Materials and methods

Sixty patients with symptomatic carotid artery disease screened consecutively at the surgical outpatient department of the Maastricht University Hospital who had been found eligible for carotid endarterectomy were asked to participate in this study. Patients who failed to provide informed consent (n=4) or underwent a combined carotid endarterectomy and coronary revascularization procedure (n=3) were excluded. Amaurosis fugax was the presenting symptom of 15 patients; 24 had T\textsc{I}As, 11 had suffered a stroke, and 3 patients presented with general cerebral hypoperfusion without focal neurological symptoms. All patients had a significant (>70%), symptomatic, carotid artery stenosis.\textsuperscript{28}

Patient characteristics are given in Table 6.1. The study was approved by the Medical Ethical Committee of Maastricht University Hospital.

<table>
<thead>
<tr>
<th>Table 6.1. Patient characteristics.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
</tr>
<tr>
<td>Age, mean (SD), y</td>
</tr>
<tr>
<td>Female/male</td>
</tr>
<tr>
<td>Current smoker</td>
</tr>
<tr>
<td>Dyslipidemia\textsuperscript{b}</td>
</tr>
<tr>
<td>Hypertension\textsuperscript{h}</td>
</tr>
<tr>
<td>Diabetes\textsuperscript{e}</td>
</tr>
<tr>
<td>TCD monitoring</td>
</tr>
<tr>
<td>Intraoperative Shunting</td>
</tr>
<tr>
<td>Patch closure of arteriotomy</td>
</tr>
<tr>
<td>Preoperative anticoagulation</td>
</tr>
<tr>
<td>Discontinuation of preoperative anticoagulation\textsuperscript{f}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Fasting cholesterol level >6.5 mmol/l and/or triglyceride level >1.95 mmol/l and/or use of antilipemic medication; \textsuperscript{b} Systolic blood pressure >160 mmHg and/or diastolic blood pressure >90 mmHg and/or use of antihypertensive medication; \textsuperscript{c} Fasting glucose level >7 mmol/l or the use of antihyperglycemic medication or insulin; \textsuperscript{d} discontinuation of anticoagulation treatment 3 to 10 days before surgery.
6.3.1 Carotid endarterectomy (CEA)

CEA was performed with the patient under normocarbic, normotensive general anesthesia using systemic heparinization (1mg heparine/kg body weight). All but one patient had been on pre-operative anticoagulation therapy; 49 were on acetylsalicylic acid and 3 on coumarin-derivatives. Thirty-three patients stopped the anticoagulation 3-10 days prior to surgery. The endarterectomy was performed through a longitudinal arteriotomy. During the entire procedure, TCD monitoring of blood flow velocity and of MES in the ipsilateral middle cerebral artery was performed, if an adequate transtemporal window was present (n=43). A Javid shunt was used selectively in case of imminent hypoperfusion (n=16) as suggested by >70% decrease of middle cerebral artery blood flow velocity. After completion of the endarterectomy, the arteriotomy was closed with a primary suture (n=27) or a patch (n=26; 24 venous, 1 dacron, 1 PTFE patch) at the discretion of the surgeon. The carotid atheroma harvested during operation was immediately processed for microscopic evaluation. During dissection, 10cc venous blood was obtained for serological studies.

6.3.2 MES-detection during CEA

During CEA, TCD monitoring of the ipsilateral middle cerebral artery was performed through the transtemporal approach with a 2-MHz probe fixed with a metal frame (Multidop X 4, DWL, Sipplingen, Germany). A satisfactory transtemporal window was present in 43 of the 53 patients. The Doppler signal was recorded on a 2-channel DAT recorder for additional offline analysis. No automatic MES detection system was used. The gain was set to the lowest possible value and the sweep time as fast as possible. The burst length equaled approximately 7.5 mm. MES were evaluated online during the surgical procedure by a technician and additionally offline by an experienced listener (W.H.M.). The criteria for MES used were (1) the typical sound and (2) the appearance as a short-lasting intensity increase in the fast Fourier transform in agreement with the report of a consensus committee. pMES were counted during dissection and tMES were registered after removal of the plaque and restoration of the flow (>5 min after clamp release). Patients with ≥2 pMES/hour were regarded pMES+, and patients with ≥6 tMES/hour were considered tMES+.

6.3.3 Carotid Plaque Histology

CEA specimens harvested during operation were divided into multiple macroscopic parts. A macroscopic sketch of the plaque was drawn with attention to the orientation of the different parts. The odd parts were snap
frozen in liquid nitrogen and stored at -80°C for future analysis. The even parts were formalin fixed and paraffin embedded. A representative 5 μm section of each paraffin embedded piece of the carotid atheroma was stained with hematoxylin and eosin for characterization of plaque (in)stability. Histological plaque instability, defined as the presence of an organized luminal thrombus and/or a ruptured fibrotic cap, was assessed by two independent investigators (T.V. and R.E.) blinded for the infectious status of the patients. In case of disagreement between the two independent assessments, the plaque was re-evaluated by the two investigators to reach a consensus.

6.3.4 Chlamydia pneumoniae serology

Venous blood drawn during operation was immediately centrifuged for 10 min at 1200 rpm and 4°C. Serum was stored at -20°C until determination of Chlamydia pneumoniae serology. IgA and IgG antibodies were determined by means of a commercially available ELISA (Labsystems, Finland). Titres were calculated form the optical density readings according to the manufacturer's instructions. Chlamydia pneumoniae IgA- and IgG-seropositivity was defined at an IgA-titre ≥1/16 and IgG-titre ≥1/64, respectively.

6.3.5 Statistical analysis

We used SPSS 10.0 for Windows for statistical analysis. The Fisher's exact test was used for comparison of prevalence of risk factors and patient characteristics between seropositive and seronegative patients, for comparison of tMES/pMES with plaque instability, and for analysis of the association between pMES, tMES or plaque instability and Chlamydia pneumoniae serology. To identify possible confounders, the independent effect of sex, age, smoking, hypertension, dyslipidemia, diabetes and discontinuation of preoperative anticoagulation on tMES/pMES was evaluated in simple logistic regression analyses. Parameters that were associated significantly with tMES/pMES in these analyses were entered as potential confounders in a multivariate logistic regression model to study the association between tMES/pMES and Chlamydia pneumoniae-serology. A 2-sided probability value of <0.05 was regarded as statistically significant.

6.4 Results

Fifty-three patients with symptomatic carotid artery stenosis were included in this study. The duration of operation averaged 110 minutes (range, 73 to 189 minutes). The average clamping and/or shunting time was 35 minutes (range, 20 to 60 minutes). MES were detected during dissection and after endarterectomy and restoration of flow, with the omission of those in the first 5
minutes after cross-clamp release to avoid false positive registration of
gaseous MES. The mean pMES detection time was 64 minutes (range, 45 to
96 minutes), and the mean tMES detection time was 23 minutes (range, 15 to
42 minutes).

One patient developed neurological symptoms the day after operation, which
on re-exploration of the carotid artery could be attributed to a fresh thrombus at
the site of the primary arterial suture. The thrombus was removed, and the
arteriotomy was closed with a venous patch. After that the patient made an
uneventful recovery and did not suffer any permanent disabilities. Three more
patients developed signs of peripheral facial nerve lesion and recovered fully
within a month after hospital discharge.

6.4.1 Carotid plaque histology

Carotid atheroma was available for histological analysis in all patients. Signs of
plaque instability, i.e., an organized luminal thrombus and/or ruptured fibrotic
cap, were seen in 23 patients (43%). The remaining patients had advanced but
stable atherosclerotic lesions, consisting of a thick fibrous cap overlying a
lipid/necrotic core with occasional intraplaque hemorrhage.

6.4.2 Micro-embolization during carotid endarterectomy

A transtemporal window was present in 43 patients (81%). Patients with no
cranial window were older than patients with a suitable window (mean age 73
versus 65 years, \(P=0.003\)) which might reflect age-related temporal bone
ossification. However, prevalence of cardiovascular risk factors, preoperative
diagnosis, operative techniques, plaque histology and Chlamydia pneumoniae
serology were comparable in both groups.

Twelve patients had \(\geq 2\) pMES per hour and were classified as pMES+. In these
patients, the pMES rate varied from 2.5 to 30.8 pMES per hour. The
occurrence of pMES correlated strongly with histological plaque instability
(Table 6.2).

In 17 patients tMES were observed. Eleven patients had a tMES rate \(\geq 6\) tMES
per hour and were designated tMES+. Thrombosis-related embolization was
not related to plaque histology (Table 6.2).
Table 6.2  Association between pMES or tMES and plaque histology.

<table>
<thead>
<tr>
<th></th>
<th>Unstable plaque, N (%)</th>
<th>Stable plaque, N (%)</th>
<th>total N</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMES +</td>
<td>11 (55)</td>
<td>1 (5)</td>
<td>12</td>
<td>0.093</td>
</tr>
<tr>
<td>pMES -</td>
<td>9  (45)</td>
<td>22 (95)</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Total pMES</td>
<td>20 (100)</td>
<td>23 (100)</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>tMES +</td>
<td>6  (30)</td>
<td>5  (22)</td>
<td>11</td>
<td>0.728</td>
</tr>
<tr>
<td>tMES -</td>
<td>14 (70)</td>
<td>16 (78)</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Total tMES</td>
<td>20 (100)</td>
<td>23 (100)</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

pMES+ indicates ≥2 pMES per hour; pMES-, <2 pMES per hour; tMES+, ≥5 tMES per hour; tMES-, <5 tMES per hour. *statistical analysis by Fisher's exact test; significance at P<0.05.

6.4.3 *Chlamydia pneumoniae* serology

Elevated levels of *Chlamydia pneumoniae* antibody titers were a common finding in our patients. Fifty-eight percent were *Chlamydia pneumoniae* IgA-seropositive (IgA ≥1/16), and 60% were *Chlamydia pneumoniae* IgG-seropositive (IgG ≥1/64). The distribution of cardiovascular risk factors and the use of an intraluminal shunt or a patch for arteriotomy closure were comparable in seropositive and seronegative patients (Table 6.3).

Table 6.3  Distribution of cardiovascular risk factors and operative techniques among *Chlamydia pneumoniae* seropositive and seronegative patients.

<table>
<thead>
<tr>
<th></th>
<th>IgG+</th>
<th>IgG-</th>
<th>P*</th>
<th>IgA+</th>
<th>IgA-</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>32</td>
<td>21</td>
<td>ns</td>
<td>30</td>
<td>23</td>
<td>ns</td>
</tr>
<tr>
<td>female/male</td>
<td>68</td>
<td>65</td>
<td>ns</td>
<td>67</td>
<td>66</td>
<td>ns</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>63%</td>
<td>76%</td>
<td>ns</td>
<td>64%</td>
<td>74%</td>
<td>ns</td>
</tr>
<tr>
<td>Hypertension</td>
<td>63%</td>
<td>76%</td>
<td>ns</td>
<td>63%</td>
<td>57%</td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes</td>
<td>13%</td>
<td>19%</td>
<td>ns</td>
<td>10%</td>
<td>22%</td>
<td>ns</td>
</tr>
<tr>
<td>Perioperative shunting</td>
<td>22%</td>
<td>14%</td>
<td>ns</td>
<td>27%</td>
<td>9%</td>
<td>ns</td>
</tr>
<tr>
<td>Patch closure</td>
<td>50%</td>
<td>48%</td>
<td>ns</td>
<td>50%</td>
<td>48%</td>
<td>ns</td>
</tr>
<tr>
<td>Discontinuation of preoperative anticoagulation</td>
<td>56%</td>
<td>67%</td>
<td>ns</td>
<td>55%</td>
<td>70%</td>
<td>ns</td>
</tr>
</tbody>
</table>

IgG+ indicates IgG seropositivity (≥1/64); IgG-, IgG seronegativity (<1/64); IgA+, IgA seropositivity (≥1/16); and IgA-, IgA seronegativity (<1/16). *statistical analysis by Fisher's exact test; statistical significance at P<0.05; ns, not significant.

6.4.4 Influence of *Chlamydia pneumoniae*-infection on carotid artery disease

Table 6.4 shows the association between *Chlamydia pneumoniae* serology and histological plaque instability, plaque-related embolization and thrombosis-
related embolization. *Chlamydia pneumoniae* seropositivity was not related to histological plaque instability or plaque-related emboli. However, IgA seropositivity was associated with thrombosis-related emboli (*P*<0.014) and IgG seropositivity showed a trend towards association with tMES (*P*<0.077). After correction for confounding covariables, only IgA seropositivity was still significantly associated with tMES (*P*<0.030). Of the potential confounders sex, age, smoking, dyslipidemia, hypertension, diabetes, and discontinuation of pre-operative anticoagulation only the latter was univariantly associated with tMES (*P*<0.030) and tended toward association with pMES (*P*<0.075); it was therefore the only confounding variable introduced in the multivariate regression analysis.

Table 6.4 Relation between *Chlamydia pneumoniae* serology (IgA and IgG seropositivity) and histological plaque instability, pMES or tMES

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>IgA+ OR 95%-CI</th>
<th>P</th>
<th>IgG+ OR 95%-CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque instability</td>
<td>1.0 0.3–3.3 0.994</td>
<td></td>
<td>0.8 0.2–2.8 0.616</td>
<td></td>
</tr>
<tr>
<td>pMES+</td>
<td>1.6 0.4–6.6 0.483</td>
<td>1.9 0.5–7.5 0.376</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8* 0.4–8.6* 0.474*</td>
<td>1.9* 0.4–9.4* 0.406*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tMES+</td>
<td>11.3 1.3–99.1 0.014</td>
<td>5.1 0.95–27.4 0.058</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.1* 1.3–114.2* 0.030*</td>
<td>4.8* 0.84–27.4* 0.078*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR indicates odds ratio. Plaque instability includes fibrous cap rupture and/or organized luminal thrombosis. Other definitions are as in Tables 6.2 and 6.3. Statistical analysis by Fisher's exact test and multivariate logistic regression analysis including 'discontinuation of pre-operative anticoagulation' in the analysis; statistical significance at *P*<0.05. * Multivariate logistic regression analysis

6.5 Discussion

Atherosclerosis is a chronic inflammatory disease. Chronic infections, especially *Chlamydia pneumoniae* infections, may play an important role in the initiation and progression of this inflammatory process. *Chlamydia pneumoniae* can induce proatherogenic changes in endothelial cells, macrophages, and smooth muscle cells. *Chlamydia pneumoniae* seropositivity has been associated with acute and chronic clinical atherosclerotic manifestations; and *Chlamydia pneumoniae* has been detected more frequently in atherosclerotic tissue compared with normal arteries. However, the vascular presence of *Chlamydia pneumoniae* has not been associated with coronary plaque morphology or plaque-related cerebral microembolization. Since the development of acute cardiovascular complications is associated with plaque instability and/or thrombotic occlusions of blood vessels, we wanted to study the association between *Chlamydia pneumoniae* serology, plaque instability, and hypercoagulability. In the present
study *Chlamydia pneumoniae* serology was not associated with plaque instability but showed a strong relation with thrombosis-related microembolization during CEA, an *in vivo* marker of hypercoagulability. Application of TCD monitoring of the ipsilateral middle cerebral artery during CEA offers the possibility to study the two basic mechanisms that contribute to cardio- and cerebrovascular events separately, i.e., plaque destabilization and (excessive) thrombosis. MES during the dissection phase of CEA have been associated with histological determinants of plaque instability (i.e., plaque rupture and/or luminal thrombosis). A pMES rate ≥2 pMES per hour has been associated with increased risk of developing cerebral ischemia. Therefore, the occurrence of MES during dissection is an *in vivo* marker of plaque instability, and patients with ≥2 pMES per hour were defined as pMES+. After endarterectomy and restoration of flow, in the absence of an unstable plaque, MES represent thromboocyte aggregations formed at the highly thrombogenic endarterectomy and clamping sites. In a series of 276 CEAs, the median embolic rate postoperatively was 1.33 MES per hour (interquartile range 0.5-6.7 MES/hour). A high embolic rate after CEA (in the upper quartile of tMES-rate, i.e., ≥6 tMES per hour) probably identifies patients with excessive thrombus formation or inadequate thrombolysis. Therefore, for the purposes of this study, a tMES rate ≥6 tMES per hour was regarded as an *in vivo* marker of hypercoagulability, and patients with ≥6 tMES per hour were designated tMES+. In agreement with previous reports, pMES were associated with histological plaque instability in this study. However tMES were not related to histology.

The average embolic count was related to operation time in our patients. Interestingly, only plaque related MES correlated with this variable. Operation time was a strong predictor of embolic count during dissection (*P*≤0.002) but not of embolic count after endarterectomy and restoration of flow (*P*≤0.873). To avoid the confounding effect of operation time on the association between *Chlamydia pneumoniae* serology and microembolization, the embolic rate (micro-emboli per hour) was used to define patients with clinically relevant plaque-related (≥2 pMES per hour) or thrombosis-related (≥6 tMES per hour) embolization.

*Chlamydia pneumoniae* serology has been associated with cerebrovascular events, carotid intima-media thickness, and sonographically detected carotid plaques. Nevertheless, a number of studies have failed to show an association between *Chlamydia pneumoniae* serology and ischemic cerebrovascular disease. However, the amount of positive evidence accumulated suggests that this association is material and not coincidental. Negative reports may have been hampered by biased study population and end point selection, limitations of the diagnostic assays used or even the unfortunate coincidence of a *Chlamydia pneumoniae* epidemic during sample
acquisition. In a randomly selected urban population, no association existed between *Chlamydia pneumoniae* serology and carotid intima-media thickness and/or the presence of sonographically detected carotid plaque. However, in patients with hypertension, coronary and peripheral arterial disease, cerebrovascular disease, and end-stage renal disease, an association between *Chlamydia pneumoniae* serology and carotid intima-media thickness or degree of stenosis could be found. In a nested case control study, Glader et al. found no association between baseline *Chlamydia pneumoniae* serology and the development of future ischemic cerebral infarction. The authors suggested that a *Chlamydia pneumoniae* epidemic at the time of patient inclusion and blood sampling might have masked a possible association between *Chlamydia pneumoniae* serology and cerebrovascular disease, a problem that could have been bypassed if antibody titres would have been detected in paired serum samples.

Despite numerous reports on the association between *Chlamydia pneumoniae* serology and cardiovascular disease, no consensus has been reached regarding the serological criteria for chronic or persistent *Chlamydia pneumoniae* infection. Various cut-off points for IgA and IgG titres or even IgA and IgG titre combinations have been used. The heterogeneity of serological assays and of serological criteria for persistent or chronic *Chlamydia pneumoniae* infections might also have contributed to conflicting results of some seroepidemiological studies. The microimmunofluorescence test is regarded as the reference method for *Chlamydia pneumoniae* serology. However, Gnarpe et al. found a good correlation between enzyme immunoassay and microimmunofluorescence test in patients with hypertension or ischemic heart disease who had a low background of *C. trachomatis* antibodies. The sensitivity, specificity, and positive and negative predictive value was 91, 80, 96 and 63 for IgG and 85, 88, 92 and 79 for IgA, respectively, with the microimmunofluorescence test regarded as gold standard. Likewise, in our laboratory, the sensitivity, specificity, and positive and negative predictive values of the enzymeimmunoassay compared with the microimmunofluorescence test were 95, 89, 98 and 77 for IgA and 95, 73, 96 and 75, respectively, for IgG in a series of 239 samples from patients with peripheral arterial disease (n=150) and healthy controls (n=89). The intertest agreement between the enzymeimmunoassay and the microimmuno-fluorescence test was very good for IgA (κ=0.679) and for IgG (κ=0.681) (T. Vainas, et al., unpublished data, 2001). Considering the adequate performance and the practical advantages of the enzyme-immunoassay compared with the microimmunofluorescence test (high throughput, objective endpoint, cost-efficiency), we had chosen the first method to determine *Chlamydia pneumoniae* serology in this study. IgA titres ≥1/16 and IgG titres ≥1/64 were considered positive in view of the results of previous studies showing an
association between *Chlamydia pneumoniae* infection and carotid artery disease.\(^5\,^6\) According to these cut-off levels, IgA seropositivity was associated with a high thrombosis-related embolic rate but not with plaque instability. Since IgA titres decline and disappear after 3-12 months of infection whereas IgG antibodies may persist for some years, it is believed that persistence of the short-lived IgA may be a better marker of chronic infection than IgG.\(^6\) Hence, chronic *Chlamydia pneumoniae* infection is associated with an *in vivo* marker of hypercoagulability in patients undergoing CEA for symptomatic carotid artery disease.

In this study *Chlamydia pneumoniae* serology was not related to plaque instability but correlated with tMES. This suggests that the reported association between *Chlamydia pneumoniae* infection and cardiovascular disease might be mediated through stimulation of thrombosis-related events rather than plaque-related phenomena by *Chlamydia pneumoniae* infection. Interestingly, immunohistochemical detection of *Chlamydia pneumoniae* in carotid atheroma of 76 patients has strongly been associated with thrombosis but not with plaque ulceration, suggesting that *Chlamydia pneumoniae* infection was independently associated with a greater risk of thrombosis on the plaques but not with plaque ulceration.\(^47\) The separate analysis of pMES and tMES in our study showed that *Chlamydia pneumoniae* seropositivity indeed is associated with tMES rather than pMES, favoring a prothrombotic and/or antithrombotic effect of *Chlamydia pneumoniae* infection in carotid artery disease. Previous studies have shown that *Chlamydia pneumoniae* infection of endothelial cells stimulates the nuclear factor-κB signal transduction pathway, inducing a 4-fold increase of tissue factor\(^15\) and stimulating the expression of plasminogen activator inhibitor 1,\(^16\) resulting in increased local thrombogenicity. Moreover, plasma fibrinogen levels are elevated in patients with chronic *Chlamydia pneumoniae* infections\(^48\) and decrease on antimicrobial treatment.\(^49\) These observations offer a novel perspective on the association between *Chlamydia pneumoniae* infection and cardiovascular events and warrant further investigation of the prothrombotic and antithrombotic effects of *Chlamydia pneumoniae* infection in atherothrombotic arterial disease.\(^50\)
6.6 References


Section 4

Genetic determinants of atherosclerosis susceptibility
Chapter 7

Synergistic effect of Toll-Like Receptor 4 and CD-14 polymorphisms on the total atherosclerosis burden in patients with peripheral arterial disease

Vainas T, Stassen FR, Bruggeman CA, Wellen RJ, van den Akker LH, Kitslaar PJ, Peña AS, Morré SA

Submitted
7.1 Abstract

Background
Genes involved in the regulation of immune responses, such as Toll Like Receptor 4 (TLR4) and CD14, show genetic variations with potential functional implications. Since atherosclerosis is an inflammatory process apparently modulated by chronic infections, we studied the effect of single nucleotide polymorphisms (SNPs) in TLR4 and CD14, on the extent of atherosclerosis in patients with peripheral arterial disease (PAD).

Materials and methods
Using an in-house developed PCR-based restriction length polymorphism assay, we determined the genotype, allele frequency and carrier trait of the TLR4 +896 A>G and the CD14 -260 C>T SNPs in 607 Dutch Caucasian patients with PAD. The extent of atherosclerosis was determined on the basis of the number of vascular territories involved, i.e. coronary, cerebral, aortic and peripheral.

Results
55% of the patients suffered from PAD only. Approximately 1/3rd of the patients had 2, and 11% had 3 vascular territories affected by atherosclerosis. The TLR4 +896 G allele frequency was 11% and the CD14 -260 T allele frequency was approximately 74%. Among PAD patients, TLR4 +896 G allele carrier-ship was univariantly associated with extensive (>2 vascular territories affected) atherosclerotic disease (OR: 2.22, P=0.020, χ² test), whereas CD14 -260 C>T carrier-ship/homozygote was not. Trend analysis showed that the TLR4 +896 G allele frequency increases with the number of vascular territories affected by atherosclerosis (P trend 0.0074). In a multivariate logistic regression analysis, including cardiovascular risk factors, TLR4- and CD14 SNPs, only the interaction variable TLR4 +896 G allele carrier-ship-CD14 -260 TT genotype survived as independent predictor of extensive atherosclerotic disease (P=0.031, OR= 4.2, 95%CI: 1.1–15.4).

Conclusion
The carrier trait 'TLR4 G allele & CD14 TT genotype', rather than each SNP individually, is associated with extent of atherosclerotic disease. Considering the importance of immune responses in atherogenesis and the genetic variation of immune regulatory genes, our data provide an explanation for inter-individual differences in susceptibility to atherosclerosis and demonstrate the need to take a wider approach analyzing relevant carrier traits instead of individual polymorphisms in relation to atherosclerosis.
7.2 Introduction

Inflammatory processes have been implicated in the pathogenesis of atherosclerosis. Responses of the innate immune system to endothelial injury are involved in the initiation of atherosclerosis and inappropriate activation of the innate and acquired immune system play a pivotal role in the propagation of the disease. Among the triggers of such atherogenic immune responses are endogenous antigens such as oxidized low density lipoprotein (LDL), and exogenous pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and exogenous and endogenous heat shock proteins (HSPs). Indeed, chronic infections, especially Chlamydia pneumoniae infections, have recently been implicated in the pathogenesis of atherosclerosis.

The innate immune system plays a key role in the defence against pathogens and, when gone awry, may contribute to the development of chronic inflammatory conditions. Pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) are involved in the elimination of pathogens through recognition of PAMPs, setting off a cascade of pro-inflammatory reactions, which, if not balanced, may exacerbate chronic inflammatory processes. TLR4 is the first TLR described in mammals and functions not only as a receptor for LPS but for agonists such as human and chlamydial HSPs as well. LPS binding is complex and requires several accessory molecules, among which, CD14, LPS binding protein (LBP) and MD-2. Candidate gene approaches investigate genetic variation including the effect of (functional) single nucleotide polymorphisms (SNPs) in genes involved in the immune response on disease susceptibility and/or severity. In polygenic and multifactorial diseases, like atherosclerosis, these approaches might identify risk factors in the context of aetiological and genetic heterogeneity. Potential candidate genes for investigating the susceptibility to and severity of atherosclerosis include PRRs such as TLR4 and CD14. Activation of these receptors results in the activation of nuclear factor-κB (NF-κB) followed by transcription of various pro-inflammatory cytokine genes like tumour necrosis factor-α (TNF-α), interleukin (IL)-1α and IL-1β. Thus, genetic variations in PRRs provide a plausible explanation for altered responsiveness of the innate immune system and may be associated with altered susceptibility to infectious and inflammatory processes and severity or outcome of disease. The recently described human TLR4 polymorphism, i.e., a missense SNP substituting an aspartic acid residue with glycine at amino acid 299 (Asp299Gly; nucleotide position TLR4 +896A>G), has been associated with hyposensitivity to LPS and reduced expression of TLR4. In the general population, this polymorphism has been associated with decreased interleukin-6 (IL-6), fibrinogen, soluble vascular adhesion molecule 1 (VCAM-1), procalcitonin and neopterin plasma levels, and
seemingly confers protection against the development of carotid and femoral atherosclerosis\textsuperscript{8,9} and acute coronary events.\textsuperscript{10} Among pravastatin users it has significantly been associated with a lower cardiovascular risk.\textsuperscript{11} A functional polymorphism in the promoter region of \textit{CD14} at position -260, the \textit{CD14} -260C>T polymorphism, which enhances the transcriptional activity of the \textit{CD14} gene, has been associated with increased carotid artery intima media thickness,\textsuperscript{12} an enhanced risk of stroke\textsuperscript{13} and acute myocardial infarction.\textsuperscript{14,15} Nevertheless, several authors demonstrated a lack of association between this polymorphism and coronary artery or cerebrovascular disease.\textsuperscript{16,17}

Although candidate gene approaches may identify signal transduction pathways of importance for a specific disease, they usually find only relative small contributions for an individual gene to the overall susceptibility to disease. Therefore, so-called carrier trait analyses are increasingly used. These strategies analyze SNPs in different genes together and investigate if potential synergic effects can be observed. A good example of the effect of such analyses is shown in a recent study by El-Omar and colleagues who evaluated the role of proinflammatory cytokine gene polymorphisms in gastric and esophageal cancers.\textsuperscript{18} They showed that combined carriage of multiple proinflammatory polymorphisms of IL-1B, IL-1 receptor antagonist, TNF-\(\alpha\), and IL-10 conferred greater risk, with ORs (and 95\% confidence intervals) of 2.8 (1.6-5.1) for one, 5.4 (2.7-10.6) for two, and 27.3 (7.4-99.8) for 3 or 4 high-risk genotypes.

In the present study we assessed the association between the \textit{TLR4} +896 A>G and \textit{CD14} (-260)C>T polymorphisms individually with extent of atherosclerosis in patients with peripheral arterial disease. Furthermore, using a multivariate logistic regression model we analysed the effect of the combination of both polymorphisms on extent of atherosclerotic disease in these patients.

\section*{7.3 Materials and methods}

\subsection*{7.3.1 Study population}

Dutch Caucasian patients, all diagnosed with peripheral arterial disease, were recruited at the surgical clinics of an university hospital and two affiliated teaching hospitals. Ankle-brachial pressure index (ABPI) measurement was used to objectify the presence of atherosclerotic disease of lower limb arteries. An ABPI<0.9 was regarded pathognomonic. In order to identify additional cardio- and cerebrovascular co-morbidities, medical charts were reviewed and attending physicians were consulted. Also, the presence/absence of AAA was ascertained in all patients by means of duplex ultrasound and/or CT-
angiography. AAA was defined as an aorta with an antero-posterior diameter >30mm. The study was approved by the local medical ethical committees of all participating centres and conformed with the principles outlined in the declaration of Helsinki. All patients gave written informed consent. Patients with acute infections, recent antibiotic use (<3 months), recent vascular surgery (<3 months), concomitant inflammatory disorders and malignancies were excluded.

7.3.2 Extent of atherosclerotic disease

In order to determine the extent of atherosclerotic disease, the documented cardiovascular, cerebrovascular, and peripheral vascular morbidity as well as the presence/absence of AAA was taken into consideration. An 'extent of atherosclerosis score' was developed ascribing a point for every vascular territory (coronary, cerebral, peripheral, and aorta) affected by symptomatic atherosclerotic disease. Since patients with PAD were included, the peripheral vascular territory was considered affected in all patients. The coronary vascular territory was considered affected in case of a history of myocardial infarction (MI), angina pectoris (AP), percutaneous transluminal coronary angioplasty (PTCA) and/or coronary artery bypass grafting (CABG). The cerebral territory was considered affected in case of a history of stroke, transient ischemic attack (TIA), amaurosis fugax and/or carotid endarterectomy. Finally, the aorta was regarded affected in case of a (history of) AAA (aortic diameter >30 mm) and/or aortic reconstruction for AAA. The sum, ranging from 1 (only PAD) to 4 (all vascular territories affected) was considered as an indication of the extent of atherosclerotic disease.

7.3.3 DNA Extraction

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) using the isopropanol isolation method. Briefly, 600µl NucliSens Lysis buffer containing 5 mmol/l guanidine thiocyanate, triton X-100, Tris-HCl (Organon Teknika, Boxtel, The Netherlands) and 1 µl glycogen, was added to 100 µl PBMCs in phosphate-buffered saline. The DNA pellets were dissolved in T10 (10 mM Tris-HCl, pH 8.0) and stored at −20°C until further analysis.

7.3.4 Genotyping of TLR4 and CD14

An in-house developed PCR-based restriction fragment-length polymorphism assay was used to detect the A>G missense mutation at nucleotide 896 bp (amino acid Asp299Gly) in the human TLR4 gene (NCBI SNP CLUSTER ID: rs4986790). Digestion with NcoI (Invitrogen Life Technologies BV, Breda,
The Netherlands) and separation on an 4.5% agarose gel containing 0.1% ethidium bromide (BiozymTC, Landgraaf, The Netherlands) of the 102bp PCR product (primerS 5'-AGC ATA CTT AGA CTA CTA CCA TG-3' and 5'-TTT ACC CTT TCA ATA GTC ACA CTC A-3') yielded fragments of 102 bp (A-allele) and/or 80 and 22 bp (G-allele).

The C>T substitution in the proximal CD14 promotor region at position -260 (NCBI SNP CLUSTER ID: rs2569190) was analysed with an in-house developed PCR assay using the primers, 5’-TCA CCT CCC CAC CTC TCT T-3’(sense) and 5’-CCT GCA GAA TCC TTC CTG TT-3’(antisense) (Invitrogen Live Technologies BV, Breda, The Netherlands). The 107bp amplification products were digested with HaeIII (New England BioLabs, England, UK) yielding either two fragments of 83 and 24 bp (C-allele) and/or an intact fragment (T-allele) of 107 bp, respectively.

7.3.5 Statistics

The χ²-test was used for comparison of TLR4 +896 A>G and CD14 -260 C>T genotype frequencies, carrier trait analyses (combined effect of the TLR4 and CD14 polymorphisms analyzed), and prevalence of risk factors between patient groups. A trend analysis was used to investigate if specific CD14 or TLR4 allele frequencies increased with the number of vascular territories affected by atherosclerosis. Multivariate logistic regression models were computed using extensive atherosclerotic disease (i.e., >2 vascular territories affected by atherosclerosis) as dependent variable and entering in a stepwise, forward conditional fashion, cardiovascular risk factors, TLR4, CD14 SNPs, and the TLR4-CD14 SNP interaction variable as independent variables. SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistics.

7.4 Results

7.4.1 Patient characteristics

A total of 607 Dutch Caucasian PAD patients were analysed. Patient characteristics are given in Table 7.1. The patients had an atherosclerotic risk factor profile, as seen in this table. It also became evident that the patients suffered from extensive atherosclerotic disease, since 273 patients (45%) presented with manifestations of atherosclerosis in at least one additional vascular territory besides peripheral, thus illustrating the systemic nature of atherosclerosis.
Table 7.1 Patient characteristics (n=807).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) Age in years</td>
<td>65 (10)</td>
</tr>
<tr>
<td>Female/male</td>
<td>184 (30%) / 423 (70%)</td>
</tr>
<tr>
<td>Smoking*</td>
<td>476 (78%)</td>
</tr>
<tr>
<td>Dyslipidemia*</td>
<td>503 (83%)</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>363 (60%)</td>
</tr>
<tr>
<td>Diabetes*</td>
<td>138 (23%)</td>
</tr>
<tr>
<td>One vascular territory affected</td>
<td>334 (55%)</td>
</tr>
<tr>
<td>Two vascular territories affected</td>
<td>204 (34%)</td>
</tr>
<tr>
<td>Three vascular territories affected</td>
<td>67 (11%)</td>
</tr>
<tr>
<td>Four vascular territories affected</td>
<td>2 (0%)</td>
</tr>
</tbody>
</table>

*a currently smoking or stopped <10 years; b fasting cholesterol level >5.1 mmol/l and/or triglyceride level >1.95 mmol/l and/or the use of antilipidemic medication; c systolic blood pressure >160mmHg and/or diastolic blood pressure >95mmHg and/or the use of antihypertensive medication; d fasting glucose level >7mmol/l or the use of antidiabetic medication or insulin.

7.4.2 TLR 4 and CD14 genotyping

Table 7.2 shows the genotype, carrier and allele frequency of the TLR4 +896 A>G SNP in the PAD patients and the healthy controls. The overall prevalence of this SNP in our PAD patients was approximately 10%. However, among our patients there seemed to be a significant relation between TLR4 +896 G allele carrier-ship and extent of atherosclerotic disease. The average extent of atherosclerosis score was higher in patients with a polymorphic allele compared to patients homozygous for the wild-type TLR-4 allele (1.8 vs. 1.5, P=0.011, Mann-Whitney U Test). TLR4 +896 G carriers had significantly more frequently (OR: 2.2, P=0.020, $\chi^2$ test) extended atherosclerotic disease (>2 vascular territories affected).

Table 7.2 Genotype, allele- and carrier frequencies of the TLR4 +896 A>G alleles in atherosclerotic patients.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>Carrier Frequency</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 affected vascular territory (PAD only)</td>
<td>334</td>
<td>307</td>
<td>27</td>
<td>0</td>
<td>8.1%*</td>
<td>4.0%</td>
</tr>
<tr>
<td>2 affected vascular territories</td>
<td>204</td>
<td>180</td>
<td>23</td>
<td>1</td>
<td>11.8%*</td>
<td>6.1%</td>
</tr>
<tr>
<td>3 affected vascular territories</td>
<td>67</td>
<td>54</td>
<td>13</td>
<td>0</td>
<td>19.4%*</td>
<td>9.7%</td>
</tr>
<tr>
<td>4 affected vascular territories</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.0%*</td>
<td>0.0%</td>
</tr>
<tr>
<td>Total</td>
<td>607</td>
<td>543</td>
<td>63</td>
<td>1</td>
<td>10.5%</td>
<td>5.4%</td>
</tr>
</tbody>
</table>

Extended atherosclerotic disease (>2 vascular territories affected, n=69) is significantly associated with TLR4 +896 G carrier-ship (OR: 2.22, P=0.020, $\chi^2$ test). Trend analysis: TLR4 +896 G-allele frequency increases with the number of vascular territories affected (1 to 2 to 3+4): P trend 0.0074 ($\chi^2$= 7.2).

Trend analysis showed that the TLR4 +896 G-allele frequency statistically significantly increased with the number of vascular territories affected by
atherosclerosis (see Table 7.2): 8.1% in patients with one affected vascular territory, 11.8% in patients with two affected territories, and 18.8% in patients with three or four affected vascular territories (P-trend 0.0074). Since only two patients had four affected vascular territories they were grouped together with those with three affected territories.

In a multivariate logistic regression model the association between the TLR4 +896 A>G SNP and extended atherosclerotic disease (>2 vascular territories affected) persisted after correction for relevant confounders (OR: 2.1, P=0.048, Table 7.3).

Table 7.3  Stepwise forward computed multivariate logistic regression model demonstrating the relation between TLR4 +896 G carrier-ship and extent of atherosclerotic disease. Dependent variable: ‘extensive atherosclerotic disease’ (i.e., >2 vascular territories affected), n=607.

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>B</th>
<th>SE</th>
<th>P</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp299Gly allele</td>
<td>0.735</td>
<td>0.371</td>
<td>0.048</td>
<td>2.085</td>
<td>1.01 – 4.31</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.205</td>
<td>0.352</td>
<td>0.001</td>
<td>3.338</td>
<td>1.68 – 6.65</td>
</tr>
<tr>
<td>Age</td>
<td>0.639</td>
<td>0.300</td>
<td>0.033</td>
<td>1.895</td>
<td>1.10 – 3.41</td>
</tr>
<tr>
<td>Constant</td>
<td>-3.251</td>
<td>0.362</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Variables excluded from the equation were: sex, smoking, diabetes, hypercholesterolemia, and positive family history.

The CD14 polymorphism was very common (Table 7.4). Approximately 22% of patients had the TT-genotype and about 50% were heterozygous for the CD14 polymorphism. In contrast to the TLR4 polymorphism, the CD14 SNP was not related to the extent of atherosclerotic disease in univariate analysis.

Table 7.4  Allele and carrier frequencies of the polymorphic CD14 (-260)C>T allele in atherosclerotic patients.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Carrier frequency</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>CC</td>
</tr>
<tr>
<td>1 affected vascular territory (PAD only)</td>
<td>334</td>
<td>88</td>
</tr>
<tr>
<td>2 affected vascular territories</td>
<td>204</td>
<td>52</td>
</tr>
<tr>
<td>3 affected vascular territories</td>
<td>67</td>
<td>16</td>
</tr>
<tr>
<td>4 affected vascular territories</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>607</td>
<td>158</td>
</tr>
</tbody>
</table>

Extent of atherosclerotic disease was not related to CD14 polymorphism.
7.4.3 Carrier trait analysis

Finally we performed a CD14-TLR4 carrier trait analysis in relation to the extent of atherosclerotic disease. Forty-four patients were carrier of the TLR4 G-allele in combination with the CD14 T-allele, and 14 patients had the TLR4 G-allele in combination with the CD14 TT genotype. Carrier trait analyses for the TLR4 and CD14 SNPs studied showed a trend towards association with extent of atherosclerotic disease, but this association failed to reach statistical significance in univariate logistic regression analyses (Table 7.5).

Table 7.5 Association between the carrier trait of TLR4- and CD14-SNP and extent of atherosclerotic disease.

<table>
<thead>
<tr>
<th>Extent of atherosclerotic disease</th>
<th>TLR4-CD14 xG-XT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TLR4-CD14 xG-TT&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2 territories affected (n=538)</td>
<td>503</td>
<td>528</td>
</tr>
<tr>
<td>&gt;2 territories affected (n=69)</td>
<td>60</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>563</td>
<td>593</td>
</tr>
</tbody>
</table>

<sup>a</sup> Univariate logistic regression analysis: \( P=0.054 \), OR: 2.156 (0.99–4.70); <sup>b</sup> Univariate logistic regression analysis: \( P=0.052 \), OR: 3.249 (0.99–10.67).

However, when in the multivariate logistic regression model cardiovascular risk factors, carriership of the TLR4 G-allele, the CD14 TT genotype, and the combination of TLR4 G-allele and CD14 TT genotype were entered as independent variables, only the interaction variable TLR4 G-allele-CD14 TT genotype (OR: 4.2, \( P=0.031 \)) survived as independent predictor of ‘extensive atherosclerotic disease’ (i.e., >2 vascular territories affected by atherosclerosis; Table 7.6).

Table 7.6 Stepwise forward computed multivariate logistic regression model demonstrating the relation between the carrier trait of TLR4 +896 G allele carriership (xG) and CD14 (-260) TT genotype (TT) and extent of atherosclerotic disease. Dependent variable: ‘extensive atherosclerotic disease’ (i.e., >2 vascular territories affected). N=807.

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>B</th>
<th>SE</th>
<th>( P )</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4(xG)-CD14(TT)</td>
<td>1.434</td>
<td>0.655</td>
<td>0.031</td>
<td>4.194</td>
<td>1.14 – 15.44</td>
</tr>
<tr>
<td>hypertension</td>
<td>1.184</td>
<td>0.352</td>
<td>0.001</td>
<td>3.269</td>
<td>1.64 – 6.52</td>
</tr>
<tr>
<td>age</td>
<td>0.087</td>
<td>0.303</td>
<td>0.023</td>
<td>1.988</td>
<td>1.10 – 3.60</td>
</tr>
<tr>
<td>constant</td>
<td>-3.210</td>
<td>0.359</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Variables excluded from the equation were: sex, smoking, diabetes, hypercholesterolemie, possible family history, TLR4 +896 G allele carrier-ship, and CD14 (-260) TT genotype.
7.5 Discussion

In the present study we assessed the effect of the common TLR4 +896 A>G and CD14 (-260) C>T polymorphisms on the extent of atherosclerosis in patients with peripheral arterial disease. Using a multivariate logistic regression model, our data showed that the carrier trait profile TLR4 G-allele in combination with the CD14 TT-genotype had the strongest effect on the extent of atherosclerotic disease.

In the present study, the overall TLR4 +896 G allele frequency in Dutch Caucasian patients with peripheral arterial disease was approximately 10%. This was comparable to our earlier reported TLR4 +896 G allele frequencies in Dutch Caucasian women with or without tubal pathology, to the TLR4 +896 G allele frequencies in male patients with angiographically documented coronary atherosclerosis, and to those in patients with meningococcal disease reported by Read et al. Surprisingly, we did observe an interesting, significant partitioning in TLR4 +896 G allele frequency, being approximately 8% in patients with PAD only, 12% in patients with 2 vascular territories affected by atherosclerosis, and being approximately 19% in patients with extensive atherosclerotic disease affecting 3 vascular territories. This represents a significant trend (χ² for trend =7.6, P=0.0074). This seems contradictory to earlier reports showing that the TLR4 polymorphism protected against the development of early carotid plaque and unstable coronary events. In contrast to these earlier studies, we did not limit our analysis of the relation between TLR4 SNPs and atherosclerosis to coronary events or carotid plaques only. Instead, in order to take into account the systemic nature of atherosclerosis we considered the clinical manifestation of atherosclerosis in the coronary, cerebral and peripheral circulation, and aorta. The early steps in atherogenesis often represent a response of the innate immune system to stimuli such as the accumulation and modification of lipoproteins in the arterial intima, whereas the progression of atherosclerosis depends on inappropriate activation of the innate and acquired immune system by both endogenous and exogenous stimuli. The TLR4 +896 A>G polymorphism may very well be associated with delayed development of early atherosclerotic plaques, theoretically through a blunted innate response, thereby explaining the inverse relationship observed between this polymorphism and carotid intima-media thickness and ultrasonographically detected, asymptomatic carotid plaque. However, once the initial steps in atherogenesis have occurred, as is the case in PAD patients with an average age of 65 years, this TLR4 polymorphism involved in the clearance of endogenous and exogenous (bacterial) atherogenic stimuli, may be associated with exacerbated development of advanced atherosclerotic lesions, as has been shown in our study. The interaction between the TLR4 polymorphism and atherosclerosis may also be influenced by the patient's pharmacotherapy. Boekholdt et al showed that the
**TLR4** +896 G allele had a significantly lower risk of cardiovascular events only for pravastatin users. Furthermore, the pravastatin related risk reduction was more pronounced for **TLR4** +896 G allele carriers.\(^{11}\)

The **CD14** promotor region polymorphism has been reported to enhance transcriptional activity of the **CD14** gene.\(^{24}\) The TT genotype is associated with higher serum levels of sCD14, increased density of **CD14** on monocytes, higher prevalence of *Chlamydia pneumoniae* infections and enhanced chlamydia-stimulated TNF-\(\alpha\) production.\(^{25,26}\) The **CD14** (-260)C>T polymorphism has been associated with carotid artery intima media thickness,\(^{72}\) increased stroke risk\(^{15}\) and acute myocardial infarction.\(^{14,15}\) In contrast, Ito *et al.* and Longobardo *et al.* showed no relation between this polymorphism and cerebrovascular disease\(^{16}\) or myocardial infarction,\(^{17}\) respectively. Likewise, using a nested case-control study within a large prospective cohort of apparently healthy individuals, Zee and co-workers demonstrated a lack of association between the **CD14** (-260)C>T polymorphism and (thromboembolic) stroke.\(^{18}\) Similarly, among the patients with peripheral arterial disease in our study, the **CD14** SNP was not related to the extent of atherosclerotic disease.

A considerable number of SNPs have been identified in innate immunity genes belonging to the TLR response pathway, among which 44 SNPs in **TLR4** and 37 in **CD14**.\(^{27}\) Therefore, when analysing the role of specific SNPs in relation to severity of a specific disease, it is imperative to consider polymorphisms with functional implications that fit into a certain pathogenic paradigm. Otherwise, bearing in mind the considerable number of SNPs, there is a considerable possibility that statistically significant associations are described that may be based on chance only. Although it is accepted that the **CD14** (-260)C>T polymorphism is functional, questions remain regarding the functionality of the **TLR4** +896 A>G polymorphism. Even though the homozygous genotype is functional,\(^{7}\) the heterozygous genotype which has been associated with atherosclerotic disease in several studies, presents no deficit in the recognition of LPS.\(^{28}\) However, this does not exclude that the heterozygous genotype is functional for other agonists which have not been tested on functionality in this heterozygous type, including human and chlamydial heat shock protein 60. Considering the complexity of the innate immune system and its high degree of genetic variation, a significant number of collateral pathways may exist for innate immune responses, which differ in their cofactor requirements and their pattern recognition specificities. Ideally, all pathways should be taken into consideration and carrier traits instead of individual SNPs should be regarded when studying genetic predisposition of the innate immune system in relation to atherosclerosis and other inflammatory diseases which are all multifactorial and polygenic diseases. To illustrate this, it has recently been shown that carriage of multiple pro-inflammatory polymorphisms conferred a greater risk of
noncardia gastric cancers, with odds ratios increasing from 2.8 for one to 27.3 for more than 3 high risk SNPs. In this study we analysed the combined effect of polymorphisms in two components of the innate immune system on the extent of atherosclerotic disease in patients with advanced atherosclerosis. Intriguingly, the combination of the CD14 SNP resulting in transcriptional activity of the CD14 gene and carriehip of the TLR4 +896 G allele was related to the extent of atherosclerotic burden in patients with PAD. Failure to take into consideration SNPs in both genes may account for the contradictory results when CD14- and TLR4 polymorphisms were studied individually with regard to atherosclerosis. Similarly, failure to correct for relevant cardiovascular risk factors is another important aspect that has to be taken into account in this kind of studies. In our population, the significance of the association between the combined TLR4-CD14 carrier trait and extent of atherosclerotic diseased was strengthened after correction for relevant risk factors (Table 7.5).

In conclusion, considering the importance of (innate) immune responses in the development of atherosclerosis, our data provide an explanation for individual susceptibility to atherosclerosis based on genetic variability of a combination of genes involved in innate immune regulation. A carrier trait of a combination of TLR4 and CD14 SNPs, rather than each polymorphism individually, was associated with extent of atherosclerotic disease using a multivariate logistic regression model.
7.6 References

Section 5

Clinical intervention trial
Chapter 8

Secondary Prevention of Atherosclerosis through
*Chlamydia pneumoniae* Eradication (SPACE trial): a
randomised clinical trial in patients with peripheral
arterial disease

Vainas T, Stassen FR, Schurink GW, Tordoir JH, Welten RJ,
van den Akker LH, Kurvers HA, Bruggeman CA, Kitslaar PJ

*Eur J Vasc Endovasc Surg.* 2005, 29:403-411
8.1 Abstract

Background
Sero-epidemiological and animal experimental studies suggest that *Chlamydia pneumoniae* infections play an important role in the development of atherosclerosis. Clinical trials have shown contradictory results regarding the efficacy of antibiotics to prevent atherosclerosis-related complications in patients with coronary artery disease. Our aim was to study the effect of a short course of azithromycin on the incidence of cardiovascular events and peripheral vascular function in patients with stable peripheral arterial disease (PAD).

Materials and methods
Five hundred and nine PAD-patients were randomised to receive a 3-day course of azithromycin (500mg daily) or placebo, with 2 years follow-up. *Chlamydia pneumoniae* serology was determined at baseline. Clinical endpoints were death (all cause mortality), coronary events (myocardial infarction, unstable angina, and/or coronary revascularization procedures), cerebral events (stroke, transient ischemic attack, and/or carotid endarterectomy) and peripheral arterial complications (increased PAD-symptoms with decreased ankle-brachial pressure index (ABPI), and/or peripheral revascularization procedures). Hemodynamic endpoint was a 0.1-point decrease of ABPI after 12 months.

Results
Five hundred and nine patients (160 women) with an atherosclerotic risk factor profile were randomised, 257 patients to azithromycin and 252 to placebo. Four hundred and forty nine patients (88%) had intermittent claudication and 60 (12%) had critical limb ischemia. By 24-month follow-up, 182 patients (36%) developed 252 complications (45 deaths, 34 coronary events, 34 cerebral events and 139 peripheral arterial complications). *Chlamydia pneumoniae* IgG titres were associated with the development of cardiovascular events. Nevertheless, the number of complications (131 in the azithromycin group vs. 121 in the placebo group) and the number of patients that developed complications (98 (38%) in the azithromycin vs. 84 (33%) in the placebo group) was comparable in both treatment groups. Life table analysis showed no effect of azithromycin on survival. Azithromycin had no effect on the hemodynamic endpoint.

Conclusion
A short-term course of azithromycin offers no benefits for survival or improved ankle pressure in PAD-patients.
8.2 Introduction

Recently, it has become evident that atherosclerosis is an inflammatory disease. Consequently, infections triggering immune responses, may play an important role in the initiation or propagation of atherosclerosis. Already in the early 20th century, before the inflammatory nature of atherosclerosis became widely accepted, it had been suggested that infections may contribute to the development of atherosclerosis. However, it was not before 1978 that it had been proven experimentally that infections may lead to the development of atherosclerosis. Subsequently, it had been observed that acute (respiratory tract) infections were associated with acute coronary events, and especially that *Chlamydia pneumoniae* infections were associated with coronary artery disease. The identification of *Chlamydia pneumoniae* in atherosclerotic plaques further supported the notion that *Chlamydia pneumoniae* infections may be associated with the development of atherosclerosis. The infection hypothesis really gained momentum when 2 groups independently reported that antibiotics may prevent cardiovascular events in patients with coronary artery disease. Since then, antibiotic trials conducted throughout the world showed contradictory results regarding the efficacy of antibiotics for prevention of atherosclerotic complications. Most of the studies showing favourable results were of small sample size, included patients with abdominal aortic aneurysms or peripheral arterial disease, or used surrogate endpoints such as flow-mediated dilation. In contrast, 3 larger studies of patients with coronary artery disease demonstrated no beneficial effect of antibiotics in the prevention of acute coronary events and/or death.

Peripheral arterial disease represents a manifestation of atherosclerosis affecting up to 20% of primary care attendees. The systemic nature of atherosclerosis is shown by the high prevalence of coronary and cerebral arterial disease in these patients. Considering the prevalence of PAD and the significant cardiovascular co-morbidity, this group of patients is very suitable to study the effect of interventions altering the progression of atherosclerosis, both in terms of clinically relevant events and in terms of easily accessible surrogate hemodynamic endpoints such as the ankle-brachial pressure index (ABPI).

*Chlamydia pneumoniae* is an obligate intracellular microbe showing a tropism for macrophages. The antichlamydial antibiotic azithromycin yields high intracellular macrophage concentrations after a 3 day course, that 10 days after the last dose still is in the bactericidal range (>32 mg/l). Furthermore, compared to other macrolide antibiotics, such as roxithromycin, azithromycin has a better gastro-intestinal tolerability. Considering the above, we tested the hypothesis that, in PAD patients, a short course of azithromycin would reduce the subsequent incidence of atherosclerotic events of the coronary.
cerebral and peripheral arterial circulation and would influence (changes in) ABPI.

8.3 Materials and methods

Patients with symptomatic peripheral arterial disease (intermittent claudication or critical limb ischaemia) were recruited at the surgical clinics of a university hospital and two affiliated teaching hospitals. Inclusion and exclusion criteria are given in Table 8.1. The study was approved by the medical ethics committees of all participating centres and conformed with the principles outlined in the declaration of Helsinki. All patients entered the study after giving written informed consent.

Table 8.1 Inclusion- and exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Symptomatic PAD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABP&lt;0.9 or decrease of ankle pressure with 25 mmHg after standardized treadmill exercise</td>
</tr>
<tr>
<td></td>
<td>Recent antibiotic use (&lt;3 months)</td>
</tr>
<tr>
<td></td>
<td>Recent vascular (coronary, cerebral, peripheral) events (&lt;6 months)</td>
</tr>
<tr>
<td></td>
<td>Recent vascular (coronary, cerebral, peripheral) intervention (&lt;12 months)</td>
</tr>
<tr>
<td></td>
<td>Renal failure</td>
</tr>
<tr>
<td></td>
<td>Liver failure</td>
</tr>
<tr>
<td></td>
<td>Life expectancy &lt;2 years</td>
</tr>
<tr>
<td></td>
<td>Malignancy</td>
</tr>
<tr>
<td></td>
<td>Inflammatory co-morbidity</td>
</tr>
</tbody>
</table>

The study was designed as a randomised, double-blind, placebo controlled, secondary prevention trial of a short course of azithromycin (3 days) in patients with PAD. The hypothesis was that the antibiotic treatment would lead to a clinically significant, i.e., 50% reduction of the combined event rate. In order to calculate the required number of patients needed to show this effect, the data reported by Dormandy et al. were used. With an annual event rate of 15%, 204 patients in every treatment group were needed to observe the desired effect after 2 years, with a power (β) of 0.95 and a type I error (α) of 0.05. To ensure sufficient events within two years, target recruitment was 500 patients.

At inclusion patients underwent physical examination. The presence of atherosclerotic risk factors was assessed and cardiac and cerebrovascular co-morbidities were documented. The ABPI was measured in the supine position after a 15 minute rest period. The ankle pressure was measured in the posterior tibial and dorsal pedal artery. The highest ankle pressure was used to determine the ABPI.

After the baseline clinical and hemodynamic evaluation, venous blood was drawn and patients were randomised to receive a 3-day course of azithromycin
(500mg daily) or an identically looking placebo. In order to assess drug tolerability and compliance, patients were asked to keep a 'treatment logbook'. The randomisation list was computer generated in blocks of 5 and randomisation was performed in 6 strata, i.e., three participating clinics by two clinical presentations (claudication or critical limb ischemia). Patients, attending surgeons and co-ordinating scientist were blinded for the experimental intervention.

8.3.1 Outcomes

The primary endpoint was the composite of "all cause mortality and/or any coronary/cerebral/peripheral arterial event". We selected all cause mortality as part of the primary outcome variable, as often the cause of death cannot be established clearly for patients dying outside the hospital. Coronary events included myocardial infarction (defined by chest pain, elevated cardiac enzymes, and/or new diagnostic q-waves in at least two contiguous leads), de novo unstable angina pectoris (defined by chest pain, supported by either ECG changes suggestive of ischemia or elevated level of cardiac enzymes, greater than normal but not diagnostic for myocardial infarction), and/or any coronary revascularization procedure (PTCA or coronary artery bypass grafting). A cerebral event was any stroke or transient ischemic attack (diagnosis based on clinical symptoms and characteristic changes on cerebral computed tomography and/or magnetic resonance imaging). A peripheral arterial event was defined as any episode of deteriorating symptoms (e.g. increased severity of ischaemic pain) that was accompanied by either a decrease in ABPI of at least 0.1 points and/or by any peripheral revascularization procedure (PTA or reconstructive vascular surgery).

8.3.2 Hemodynamic endpoint

In addition to aforementioned clinical endpoints, changes in ABPI over a 12-month period were analysed. A change in ABPI of at least 0.1 point was considered significant (significant increase or decrease).

8.3.3 Chlamydia pneumoniae serology

Chlamydia pneumoniae IgA titres were determined by a commercially available enzyme immunoassay (Labsystems, Finland) as previously described. Titres were calculated from optical density readings according to the manufacturer's instructions and were expressed in enzyme immunonounds (EIUs). Although no consensus has been reached so far regarding the definition of Chlamydia pneumoniae seropositivity, patients with an IgA titre ≥16 EIUs were considered seropositive in view of previously published studies showing an
association between *Chlamydia pneumoniae* seropositivity and atherosclerosis using this cut off point.\textsuperscript{23-25}

### 8.3.4 Statistics

The $\chi^2$-test was used for analysis of dichotomous variables and the Mann-Whitney $U$-test for analysis of continuous variables. Wilcoxon signed ranks test was used to compare average ABPI at inclusion and at 12 months. Kaplan-Meier with log rank statistics was used for the analysis of event free survivals in both treatment groups. Analyses were carried out on an intention to treat basis. All statistics were performed on SPSS 10.0 for windows software package (SPSS Inc., Chicago, IL, USA).

### 8.4 Results

Five hundred and nine patients were randomised. As expected a high proportion of our patients had (multiple) conventional cardiovascular risk factors (Table 8.2). Efforts were made to assure that all patients received best medical (secondary preventive) treatment. This implies that all patients were given antplatelet therapy, that patients with untreated hyperglycemia or hypertension were referred to a physician for serum glucose and blood pressure regulation, and that patients with cholesterol >5 mmol/l were treated with statins. The randomisation yielded two comparable groups (azithromycin group: n=257; placebo group: n=252) with respect to the prevalence of cardiovascular risk factors (Table 8.2), severity of PAD (Table 8.3), type of vascular treatment at inclusion and degree of vascular co-morbidity (Table 8.3), baseline ABPI (Table 8.3) and *Chlamydia pneumoniae* serology (Table 8.4).

Patients were followed for a median of 24 months (range: 1 - 44 months).

<table>
<thead>
<tr>
<th>Table 8.2 Patient characteristics.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total group</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>Average age (SD), years</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Cardiovas. Fam. History(^a)</td>
</tr>
<tr>
<td>Smoking(^b)</td>
</tr>
<tr>
<td>Diastolic(^c)</td>
</tr>
<tr>
<td>Hypertension(^d)</td>
</tr>
<tr>
<td>Diabetes(^e)</td>
</tr>
</tbody>
</table>

\(^a\) first degree relative with first ischemic cardiovascular event before age of 70, \(^b\) currently smoking or stopped <10 year ago, \(^c\) fasting cholesterol level >5.0 mmol/l and/or triglyceride level >1.95 mmol/l and/or use of antilipemic medication, \(^d\) systolic blood pressure >160 mmHg and/or diastolic blood pressure >95 mmHg and/or use of antihypertensive medication, \(^e\) fasting glucose level >7mmol/l and/or use of antihyperglycemic medication/insulin. ns: not significant.
Table 5.3  Cardiovascular status of the PAD patients at inclusion. This table shows the degree of PAD, ABPI, the planned vascular treatment and the cardiovascular co-morbidity of the patients at inclusion in the study.

<table>
<thead>
<tr>
<th>Degree of PAD (Fontaine class)</th>
<th>Total</th>
<th>Azithromycin</th>
<th>Placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudication (Fontaine II)</td>
<td>449 (88%)</td>
<td>227 (88%)</td>
<td>222 (88%)</td>
<td>ns</td>
</tr>
<tr>
<td>Critical limb ischemia (Fontaine III/IV)</td>
<td>60 (12%)</td>
<td>30 (12%)</td>
<td>30 (12%)</td>
<td>ns</td>
</tr>
<tr>
<td>Average (SD) ABPI</td>
<td>0.63 (0.18)</td>
<td>0.63 (0.18)</td>
<td>0.62 (0.18)</td>
<td>ns</td>
</tr>
<tr>
<td>Vascular intervention plan at inclusion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conservative</td>
<td>344 (68%)</td>
<td>178 (60%)</td>
<td>166 (68%)</td>
<td>ns</td>
</tr>
<tr>
<td>Balloon Angioplasty (PTA)</td>
<td>92 (18%)</td>
<td>40 (16%)</td>
<td>52 (21%)</td>
<td>ns</td>
</tr>
<tr>
<td>Reconstructive vascular surgery</td>
<td>73 (14%)</td>
<td>39 (15%)</td>
<td>34 (13%)</td>
<td>ns</td>
</tr>
<tr>
<td>Vascular co-morbidity:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD</td>
<td>163 (32%)</td>
<td>79 (31%)</td>
<td>84 (33%)</td>
<td>ns</td>
</tr>
<tr>
<td>CVD</td>
<td>76 (15%)</td>
<td>41 (16%)</td>
<td>35 (14%)</td>
<td>ns</td>
</tr>
<tr>
<td>AAA</td>
<td>50 (10%)</td>
<td>24 (9%)</td>
<td>26 (10%)</td>
<td>ns</td>
</tr>
<tr>
<td>Combined CAD &amp; CVD</td>
<td>36 (7%)</td>
<td>18 (7%)</td>
<td>18 (7%)</td>
<td>ns</td>
</tr>
</tbody>
</table>

CAD: coronary artery disease; CVD: cerebrovascular disease; AAA: abdominal aortic aneurysm; PAD: peripheral arterial disease; ABPI: ankle-brachial pressure index; ns: not significant.

Among the patients, 88% suffered from intermittent claudication and 12% had critical limb ischemia, i.e., resting pain and/or ischemic tissue loss (Table 8.3). The baseline treatment plan was conservative in the majority of cases (Table 8.3). Approximately one third of the patients were, at inclusion, scheduled to undergo an early intervention, either catheter based (18%) or vascular surgery (14%), that was not considered a peripheral arterial endpoint. A significant proportion of patients suffered from cardiovascular co-morbidities, i.e., 32% had co-existent coronary artery disease, 15% had cerebrovascular disease and 10% were diagnosed with or had been treated for an abdominal aortic aneurysm. The average (SD) ABPI was 0.63 (0.18). The average (SD) Chlamydia pneumoniae IgA titre was 28 (30) EIU and 52% of patients were considered seropositive (having an IgA>16 EIU) (Table 8.4).

Table 8.4  Chlamydia pneumoniae IgA titres and seropositivity.

<table>
<thead>
<tr>
<th></th>
<th>Entire population N=509</th>
<th>Azithromycin N=257</th>
<th>Placebo N=252</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average (SD) IgA, EIU</td>
<td>27.6 (39.8)</td>
<td>26.6 (28.7)</td>
<td>28.7 (32.8)</td>
<td>0.86*</td>
</tr>
<tr>
<td>N (%) IgA+</td>
<td>265 (52)</td>
<td>139 (54)</td>
<td>126 (50)</td>
<td>0.42*</td>
</tr>
</tbody>
</table>

* Mann-Whitney U test, "χ²-test. EIU. Enzyme Immuno Units; IgA+ indicates Chlamydia pneumoniae IgA seropositivity (IgA>16 EIU).
Azithromycin was well tolerated by the patients and self-reported compliance was excellent. Approximately 95% of patients completed the assigned treatment, i.e., 242/257 (94%) azithromycin treated patients and 242/252 (95%) placebo treated patients. Fifteen patients in the azithromycin group and 10 patients in the placebo group discontinued treatment mainly due to (mild) gastro-intestinal complaints (diarrhea, nausea, vomiting, flatulence, stomach-pain). One patient in the azithromycin group developed a skin rash.

8.4.1 Follow-up, cardiovascular events and azithromycin

In total, 182 patients (36%) developed 252 events during follow-up (Table 8.5). Thirty-three patients (6%) developed 34 coronary events, 33 patients (6%) had 34 cerebrovascular events, 108 patients (21%) developed 139 peripheral arterial events and 45 patients (9%) died. Neither the total number of cardiovascular complications, nor the number of patients developing an event differed between groups. Ninety-eight patients (38%) treated with azithromycin developed 131 complications vs. 84 patients (33%) developing 121 cardiovascular events in the placebo group (Mann-Whitney U test for comparison of total number of events, \( P = 0.37 \), and \( \chi^2 \)-test for comparison of fractions of patients reaching the combined endpoint, \( P = 0.26 \)). In addition, the fraction of patients developing multiple events did not differ between either group (\( \chi^2 \)-test, \( P = 0.57 \)). Cumulative freedom from the combined endpoint (death and/or any cardiovascular event, Figure 8.1) or from death, coronary, cerebrovascular, or peripheral event separately was not influenced by azithromycin (Kaplan Meier with Log rank test, \( P = 0.32 \) for survival differences in the combined endpoint).

Table 8.5 Follow-up data regarding the effect of azithromycin on cardiovascular and hemodynamic endpoints.

<table>
<thead>
<tr>
<th>Event Type</th>
<th>Azithromycin group</th>
<th>Placebo group</th>
<th>( P^a )</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( N (%) ) events</td>
<td>( N (%) ) patients</td>
<td>( N (%) ) events</td>
<td>( N (%) ) patients</td>
</tr>
<tr>
<td>Coronary events</td>
<td>19 (7)</td>
<td>15 (8)</td>
<td>14 (9)</td>
<td>0.41</td>
</tr>
<tr>
<td>Cerebral events</td>
<td>18 (7)</td>
<td>16 (6)</td>
<td>16 (6)</td>
<td>0.75</td>
</tr>
<tr>
<td>Peripheral events</td>
<td>74 (23)</td>
<td>65 (20)</td>
<td>50 (20)</td>
<td>0.46</td>
</tr>
<tr>
<td>Death</td>
<td>20 (8)</td>
<td>25 (10)</td>
<td>25 (10)</td>
<td>0.40</td>
</tr>
<tr>
<td>All events</td>
<td>131 (38)</td>
<td>121 (33)</td>
<td>84 (33)</td>
<td>0.37</td>
</tr>
<tr>
<td>Median (range)</td>
<td>24.3 (0.4-1)</td>
<td>24.9 (0.4-5)</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Average (SD) ABPI at inclusion</td>
<td>0.64 (0.18)</td>
<td>0.62 (0.16)</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Average (SD) ABPI at 12 months</td>
<td>0.71 (0.19)</td>
<td>0.70 (0.22)</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Average (SD) change in ABPI in 12 months</td>
<td>0.07</td>
<td>0.08</td>
<td>0.71</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Mann-Whitney U test for comparison of number of events or average follow-up period between groups, and \( ^b \) \( \chi^2 \)-test for comparison of fraction of patients reaching events between groups.
8.4.2 ABPI and azithromycin

The average (SD) ABPI at inclusion was 0.63 (0.18). This increased significantly to 0.70 (0.21) in the total group after one year (Wilcoxon signed rank test, \( P=0.001 \)). This increase in ABPI resulted mainly from interventions (PTA or surgery). For the patients who did not undergo any intervention within the first year, the ABPI increased slightly, though significantly from 0.66 to 0.68 (Wilcoxon signed rank test, \( P=0.009 \)). Given the reproducibility of ABPI measurements this difference may not reflect an actual clinical improvement. In contrast, the ABPI increased from 0.58 (0.17) to 0.74 (0.23) in those patients who had an intervention within one year after randomisation. This difference is both clinically and statistically significant (Wilcoxon signed rank test, \( P=0.001 \)). Whether the patients had undergone an intervention or not, azithromycin did not affect (the change in) ABPI at one year (Mann-Whitney \( U \) Test, \( P=0.71 \)) (Table 8.5). The number of patients showing a significant increase or decrease in ABPI (by 0.1 point) did not differ significantly between azithromycin- and placebo-treated patients (\( \chi^2 \)-test, \( P=0.21 \)). Exclusion of patients with critical limb ischemia (12%) from the analyses did not alter any of the above associations.

8.4.3 *Chlamydia pneumoniae* serology, extent of PAD and future events

The mean *Chlamydia pneumoniae* IgA-titre and the prevalence of *Chlamydia pneumoniae* seropositivity (IgA >16EIUs) were comparable in the azithromycin and placebo group (Table 8.4). *Chlamydia pneumoniae* serology was not
related to severity of peripheral arterial disease (Fontaine class or ABPI) at inclusion. However, patients who reached a cardiovascular (combined) endpoint (Mann-Whitney U test, \( P = 0.02 \)), and especially a peripheral arterial endpoint (Mann-Whitney U test, \( P = 0.01 \)) had a higher average IgA titre compared to patients who remained free of events (Table 8.6). *Chlamydia pneumoniae* IgA seropositivity was related to the development of the combined endpoint (\( \chi^2 \)-test, \( P = 0.03 \)), and of peripheral arterial events more specifically (\( \chi^2 \)-test, \( P = 0.05 \)) (Table 8.6).

Despite the association between *Chlamydia pneumoniae* serology and the occurrence of events, azithromycin treatment did not reduce the risk of any event in the total patient group or in the subgroup of seropositive patients.

**Table 8.6** Prevalence of *Chlamydia pneumoniae* IgA seropositivity (IgA >16 EIU) and average (SD) *Chlamydia pneumoniae* IgA titre in the patients in relation to cardiovascular endpoints.

<table>
<thead>
<tr>
<th></th>
<th>IgA seropositivity</th>
<th>Average (SD) IgA titre (EIUs)</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (N=509)</td>
<td>52</td>
<td>28 (30)</td>
<td></td>
</tr>
<tr>
<td>Reaching Combined endpoint (N=182)</td>
<td>59</td>
<td>32 (34)</td>
<td>0.02</td>
</tr>
<tr>
<td>Free of combined endpoint (N=327)</td>
<td>49</td>
<td>25 (28)</td>
<td></td>
</tr>
<tr>
<td>Reaching Coronary event (N=33)</td>
<td>50</td>
<td>29 (34)</td>
<td>0.69</td>
</tr>
<tr>
<td>Free of coronary event (N=476)</td>
<td>52</td>
<td>28 (31)</td>
<td></td>
</tr>
<tr>
<td>Reaching Cerebral event (N=32)</td>
<td>60</td>
<td>27 (27)</td>
<td>0.49</td>
</tr>
<tr>
<td>Free of Cerebral event (N=477)</td>
<td>51</td>
<td>28 (31)</td>
<td></td>
</tr>
<tr>
<td>Reaching PAD event (N=108)</td>
<td>61</td>
<td>36 (37)</td>
<td>0.01</td>
</tr>
<tr>
<td>Free of PAD event (N=401)</td>
<td>49</td>
<td>25 (28)</td>
<td></td>
</tr>
<tr>
<td>Death (N=45)</td>
<td>59</td>
<td>32 (30)</td>
<td>0.26</td>
</tr>
<tr>
<td>Free of death (N=464)</td>
<td>51</td>
<td>27 (31)</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \chi^2 \)-test, \( ^b \)Mann-Whitney U test.

### 8.5 Discussion

Our data shows that a short course of azithromycin does not reduce the risk of death or cardiovascular events in patients with lower limb ischemia secondary to occlusive peripheral atherosclerotic disease, irrespective of their serological status. Furthermore, azithromycin treatment did not affect the ABPI in these patients.

A number of randomised clinical trials assessing the effect of antibiotics on atherosclerosis related complications or biochemical and hemodynamic endpoints have been published (Table 8.7). Generally, in those studies reporting a benefit from antibiotics, a small number of patients have been
included or followed for only a limited time, so that only a modest number of events has been observed under antibiotic exposure. Larger trials with longer follow-up periods have, in accordance with our data, unequivocally shown that antibiotics have no effect in reducing the risk for clinical cardiovascular endpoints. In total, including the present study, 12,901 patients have been included in the randomised clinical antibiotic trials with clinical cardiovascular endpoints (Table 8.7). The studies reporting beneficial effects of antibiotics on cardiovascular risk prevention have included 1583 patients, and 858 of these were randomised to receive the experimental treatment. In contrast, 11,318 patients were included and 5,660 patients were randomised to receive an antibiotic in the negative studies. In order to demonstrate the potentially beneficial effects of antibiotics on atherosclerosis using a limited number of patients (and cardiovascular events), several authors have chosen to study surrogate endpoints that might be more sensitive to the anti-atherogenic effects of antibiotics, such as carotid intima media thickness, baseline NO production, flow-mediated dilatation, aortic expansion rate, matrix metalloproteinase metabolism, or presence of Chlamydia pneumoniae DNA in vascular tissue.

Table 8.7 Overview of randomised clinical trials of antibiotics in cardiovascular disease.

<table>
<thead>
<tr>
<th>Trial/Author</th>
<th>Diagnosis</th>
<th>Treatment</th>
<th>Follow-up</th>
<th>N</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gupta et al.</td>
<td>CAD</td>
<td>azithromycin</td>
<td>3-6 d</td>
<td>18 mo</td>
<td>60</td>
</tr>
<tr>
<td>ISAR-3</td>
<td>stent</td>
<td>roxithromycin</td>
<td>4 w</td>
<td>12 mo</td>
<td>1,010</td>
</tr>
<tr>
<td>CLARIFY</td>
<td>ACS</td>
<td>clarithromycin</td>
<td>3 mo</td>
<td>18 mo</td>
<td>1,48</td>
</tr>
<tr>
<td>STAMINA</td>
<td>ACS</td>
<td>azithromycin</td>
<td>3 d</td>
<td>12 mo</td>
<td>325</td>
</tr>
<tr>
<td>Wiesli et al</td>
<td>PAD</td>
<td>roxithromycin</td>
<td>3 d</td>
<td>1 w</td>
<td>107</td>
</tr>
<tr>
<td>ROXIS</td>
<td>ACS</td>
<td>azithromycin</td>
<td>30 d</td>
<td>6 mo</td>
<td>202</td>
</tr>
<tr>
<td>ACADEMIC</td>
<td>CAD</td>
<td>azithromycin</td>
<td>3 mo</td>
<td>24 mo</td>
<td>302</td>
</tr>
<tr>
<td>ANTIBIO</td>
<td>ACS</td>
<td>roxithromycin</td>
<td>6 w</td>
<td>12 mo</td>
<td>872</td>
</tr>
<tr>
<td>WIZARD</td>
<td>CAD</td>
<td>azithromycin</td>
<td>3 mo</td>
<td>24 mo</td>
<td>7,722</td>
</tr>
<tr>
<td>AZACS</td>
<td>ACS</td>
<td>azithromycin</td>
<td>5 d</td>
<td>24 mo</td>
<td>1,439</td>
</tr>
<tr>
<td>Sander et al</td>
<td>CVD</td>
<td>roxithromycin</td>
<td>4 w</td>
<td>48 mo</td>
<td>272</td>
</tr>
<tr>
<td>SPACE</td>
<td>CAD</td>
<td>azithromycin</td>
<td>3 d</td>
<td>24 mo</td>
<td>509</td>
</tr>
</tbody>
</table>

N of patients in positive clinical endpoint trials: 1,583
N of patients in negative clinical endpoint trials: 11,318
N of patients in all clinical endpoint trials: 12,901

Ab: antibiotic; AB: antibiotic group; ACS: acute coronary syndromes; amoxi: amoxicillin; azi: azithromycin; CAD: stable coronary artery disease; clar: clarithromycin; CVE: cardiovascular event; CVD: cerebrovascular disease; d: days, mo: months; PAD: peripheral arterial disease; PL: placebo group; roxi: roxithromycin; w: weeks. =: no effect; ↓: reduced.

The demonstration of (a lack of) beneficial effects in cardiovascular event reduction by the clinical trials does not necessarily (dis)prove the infectious
concept of atherosclerosis. Accruing sero-epidemiological, histological, in vitro, and animal experimental data strongly suggest that infections play a (modulatory) role in atherogenesis in general and in the development of peripheral arterial disease in particular. In our study population, the baseline Chlamydia pneumoniae serology was not related to the extent of atherosclerotic disease in terms of clinical severity or ABPI at inclusion in the study. However, we observed an association between Chlamydia pneumoniae serology and development of future cardiovascular events and especially peripheral arterial events during follow-up. In spite of that, the 3-day azithromycin treatment did not result in a significant reduction of events in this population. In line with this findings, antibiotic treatment has proven to have no effect on Chlamydia pneumoniae titres in cardiovascular patients.

The lack of beneficial effects from antibiotics in the prevention of atherosclerosis-related complications may be related to inadequate study medication and/or sub-optimal patient selection. We chose azithromycin as this was the most commonly used antibiotic in the trials with cardiovascular endpoints. Azithromycin has proven to be well tolerated and can be used safely in patients with atherosclerotic disease. In vitro work suggested that vascular Chlamydia pneumoniae strains were susceptible to azithromycin as well as to roxithromycin and doxycycline. Instinctively one would expect that higher doses of antibiotics or longer treatment duration should have been more effective in reducing cardiovascular risk. Surprisingly, benefit from azithromycin treatment has been shown by studies with short course treatments (3-6 days, total azithromycin dose varying between 1500 and 3000mg), whereas trials with prolonged azithromycin treatment regimes did not show any beneficial effects. Irrespective of the duration of a single antibiotic course, published data suggest that if there is any effect, it may wear off with time. A preliminary report of the ROXIS trial showed a significant reduction of the primary endpoint (cardiac ischaemic death, myocardial infarction, and severe recurrent ischaemia) by roxithromycin at 31 days whereas this effect had worn off at 6 months. Similarly, roxithromycin seemed to reduce intima-media thickness progression in patients with cerebrovascular disease during the first two years after treatment, but this effect was lost after 4 years. A temporary effect of antibiotic treatment has also been suggested by the data of the WIZARD trial showing a reduced death or myocardial infarction risk at six months after randomisation but not after a longer follow-up. Bearing in mind that our study population was too small to observe a sufficient number of early events (i.e., within six months from randomisation), our data did not support an early effect of azithromycin on cardiovascular events that waned over time. Antibiotics may have only a transient effect, as patients may contract recurrent Chlamydia pneumoniae infections or suffer from Chlamydia pneumoniae reactivation, after the microbe has been forced into a latent state, instead of being eliminated by
the antibiotic treatment. Indeed, in an *in vitro* continuous infection model of
epithelial cells, prolonged azithromycin treatment reduced but did not
completely eliminate *Chlamydia pneumoniae*.44 Furthermore, *Chlamydia
pneumoniae* carried within circulating monocytes has shown to be refractory to
standard anti-chlamydial treatments such as azithromycin.45 It remains to be
seen whether longer-duration antibiotic treatments or repeated antibiotic
treatment cycles would result in a significant cardiovascular risk reduction.
In addition to *Chlamydia pneumoniae*, several other pathogens have been
implicated in atherogenesis such as *Helicobacter pylori,46* cytomegalovirus,47
and herpes simplex virus.48 In fact, it seems that the total pathogen burden
more than an individual micro-organism is associated with increased risk of
developing cardiovascular disease.41,49 Thus, treatments aimed at *Chlamydia
pneumoniae* may not cover all contributory pathogens, explaining the failure of
current antibiotic regimes.
A critical issue of the randomised clinical trials is the selection of patients.
Several authors have chosen to include only *Chlamydia pneumoniae*
seropositive patients.11-13,37 High *Chlamydia pneumoniae* antibody titres have
been associated with presence of atherosclerosis and the development of
cardiovascular events. However, antibiotics do not seem to affect *Chlamydia
pneumoniae* titres.20,31,32,36,42 This matter is further complicated by the fact that
no uniform criteria for *Chlamydia pneumoniae* seropositivity have been
formulated.22 Therefore, since the prevalence of *Chlamydia pneumoniae*
antibodies in the general population is high, we chose to include patients
regardless of their serological status.50
Atherosclerotic disease status at entry may be important for the outcome of
antibiotic intervention trials. Experimental data suggests that *Chlamydia
pneumoniae* may promote all stages of atherosclerosis development, i.e.,
plaque growth, rupture, thrombosis and neointimal formation after vascular
intervention.51 In previously published trials, patients with acute coronary
syndromes (i.e., acute myocardial infarction, unstable angina or non-q wave
infarcts), stable atherosclerotic disease of the coronary, cerebral and peripheral
arteries, or after coronary stent placement have been included, and treated
with variable success with antibiotics (see Table 8.7). Only neointimal formation
after stent-placement seems to be inhibited by roxithromycin, but this result has
come from only one study and needs to be verified by others. We attempted to
assess the influence of antibiotics on the progression of atherosclerosis in
patients with PAD. To avoid the influence of tissue destruction following acute
vascular events or surgery on inflammatory responses affecting atherosclerosis
we included only patients with stable PAD free from recent events and
interventions.
In summary, this is currently the largest study assessing the effect of
azithromycin on patients with peripheral arterial disease. Although *Chlamydia
pneumoniae serology was related to the development of cardiovascular events, the 3-day azithromycin treatment did not reduce the prevalence of cardiovascular complications, and has therefore no place in the secondary prevention of patients with stable peripheral arterial disease.
8.6 References

19. Gottlieb SS. Dead is dead—artificial definitions are no substitute. Lancet. 1997;349:862-3.


Section 6

General discussion and Summary
Chapter 9

General discussion

Parts of this chapter has been published as


9 General discussion

The data presented in this thesis conforms to the notion that atherosclerosis is an inflammatory process. It confirms and strengthens the relationship between C-reactive protein and Chlamydia pneumoniae infection on the one hand and atherosclerotic disease on the other, and offers moreover some novel insights in this relationship.

9.1 CRP & atherosclerosis

9.1.1 Serum hsCRP as marker of cardiovascular risk and of extent of atherosclerotic disease.

The pentraxin CRP has consistently been associated with (coronary) atherosclerotic disease. It has been shown that patients with coronary artery disease\textsuperscript{1-7} and cerebrovascular disease\textsuperscript{8,9} have elevated levels of serum CRP, measured highly sensitively (hsCRP). Additionally, hsCRP has been identified as an independent marker of cardiovascular risk in patients with coronary artery disease\textsuperscript{10-21} or peripheral arterial disease\textsuperscript{22} and, most intriguingly, in apparently healthy middle aged and elderly individuals.\textsuperscript{23-37} Regarding patients with PAD, it has been shown that hsCRP is inversely related to ankle-brachial pressure index,\textsuperscript{38} walking performance\textsuperscript{39} and endothelial function.\textsuperscript{40} Studies assessing serum hsCRP in patients with abdominal aortic aneurysms were not available. We analysed the serum hsCRP profile in patients with PAD (chapter 2) and in patients with asymptomatic abdominal aortic aneurysms (chapter 3). In both patient groups, the average serum hsCRP was in the range conferring increased risk for future cardiovascular events. Intriguingly, serum hsCRP was related to the extent of both diseases. Serum hsCRP showed a direct relationship with the diameter of the aneurysm in patients with AAA and an inverse relationship with ankle-brachial pressure index (as a marker of extent of peripheral arterial disease) in PAD patients. Following the latter patients prospectively we were able to establish an association between baseline hsCRP level and future cardiovascular events.

Considering the available literature and our data, it may be concluded that serum hsCRP is elevated in patients with any of the clinical manifestations of atherosclerosis (i.e., coronary artery disease, cerebrovascular disease, peripheral arterial disease and abdominal aortic aneurysms), that it is related to the extent of any type of atherosclerotic vascular disease and that it is an important predictor of cardiovascular risk in healthy individuals as well as in patients with any type of atherosclerotic vascular disease, adding prognostic value to conventional risk estimation models.\textsuperscript{41}
9.1.3 Pro-atherogenic properties and vascular production of CRP

The consistent association between serum hsCRP and extent and progression of atherosclerotic disease has led to the question whether CRP is merely a marker of atherosclerosis or an actual participant in the development of the disease. As discussed in chapter 1, many in vitro studies have shown that CRP has pro-atherogenic properties on all cell types involved in the development of atherosclerosis, i.e., endothelial cells, smooth muscle cells and macrophages. Most of these in vitro effects have been demonstrated by using CRP concentrations ranging from 5 to 900 mg/l, which is far above the serum concentration of CRP used to denote high risk for future events (3 mg/l). It has therefore been questioned whether CRP is able to elicit these effects in the vascular wall. Accumulation in the vessel wall by uptake of serum CRP, may yield high local CRP concentrations, overcoming the discrepancy between the ‘low’ serum CRP concentration and the amounts needed for CRP to exert its pro-atherogenic in situ properties. Alternatively, local production of CRP by cells of the vessel wall may support local paracrine loops and thus mediate the potential in situ pro-atherogenic effects of CRP that has been observed in the in vitro studies. Ample evidence for the extra-hepatic production of CRP has been gathered suggesting that a variety of cells and tissues produce CRP in response to stress as part of a localized immunological reaction. It has been shown that neuronal cells, alveolar macrophages, epithelial cells, cardiomyocytes, lymphocytes and monocytes produce CRP. Production of CRP has been demonstrated in coronary atherosclerotic plaques and venous coronary artery bypass grafts, probably by macrophages and smooth muscle cells. In section 2 we demonstrated that femoral atherosclerotic plaques (chapter 2) and abdominal aortic aneurysms (chapter 3) are also capable of CRP production suggesting that vascular CRP production is a generalized phenomenon in the diseased arteries. This notion is supported by two recent studies showing that human vascular smooth muscle cells and endothelial cells produce CRP when stimulated with IL-6 and IL-1. Intriguingly, LPS was also able to stimulate vascular smooth muscle cells to produce CRP offering, in theory, a biological substrate for an interaction between infections and CRP as novel risk factors for atherosclerotic disease.

9.2 Infection and atherosclerosis

Following Saikku and colleagues, who first described an association between Chlamydia pneumoniae antibodies and coronary artery disease, a large number of sero-epidemiological studies exploring the relation between Chlamydia pneumoniae serology and atherosclerotic disease has been published, with conflicting results. This may be related to the fact that various
serological assays and different criteria for the serological detection of chronic active or persistent *Chlamydia pneumoniae* infection have been used. The microimmunofluorescence test (MIF) has traditionally been used for the detection of *Chlamydia pneumoniae* antibodies, it is considered a sensitive and specific technique for the detection of acute *Chlamydia pneumoniae* infections and is regarded as the gold standard for the detection of *Chlamydia pneumoniae* antibodies. However, the MIF test has considerable limitations. It is a semi-quantitative assay, not suited for automated sample processing which would be desired for screening purposes. Furthermore, it is labour intensive and time consuming, has a subjective outcome measure and is, therefore, poorly reproducible. In chapter 4, a commercially available enzyme immunoassay (EIA), a technically less demanding test with an objective endpoint, suited for high throughput and automated sample processing, was compared to the MIF. Our data showed that in patients with cardiovascular disease, the EIA yielded results comparable to the MIF, when measures were taken to obtain results in the linear portion of the detection curve by retesting sera with high titres at a higher predilution. Additionally, in our hands the EIA showed a better reproducibility than the MIF. Considering the practical advantages of EIA and the limited microbiological background and experience of cardiovascular investigators involved in *Chlamydia pneumoniae* related cardiovascular research, the use of EIA instead of MIF is recommended, in order to improve the external validity of these studies. Despite the large numbers of sero-epidemiological trials assessing the association between *Chlamydia pneumoniae* and atherosclerosis, no consensus regarding the serological detection of clinically relevant (vascular) *Chlamydia pneumoniae* infection has been reached. In chapter 5 we explored the correlation between *Chlamydia pneumoniae* antibodies and presence of *Chlamydia pneumoniae* DNA and/or proteins in atherosclerotic vascular tissue, in order to determine serological criteria for vascular *Chlamydia pneumoniae* infection. Our results indicated that, both, *Chlamydia pneumoniae* IgA and IgG-antibodies were poorly associated with the presence of *Chlamydia pneumoniae* antigens or DNA in the atherosclerotic vessel wall. This suggests that serum antibody titre measurement may be inadequate to detect patients with *Chlamydia pneumoniae* infested atherosclerotic plaques. On the other hand, although *Chlamydia pneumoniae* traces are generally found in atherosclerotic sites and not in healthy vascular tissue, our data presented in chapter 5, have shown, in accordance with other studies, that the presence of *Chlamydia pneumoniae* DNA or proteins was not related to plaque histology. This casts doubts on the theory that infestation of the vascular wall with *Chlamydia pneumoniae* may stimulate advanced atherosclerotic lesion progression through *in situ* processes.
The association between *Chlamydia pneumoniae* antibody titres and clinical signs of atherosclerotic disease has been explored in numerous cross sectional and longitudinal sero-epidemiological studies.56 Although a few failed to verify the association between infection and atherosclerosis, the majority of these studies supported an association between *Chlamydia pneumoniae* serology and clinical manifestations of atherosclerotic disease. A recent meta-analysis, involving studies published between January 1997 and December 2000, showed that a positive *Chlamydia pneumoniae* titre is related to atherosclerosis with a pooled odds ratio of 1.6 (95% confidence interval CI:1.3-2.0).60 This meta-analysis suggested that study design (cross-sectional versus nested case control studies) was a source of variability, as the association between infection and atherosclerosis was stronger in cross sectional studies. Additionally, this meta-analysis suggested that the duration of follow-up in the prospective studies was inversely related to the strength of this association. Data from our randomised clinical trial (chapter 8) supported a relation between baseline *Chlamydia pneumoniae* serology and future cardiovascular events among patients with peripheral arterial disease.

The fact that *Chlamydia pneumoniae* serology is associated with clinical manifestations of atherosclerotic disease whereas any sign of infection, whether serologic (antibodies)61,62 or histologic (vascular presence of DNA/proteins),63,64 does not seem to be related to atherosclerotic plaque histology may suggest that in humans, *Chlamydia pneumoniae* does not stimulate advanced atherosclerotic lesion progression via the *in situ* processes that have been described in chapter 1. In contrast, the association between infection and atherosclerosis might be mediated through the systemic effects of (non)vascular *Chlamydia pneumoniae* infection and/or by the host response to *Chlamydia pneumoniae* infection. We have shown in this respect that *Chlamydia pneumoniae* serology is associated with hypercoagulability but not with histological plaque instability in patients with carotid artery disease (chapter 6). This conforms with the finding that, in patients with *Chlamydia pneumoniae* infections, plasma fibrinogen levels are elevated65 and that these levels decrease upon anti-microbial treatment.66 *Chlamydia pneumoniae* infection might also contribute to the development of atherosclerosis through stimulation of cytokine production,67 through interference with lipid metabolism68,69 and by increased gelatinolytic activity. Furthermore, it has been shown that antibodies elicited against chlamydial HSP60 cross-react with human autologous HSP60 and can cause lysis of stressed endothelial cells that express human HSP60 on their surface.70 In other words, although *in vitro* studies have suggested that infection of vascular cells with *Chlamydia pneumoniae* is associated with atherosclerosis progression, in humans *Chlamydia pneumoniae* may have clinically significant pro-atherogenic effects through systemic rather than *in situ* phenomena.
Although *Chlamydia pneumoniae* has been studied most extensively in relation to atherosclerosis, a large number of viruses and other bacteria have been related to atherosclerosis development (see chapter 1.8). In fact, recent evidence suggests that total pathogen burden rather than exposure to individual micro-organisms is related to cardiovascular events. 71-73 These data suggest that the interaction between individuals and the microbiological environment is more complex than traditionally anticipated. In most cases of acute infections, the link between a certain micro-organism and the disease is obvious, mostly fulfilling Koch's postulates. However, in the case of chronic degenerative diseases such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease or chronic obstructive pulmonary disease, micro-organisms may interact in many, not necessarily specific ways with the host (e.g., direct effects on infected cells, induction of auto-immune reactions, stimulation of chronic inflammatory responses), making it difficult to prove a causal or at least modulatory involvement of the micro-organisms in disease initiation or progression.

9.3 Interaction of inflammation and infectious organisms in the development of atherosclerosis

Since CRP and *Chlamydia pneumoniae* - and in fact total pathogen burden - has been related to atherosclerotic disease progression, it is plausible that (*Chlamydia pneumoniae*) infections may interact with CRP in the stimulation of atherosclerosis progression. Data from the Helsinki Health Study has shown that persistently elevated antibodies against *Chlamydia pneumoniae* and elevated serum CRP had a synergistic effect on coronary risk. 74 In fact, chronic active or persistent infections may give rise to chronically elevated serum CRP concentrations through maintenance of a sustained inflammatory response. In addition, the fact that *Chlamydia pneumoniae* is present in atherosclerotic plaques and that CRP is produced in atherosclerotic arteries may suggest that infestation of the vascular cells with *Chlamydia pneumoniae* (or other micro-organisms) may stimulate vascular CRP production. *In vitro* experiments have shown that incubation of human smooth muscle cells with bacterial endotoxin stimulated CRP production. 52 Although in our prospective trial, both, baseline serum *hsCRP* (chapter 2) and baseline *Chlamydia pneumoniae* serology (chapter 8) were related to future events, serum *hsCRP* concentration and *Chlamydia pneumoniae* serology were not correlated. As we had chosen to include patients with no clinical signs of respiratory tract infection in our trial, total pathogen burden rather than infection with *Chlamydia pneumoniae* should more likely be related to active inflammatory response and serum *hsCRP* concentration.
9.4 The role of host factors on the pro-atherogenic properties of inflammation and infection

Taking into account the high incidence of Chlamydia pneumoniae antibodies in the general population, it seems likely that genetic factors may affect individual susceptibility for the pro-atherogenic effects of infections and, thus, determine the outcome and severity of chronic degenerative inflammatory diseases. Toll Like Receptor 4 (TLR4) and CD14 are among the genes involved in the regulation of immune responses, showing genetic variations with potential functional implications. The recently described human TLR4 polymorphism, TLR4 +696A>G, has been associated with hyporesponsiveness to LPS and seems to confer protection against the development of ultrasound detected carotid artery disease and acute coronary syndromes. Among pravastatin users this polymorphism has significantly been associated with a lower cardiovascular risk. A functional polymorphism in the promoter region of CD14 at position -260, the CD14 (-260)C>T polymorphism, that enhances the transcriptional activity of the CD14 gene, has been associated with carotid artery intima media thickness, and increased risk of stroke or acute myocardial infarction. In contrast, several authors demonstrated a lack of association between this polymorphism and coronary artery or cerebrovascular disease. The data presented in chapter 7 show that among patients with peripheral arterial disease the carrier trait TLR4 G-allele & CD14 TT genotype, rather than individual polymorphisms, is associated with extent of atherosclerotic disease. Considering the complexity of the innate immune system and its high degree of genetic variation, a significant number of collateral pathways may exist for innate immune responses, which differ in their cofactor requirements and their pattern recognition specificities. Ideally, all pathways should be taken into consideration and carrier traits instead of individual single nucleotide polymorphisms should be regarded when studying genetic predisposition of the innate immune system in relation to atherosclerosis or other multifactorial and polygenic inflammatory diseases. Our data illustrate the significance of taking a wider approach analyzing relevant carrier traits instead of individual polymorphisms in relation to atherosclerosis, and provide an explanation for inter-individual differences in susceptibility to atherosclerosis based on genetic variability of a combination of genes involved in innate immune regulation.

9.5 Interventions based on the inflammatory and infectious aspects of atherosclerosis

The novel insights into the pathogenesis of atherosclerosis offer new potential targets for (prophylactic) treatment of patients with atherosclerotic (cardio)vascular disease.
9.5. a Role of anti-inflammatory drugs in atherosclerotic disease

In view of the potent pro-atherogenic properties of CRP described in chapter 1.7, the strong association between serum hsCRP and cardiovascular disease and the active production of CRP in the atherosclerotic vessel wall may imply that CRP is not merely a biomarker of atherosclerotic disease but an actual partaker in atherosclerotic lesion development. Although anti-CRP treatment strategies as such have not been described yet, clinical evidence suggest that the potent protective effects of aspirin and statins may be related to lowering serum CRP.24,86,87 This is in accordance with recent data of Ricker et al. showing that when statin treatment resulted in low serum CRP levels it was associated with significant improvement of event free survival in patients with acute coronary syndromes irrespective of (LDL)cholesterol levels, suggesting that strategies designed to reduce inflammation may improve cardiovascular outcome.86

9.5. b The role of antibiotics in the treatment of atherosclerotic disease

The sero-epidemiological and experimental data suggesting a possible association between Chlamydia pneumoniae infection and atherosclerosis triggered the interest of (cardio)vascular clinicians for the role of anti-chlamydial antibiotics as a new treatment modality for patients with atherosclerotic vascular disease. Indeed, two early small randomised clinical trials suggested that antibiotics may reduce cardiovascular risk in patients with coronary artery disease.89,90 Prompted by these results, several investigators have tried to evaluate the effect of antibiotics on cardiovascular risk by observational case-control studies. A number of these studies demonstrated a survival benefit from exposure to anti-chlamydial antibiotics,91-94 although others refuted it.95-99 Considering the methodological shortcomings of these retrospective studies, randomised clinical trials were needed to determine the effectiveness of antibiotics in atherosclerotic disease prevention. Following the two initial positive pilot studies89,90 a number of randomised clinical trials have been designed and carried out worldwide with contradictory results.100-120 In general, favourable results were shown in studies with small sample size and/or limited follow-up.89,90,107,108,121 Trials that have included patients with abdominal aortic aneurysms105,106 or studied surrogate endpoints such as carotid intima media thickness,121 basal NO production,103 flow mediated dilatation,110,115 aortic expansion rate105,106 and matrix metalloproteinase metabolism.111 In chapter 8 the results of our randomised clinical trial are presented, showing that a short term antibiotic prophylactic treatment did not offer any benefits in terms of clinical events or (changes in) ankle-brachial pressure index in patients with peripheral arterial disease. Similarly, recently published results of larger studies, involving patients with coronary artery disease,112-114,119,120 demon-
strated no beneficial effect of antibiotics in the prevention of cardiovascular events or death.

Table 9.1 lists all the randomised clinical trials carried out to assess the effects of antibiotics on cardiovascular endpoints in patients with atherosclerotic disease of the coronary, cerebral and/or peripheral circulation.

Table 9.1 Overview of randomised clinical trials assessing the effect of antibiotics on cardiovascular events in patients with atherosclerotic disease.

<table>
<thead>
<tr>
<th>Trial/Author</th>
<th>Diagnosis</th>
<th>Treatment</th>
<th>Follow-up</th>
<th>N</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gupt a et al.</td>
<td>CAD</td>
<td>azi</td>
<td>3-8 d</td>
<td>18 mo</td>
<td>60 40 20 ↓ CVE</td>
</tr>
<tr>
<td>ISAR-3</td>
<td>stent</td>
<td>roxi</td>
<td>4 w</td>
<td>12 mo</td>
<td>1,010 506 504 ↓ restenosis</td>
</tr>
<tr>
<td>CLARIFY</td>
<td>ACS</td>
<td>clari</td>
<td>3 mo</td>
<td>18 mo</td>
<td>148 74 74 ↓ CVE</td>
</tr>
<tr>
<td>STAMINA</td>
<td>ACS</td>
<td>azi</td>
<td>3 d</td>
<td>12 mo</td>
<td>325 111 107 ↓ CVE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>amoxi</td>
<td>1 w</td>
<td></td>
<td>107</td>
</tr>
<tr>
<td>Wiesl et al.</td>
<td>PAD</td>
<td>roxi</td>
<td>4 w</td>
<td>20 mo</td>
<td>40 20 20 ↓ PAD events</td>
</tr>
<tr>
<td>ROXIS</td>
<td>ACS</td>
<td>roxi</td>
<td>30 d</td>
<td>6 mo</td>
<td>202 102 100 = CVE</td>
</tr>
<tr>
<td>ACADEMIC</td>
<td>CAD</td>
<td>azi</td>
<td>3 mo</td>
<td>24 mo</td>
<td>302 150 152 = CVE</td>
</tr>
<tr>
<td>ANTIBIO</td>
<td>ACS</td>
<td>roxi</td>
<td>6 w</td>
<td>12 mo</td>
<td>872 433 439 = CVE</td>
</tr>
<tr>
<td>WIZARD</td>
<td>CAD</td>
<td>azi</td>
<td>3 mo</td>
<td>24 mo</td>
<td>7,722 3,866 3,856 = CVE</td>
</tr>
<tr>
<td>AZACS</td>
<td>ACS</td>
<td>azi</td>
<td>5 d</td>
<td>24 mo</td>
<td>1,439 716 723 = CVE</td>
</tr>
<tr>
<td>Berg et al.</td>
<td>CAD</td>
<td>clari</td>
<td>16 d</td>
<td>24 mo</td>
<td>473 238 235 = CVE</td>
</tr>
<tr>
<td>SPACE</td>
<td>PAD</td>
<td>azi</td>
<td>3 d</td>
<td>24 mo</td>
<td>509 257 252 = CVE</td>
</tr>
<tr>
<td>ACES</td>
<td>CAD</td>
<td>azi</td>
<td>12 mo</td>
<td>47 mo</td>
<td>4,012 2,004 2,006 = CVE</td>
</tr>
<tr>
<td>PROVE-IT-TIM</td>
<td>ACS</td>
<td>gati</td>
<td>24 mo</td>
<td>24 mo</td>
<td>4,162 2,076 2,086 = CVE</td>
</tr>
</tbody>
</table>

N of patients in positive clinical endpoint trials 1,583 858 725 ↓ CVE
N of patients in negative clinical endpoint trials 19,893 9,842 9,851 = CVE
N of patients in all clinical endpoint trials 21,276 10,700 10,576

Ab: antibiotic; AB: antibiotic group; ACS: acute coronary syndromes; amoxi: amoxicillin; azi: azithromycin; CAD: stable coronary artery disease; clari: clarithromycin; CVE: cardiovascular event; d: days; gati: gatifloxacin; mo: months; PAD: peripheral arterial disease; PL: placebo group; roxi: roxithromycin; w: weeks; "": no effect; ↓: reduced.

Overall these data suggest that antibiotics are not able to reduce cardiovascular risk in atherosclerotic patients. Indeed, the latest meta-analysis involving all randomized clinical trials that have included patients with coronary artery disease showed a lack of effect of antibiotics on cardiovascular risk. In light of the strong evidence in favour of involvement of *Chlamydia pneumoniae* in the development of atherosclerosis, the negative results of the clinical trials may be related to the inability to select patients with clinically relevant (vascular) *Chlamydia pneumoniae* infection, to insufficient study medication, or to wrong timing of antibiotic treatment during the course of atherosclerosis development. In fact, effective treatment of chronic (vascular) *Chlamydia pneumoniae* infection may be more troublesome than initially anticipated. Recent *in vitro* evidence suggests that *Chlamydia pneumoniae* carried within
macrophages is refractory to anti-chlamydial antibiotics and that even prolonged antibiotic treatment failed to completely eliminate *Chlamydia pneumoniae* from infected epithelial cells. It may therefore not be surprising that the prolonged treatment strategies, varying from 3 to 24 months, employed in the recently published mega-trials have not resulted in a significant reduction of cardiovascular events. Considering the fact that in animals the *Chlamydia pneumoniae* related progression of atherosclerosis can only be prevented by antibiotics when they are administered within 5 days of inoculation of the animals, and that in humans the majority of *Chlamydia pneumoniae* respiratory tract infections remain clinically unnoticed whereas *Chlamydia pneumoniae* antibody titres rise already during early childhood, it might be concluded that antibiotics may only contribute to cardiovascular risk reduction when administered early in life as part of a primary preventive strategy.

9.6 Synopsis and perspectives for the future

The long standing theory that inflammation and infections play a role in atherosclerosis development has been supported by substantial recent scientific evidence. The data presented in this thesis fit into this recent inflammatory paradigm for atherosclerosis development.

It has been demonstrated that the acute phase reactant CRP was related to the extent of peripheral arterial disease and of abdominal aortic aneurysms. Furthermore, the serum CRP concentration proved to be an independent strong predictor of future cardiovascular events in patients with PAD. Additionally, it was shown that CRP is produced by atherosclerotic vascular tissue. Since the cardiovascular protective effect of statin therapy is more pronounced when it results in lower plasma CRP levels, unravelling the mechanisms governing vascular CRP production and answering the question whether statins interfere herewith become interesting targets for future work in this field.

Considering the reported limitations in the detection of *Chlamydia pneumoniae* in patients with cardiovascular disease, an easy-to-use and reliable assay for the detection of *Chlamydia pneumoniae* antibodies, the enzyme immunoassay, was evaluated and showed excellent agreement with the gold standard serological assay (microimmunofluorescence). Furthermore, it was shown that no correlation existed between serum antibodies and presence of *Chlamydia pneumoniae* particles in the vascular wall, demonstrating the inability to detect patients with clinically relevant (cardiovascular) *Chlamydia pneumoniae* infections. Setting out standards for the detection of cardiovascularly relevant *Chlamydia pneumoniae* infections is an important hurdle to be taken, in order to further studies on the role of infections in atherosclerosis. Despite this
limitation, we were able to show that *Chlamydia pneumoniae* serology was related to future cardiovascular events in patients with peripheral arterial disease. Moreover, using patients with carotid artery disease as an *in vivo* model that allows differentiation of atherosclerotic plaque instability from hypercoagulability, our data suggested that the relation between *Chlamydia pneumoniae* serology and future events may be related to the pro-coagulant effects of *Chlamydia pneumoniae* infection rather than to induction of plaque instability per se.

Bearing in mind the variation of genes involved in regulation of immune responses, we assessed the influence of two common polymorphisms of inflammatory genes (CD14 and TLR-4) on atherosclerotic disease progression. Our data indicated that the combined trait of the TLR4-G allele and the *CD14 TT*-genotype had the strongest effect on the extent of atherosclerotic disease, whereas each of these polymorphisms individually was not related to severity of atherosclerotic disease. This illustrates the need to consider the variation of, if possible, all genes involved in the regulation of the immune system, in order to understand the inter-individual differences in susceptibility to the pro-atherogenic effects of various infectious and inflammatory stimuli.

The novel insights into the pathogenesis of atherosclerosis suggesting that infectious and inflammatory processes play an important role in the development of atherosclerotic disease offered potential new targets for medical intervention. The last part of this thesis describes a randomized clinical trial designed to assess the effect of a 3 day course of azithromycin on the progression of atherosclerosis in patients with peripheral arterial disease. The generated data showed that the short antibiotic treatment has no effect on ankle brachial pressure index or on cardiovascular survival in patients with peripheral arterial disease. Combined with the results of recently published mega trials including thousands of patients with coronary artery disease, these data suggest that antibiotics have no place in the secondary prevention of patients with atherosclerotic disease. Although it appears that anti-inflammatory statin treatment may find its way into the therapeutic arsenal of cardiovascular physicians, it remains to be seen whether selected subgroups of patients may benefit from antibiotics as part of a secondary or even primary preventive strategy.
9.7 References


Summary
Summary

In this thesis the inflammatory and infectious features of atherosclerosis are scrutinized. Chapter 1 lays out the theoretical background of the studies described here. A comprehensive overview of the pathogenesis and epidemiology of atherosclerotic disease is given with special emphasis on the inflammatory nature of this process and the potential involvement of infectious microbes herein. Also a short historical overview of the developments in atherosclerotic cardiovascular medicine is given, commemorating the tremendous achievements of the past in this field.

Section 2 (inflammation and atherosclerosis, chapters 2 & 3) deals with the relation between CRP and (extent of) atherosclerotic disease. In Chapter 2, serum CRP was measured highly sensitive in patients with peripheral arterial disease, showing an inverse relation to ankle-brachial pressure index. Baseline serum CRP was also related to future cardiovascular events. Furthermore, femoral plaques have shown to be able to produce CRP. Immunohistochemical analysis located the vascular CRP production on macrophages, T-cells and smooth muscle cells. Similarly, in chapter 3 it was shown that serum CRP was related to the dimension of abdominal aortic aneurysms and that aneurysmal tissue produces CRP.

In section 3 (infection and atherosclerosis, chapters 4–6) the role of Chlamydia pneumoniae infection in peripheral vascular disease was studied. In chapter 4, Chlamydia pneumoniae IgA and IgG antibodies were measured with a commercially available enzyme immunoassay and with the micro-immunofluorescence test, which has been regarded the gold standard for detection of Chlamydia pneumoniae antibodies. Our data showed that both tests yielded comparable results for the detection of Chlamydia pneumoniae IgA- and IgG-antibodies in patients with cardiovascular disease. In chapter 5, Chlamydia pneumoniae antibodies were related to the presence of Chlamydia pneumoniae DNA or proteins in atherosclerotic plaques of patients undergoing reconstructive vascular surgery of the aorta, carotid or femoropopliteal arteries. These data demonstrated a lack of association between Chlamydia pneumoniae serology and presence of Chlamydia pneumoniae particles in the vascular wall, rendering serological detection of vascular Chlamydia pneumoniae infection virtually impossible. In chapter 6 we were able to demonstrate that Chlamydia pneumoniae serology was related to hypercoagulability rather than plaque rupture per se, relating Chlamydia pneumoniae antibodies to micro-embolic signals in the middle cerebral artery in patients undergoing carotid endarterectomy for symptomatic carotid artery disease.

In section 4 (chapter 7), common polymorphisms of two pattern-recognition receptor genes, i.e., the Toll-like receptor 4 (TLR4) +896 A>G and the CD14 –
260 C>T single nucleotide polymorphism were analyzed in relation to total atherosclerosis burden in patients with peripheral arterial disease. Multivariate logistic regression analysis showed that combination of the TLR4 +896A>G allele carriergship and CD14 -260 TT homozygocity was an independent predictor of extensive atherosclerotic disease. This provided an explanation for inter-individual susceptibility to atherosclerosis based on genetic variability of a combination of genes involved in innate immune regulation. Considering the inflammatory nature of atherosclerosis and the potential modulatory role of (Chlamydia pneumoniae) infections in the development of cardiovascular events, new potential therapeutic (anti-inflammatory or antimicrobial) targets have evolved. In section 5 the effect of a short course of antibiotics in the progression of atherosclerosis in patients with peripheral arterial disease was studied in a randomised clinical trial (chapter 8). Although Chlamydia pneumoniae serology was related to future cardiovascular events, no beneficial effect of a 3-day course of azithromycin on cardiovascular events or changes in ankle-brachial pressure index were observed. In chapter 9, the main results of this thesis are discussed in relation to the literature.

In conclusion, the data presented are in concord with the inflammatory nature of atherosclerosis. It has been shown that CRP is produced by diseased vascular tissue and that serum CRP is related to the extent of atherosclerotic disease and to future cardiovascular events. Furthermore, it has been suggested that Chlamydia pneumoniae infection can induce a clinically relevant hypercoagulable state, as Chlamydia pneumoniae antibodies were associated with thrombosis related micro-embolization after carotid endarterectomy and with future cardiovascular events in patients with peripheral arterial disease. Despite these associations, a short-term antibiotic treatment had no effect on future events and changes in ankle-brachial pressure index in patients with peripheral arterial disease.
Samenvatting
Samenvatting

In dit proefschrift worden de inflammatoire en infectieuze aspecten van atherosclerose bestudeerd. Deel 1 (Introduction, Hoofdstuk 1) geeft een theoretische achtergrond voor de beschreven studies aan de hand van een overzicht van de pathogenese en epidemiologie van atherosclerotische ziekte. Hierbij ligt de nadruk op de inflammatoire kenmerken van atherosclerose en de mogelijke rol van infectieuze micro-organismen hierin. Tevens wordt een historisch overzicht betreffende de ontwikkelingen in cardiovasculaire geneeskunde gepresenteerd.

Deel 2 (Inflammation and atherosclerosis, hoofdstuk 2 en 3) gaat over de relatie tussen C-reactieve protein (CRP) en de uitgebreidheid van atherosclerotische ziekte. In hoofdstuk 2 wordt een onderzoek beschreven naar CRP met hoge gevoeligheid (hsCRP) gemeten in het serum van patiënten met perifere arterieel vaatlijden. Er bleek sprake van een omgekeerde relatie tussen de hoogte van hsCRP en de hoogte van de enkel-arm-index, dat een gevoelige maat is voor de uitgebreidheid van perifere arterieel vaatlijden. Daarnaast bleek de hsCRP serum concentratie gerelateerd aan het optreden van voorkomende cardiovasculaire complicaties. Uit aanvullende studies bleek dat atherosclerotische plaques uit de liesslagader CRP kunnen produceren. Immunohistochemische analyses suggereerden dat deze CRP productie plaatsvindt in macrophagen, T-cellen en gladde spiercellen. Op overeenkomstige wijze toonde de studie beschreven in hoofdstuk 3 dat de hoogte van hsCRP direct gerelateerd was aan de diameter van aneurysmata van de aorta abdominale en dat CRP geproduceerd werd door het aneurysmaweeleefsel zelf.

De rol van infectie met het micro-organisme Chlamydia pneumoniae in relatie tot perifere vaatlijden wordt in deel 3 (Infection and atherosclerosis, hoofdstuk 4–6) beschreven. In hoofdstuk 4 wordt beschreven hoe Chlamydia pneumoniae IgA- en IgG-antilichamen werden gemeten met een commercieel verkrijgbare ELISA en met de micro-immunofluorescentietest, welke als gouden standaard gezien wordt voor het detecteren van Chlamydia pneumoniae antilichamen. Onze data toonden dat beide testen vergelijkbare resultaten opleverden met betrekking tot de detectie van Chlamydia pneumoniae IgA- en IgG-antilichamen in patiënten met cardiovasculaire aandoeningen. De relatie tussen Chlamydia pneumoniae antilichamen in het serum en de aanwezigheid van Chlamydia pneumoniae DNA of proteïnen in atherosclerotisch weefsel van patiënten die een vaatoperatie ondergingen aan de acrta, hals-, liess- of knieslagader wordt in hoofdstuk 5 beschreven. Er werd geen associatie gevonden tussen Chlamydia pneumoniae serologie en aanwezigheid van Chlamydia pneumoniae partikels in de vaatwand. Serologische detectie van vasculaire infectie met Chlamydia pneumoniae is dus waarschijnlijk niet mogelijk. In hoofdstuk 6 wordt beschreven dat
Chlamydia pneumoniae serologie geassocieerd bleek te zijn met hypercoagulabiliteit en niet met plaque-instabiliteit. Deze conclusie werd gebaseerd op de gevonden associatie tussen Chlamydia pneumoniae seropositiviteit en het aantal thrombus gerelateerde micro-embolieën in de arteria cerebri media bij patiënten die een endarteriectomie van de halsslagader ondergingen in verband met een symptomatische a.carotis stenoese.

Deel 4 (Genetic determinants of atherosclerosis susceptibility, hoofdstuk 7) beschrijft een studie waarin vaak voorkomende DNA-polymorfismen van twee receptoren die onderdeel uitmaken van het aangeboren immuunsysteem, namelijk de Toll-like receptor 4 (TLR-4) +896 A>G en de CD14 -260 C>T polymorfismen, geanalyseerd werden in relatie tot de algemene uitgebreidheid van atherosclerose in patiënten met perifere arterieel vaatlijden. Multivariaat logistische regressie analyse toonde dat de combinatie van het TLR4 Asp299Gly G-allel dragerschap en CD14 -260 TT homozygotie een onafhankelijke voorspeller was van uitgebreide atherosclerotische ziekte. Deze uitkomst bood een verklaring voor de inter-individuele ontvankelijkheid voor atherosclerose gebaseerd op genetische variabiliteit van een gencombinatie die van belang is voor de immuunregulatie.

Het inflammatoire karakter van atherosclerose en de mogelijk modulerende rol van (Chlamydia pneumoniae) infecties bij cardiovasculaire ziekten, vormen de theoretische basis voor nieuwe potentiële therapeutische (anti-inflammatoire of anti-microbiële) strategieën. In een prospectief opgezet gerandomiseerde studie werd het effect van een korte antibioticakuur bestudeerd op de progressie van atherosclerose in patiënten met perifere arterieel vaatlijden (Clinical intervention trial; deel 5, hoofdstuk 8). Hoewel in deze studie de initiële Chlamydia pneumoniae serologie gerelateerd was aan later optredende cardiovasculaire gebeurtenissen, werd er door het geven van een 3-daagse kuur azithromycine geen gunstig effect gemeten op toekomstige cardiovasculaire gebeurtenissen of op veranderingen in de enkel-arm-index, als maat van progressie van perifeer vaatlijden.

Tot slot worden in deel 6 (Discussion, hoofdstuk 9) de belangrijkste resultaten van dit proefschrift bediscussieerd in relatie tot bevindingen uit de literatuur. De gepresenteerde eigen data zijn in overeenstemming met het inflammatoire karakter van atherosclerose. De resultaten van dit proefschrift tonen dat CRP geproduceerd wordt door aneurysmatisch en atherosclerotisch vasculair weefsel en dat CRP, gemeten in het serum, gerelateerd is aan de uitgebreidheid van atherosclerotische ziekte en aan toekomstige cardiovasculaire complicaties. Daarnaast suggereren de hier beschreven studies dat een infectie met Chlamydia pneumoniae een klinisch relevante hypercoagulabele status kan induceren, daar Chlamydia pneumoniae antilichamen geassocieerd bleken met het optreden van micro-embolieën na
endarteriectomie van de halsslagader en met toekomstige cardiovasculaire gebeurtenissen bij patiënten met perifeer arterieel vaatlijden. Ondanks deze associaties bleek een korte antibioticakuur, bij patiënten met perifeer arterieel vaatlijden, niet effectief in het voorkomen van toekomstige cardiovasculaire complicaties of progressie van perifeer arterieel vaatlijden zoals weergegeven door verandering van de enkel-arm index.
Περίληψη
Περίληψη

Στην παρούσα διατριβή εξετάζονται τα φλεγμονώδη και μολυσματικά χαρακτηριστικά της αθηροσκλήρωσης. Το πρώτο κεφάλαιο θέτει το θεωρητικό υπόβαθρο των μελετών που περιγράφονται εδώ. Δίδεται μια σφαιρική εικόνα της παθογένεσης και επιδημιολογίας της αθηροσκληρωτικής νόσου, με ειδική έμφαση στην φλεγμονώδη φάση αυτής της διαδικασίας και την πιθανή συμμετοχή σε αυτήν μολυσματικών μικροβίων. Επίσης γίνεται μια σύντομη ιστορική ανάδρομη των εξελίξεων στον κλάδο της καρδιαγγειακής ιατρικής, μεταναστεύοντας τις τεράστιες επιτεύγματα του παρελθόντος σε αυτόν τον τομέα.

Το δεύτερο τμήμα (φλεγμονή και αθηροσκλήρωση, κεφάλαια 2 & 3) προγραμματίζεται τη σχέση μεταξύ της πρωτεΐνης CRP και της (έκτασης της) αθηροσκληρωτικής νόσου. Στο κεφάλαιο 2, η CRP ορού μετρήθηκε σε ασθενείς με αποφαστική νόσος των περιφερειακών αρτηριών, παρουσιάζοντας μια αντίστροφη σχέση προς το δείκτη κλίμα-κλήρους. Επιπλέον, η CRP ορού, συσχετίστηκε με μελλοντικά καρδιαγγειακά επεισόδια. Οι μηριαίες αθηροσκληρωτικές πλάκες έδειξαν ότι μπορούν να παράγουν CRP. Ανασχηματική ανάλυση εντάσσει την αγγειακή παραγωγή CRP σε μακροφάγα, T-λευκοκύτταρα και λεία μυκώνες. Παρατηρώθηκε, ότι το κεφάλαιο 3 καταδείχτηκε ότι η CRP ορού σχετίζεται με την έκταση των κοιλιακών αορτικών ανευρισμάτων και οτι ανευρισματικός ιστός δύναται να παράγει CRP. Στο τρίτο τμήμα (μόλυνση και αθηροσκλήρωση, κεφάλαια 4-6) εξετάστηκε ο ρόλος της μόλυνσης από Chlamydia pneumoniae στη νόσο των περιφερειακών αρτηριών. Στο κεφάλαιο 4, μετρήθηκαν αντισώματα ανοσοασφαλίζοντας στην εισαγωγή A ή G στα Chlamydia pneumoniae με ενζυμο-αναλογική δοκιμή και με χρήση microimmunofluorescence test, που θεωρείται ο χρυσός κανόνας για την ανίχνευση των αντισωμάτων των χλαμυδιών. Τα δεδομένα μας έδειξαν ότι τα δύο test έδωσαν συγκρίσιμα αποτελέσματα για την ανίχνευση των αντισωμάτων χλαμυδιών, σε ασθενείς με καρδιαγγειακή νόσο. Στο κεφάλαιο 5, αντισώματα χλαμυδιών σχετίστηκαν με την παρουσία DNA ή πρωτεΐνων χλαμυδιών στις αθηροσκληρωτικές πλάκες ασθενών που υπεβλήθησαν σε αγγειοσειρουργική αποκατάσταση της ασθήματος καρδιών ή μηριαίων ανακομιδών αρτηριών. Τα δεδομένα αυτά, εξελίχθηκαν μια έλλειψη συσχετισμού μεταξύ των αντισωμάτων χλαμυδιών και της παρουσίας συμπαθών χλαμυδιών στο αγγειακό τοίχωμα, καθιστώντας σχεδόν ασφαλή την ανίχνευση της αγγειακής μόλυνσης στο χλαμύδια με τέσσερεις ορούς.

Στο κεφάλαιο 6, μπορείται να καταδείχουμε ότι τα αντισώματα χλαμυδιών σχετίζονται περισσότερο με την υπερτηπητικήτητα του αίματος παρά με την ρήξη της αθηροσκληρωτικής πλάκας καθαυτής, συσχετίζοντας τα αντισώματα χλαμυδιών με σημεία μικροεμβολίας στη μέση εγκεφαλική αρτηρία, σε ασθενείς που υποβάλλονται σε επεμβάσεις της καρδιώτικος αρτηρίας λόγω νόσου της αρτηρίας αυτής με εκδηλώσεις συμπυκνώματων.
Στο τέταρτο τμήμα (κεφάλαιο 7), αναλύθηκαν κοινοί πολυμορφισμοί δύο γονιδίων υποδοχέων αναγνώρισης μορφών, του Toll-like receptor 4 (TLR4)+896 A>G και του CD14-260 C>T μονονουκλεοτιδικό πολυμορφισμού, σε σχέση με το συνολικό αθηροματικό φόρτο σε ασθενείς με νόσο των περιφερειακών αρτηριών. Πολυπληθής λογιστικής ανάλυσης καμπύλης εδείχε ότι ο συνδιασμός του TLR4 +896A>G αλληλόμορφου γονίδιου και ομοζυγοτισμού για CD14 -260 TT ήταν ανεξάρτητος παράγοντας εκτεταμένης αθηροακληρωτικής νόσου. Ετσι δόθηκε μια εξήγηση για την ατομική προδιάθεση στην αθηροακληρωτική που βασίζεται στην γενετική ποικιλία ενός συνδιασμού γονίδιων που εμπλέκονται στην εγγενή ανοσορύθμιση.

Λαμβάνοντας υπόψη την φλεγμονώδη φύση της αθηροακληρωτικής και τον πιθανότως ρυθμιστικό ρόλο μολύνσεων (από χλαμώδια) στην εκδηλώση καρδιαγγειακών επεισοδίων, εξελίχθηκαν νέοι πιθανώς θεραπευτικοί (αντιφλεγμονώδεις ή αντικρουτακοί) στόχοι. Στο πέμπτο τμήμα, μελετήθηκε η επιδράση μιας βραχείας χορήγησης αντιβιοτικών στην εξέλιξη της αθηροακληρωτικής σε ασθενείς με νόσο των περιφερειακών αρτηριών, με τυχαίο αποτελέσματα κλινικής συμπτωματικής (κεφάλαιο 8). Αν και τα αντισώματα χλαμώδεων συνέδεθηκαν με μελλοντικά καρδιαγγειακά επεισόδια, δεν παρατηρήθηκε κάποια ευνοϊκή επίπτωση στα καρδιαγγειακά επεισόδια ή αλλαγές στο διείσοδο πίεσης κηφήνης-βραχιόνος, μετά από χορήγηση αζόρμοικης για 3 ημέρες.

Στο κεφάλαιο 9 παρουσιάζονται τα κύρια αποτελέσματα αυτής της διατριβής σε σχέση με τη διεθνή επιστημονική βιβλιογραφία.

Συμπερασματικά, τα δεδομένα που παρουσιάζονταν σε αυτήν την διατριβή συνάδουν με την φλεγμονώδη φύση της αθηροακληρωτικής. Καταδεικνύθηκε ότι η CRP παράγεται στα νοσούντα γγειακό αιτά και ότι η CRP ορούς σχετίζεται με την έκταση της αθηροακληρωτικής νόσου και μελλοντικά καρδιαγγειακά επεισόδια. Επίσης υποστηρίζεται ότι η μόλυνση από χλαμώδια μπορεί να προκαλεί κλινική κατάσταση υπερηρητικοτήτας του σύμπτωμα, καθώς τα αντισώματα χλαμώδεων σχετίζονταν με θρομβωτική μικροεμβόλομα, μετά από εκτομή αθηροακληρωτικής πλάκας της καρσπίδας αρτηρίας, και με μελλοντικά καρδιαγγειακά επεισόδια σε ασθενείς με αποφρακτική αθηροακληρωτική νόσο των περιφερειακών αρτηριών. Παρά τους συσχετισμούς αυτούς, μια θεραπεία θεραπευτική με αντιβιοτικά δεν είχε επιπτώσεις σε μελλοντικά καρδιαγγειακά συμβάντα και στον δείκτη κηφήνης-βραχιόνος, σε ασθενείς με αποφρακτική νόσο των περιφερειακών αρτηριών.
Acknowledgements
Acknowledgements

One may suggest that since Greece won the European Football Championship and the Eurovision Song contest in one year, it should not be too difficult for a Greek like me to earn a PhD. Indeed, after a long struggle I have finally been able to complete my thesis. Marvelous as this adventure may have been, it would not have been possible without the help of many.

I would have nothing to say or write without the inspiring co-operation of the hundreds of patients who subjected themselves to my tedious enquiries. While they gave me the chance to satisfy my curiosity and further my career, they gained nothing than my gratitude and this simple word of thanks.

Professor Kitslaar, among the medical students you had earned the reputation of being very demanding. During the last 6 years you evolved from an authoritarian teacher to an inspirational mentor, always there to tame my youthful enthusiasm and keeping me on the right track. Your methodological approach put structure into my chaotic thinking, and your critique helped me formulate hypotheses more accurately.

Professor Bruggeman, from the first day of my appointment you tried to make me feel at ease and, indeed, soon I found my place in your lab. The weekly progress meetings and journal discussions formed an important guidance and helped me keep faith in the completion of my work, even though occasionally it seemed like a lost case to me. Together with Professor Kitslaar you created the conditions that enabled me to complete my PhD thesis. I cannot thank you enough for all your support.

Dr Stassen, our best brainstorm sessions were followed by the most hideous hangovers. I wonder how many brilliant ideas have been lost in the bottom of countless beer glasses. I am sure that if we would write down half of the ideas generated in alcohol I could have written 3 PhD theses in half the time. I am certainly looking forward to many more 'scientific meetings' in the future.

Dr Kurvers played a key role in the design of the initial research project. It's a shame that we didn't have the chance to collaborate more closely during the course of my research appointment. I know it would have increased my scientific output significantly.

Dr Welten, I guess that neither you nor I had realised fully, the implications of your exclamation: "Doc you go to England" after you had arranged a clinical attachment for me at Leicester Royal Infirmary under supervision of Sir Professor Bell. My time in England inspired me to go into vascular research. On my return to Maastricht, you played an important role in the realisation of our clinical studies, which would not have been possible without the patients recruited from your clinic.

The weekly visits to Sittard were a welcome change from the frenzy of academia. Dr Van den Akker, your down to earth, no nonsense approach of the
vascular patient was a refreshing reminder that I shouldn’t take my scientific work too seriously. After all, a salvaged limb is worth a thousand publications.

Dr Jan Tordoir and Dr Geert Willem Schurink provided me not only with many patients for the studies but also with invaluable advice. The aneurysm work described in chapter 3 would not have been possible without the inspiring involvement and sharp reasoning of Geert Willem. I am looking forward to continue working with you on this matter in the future.

Professor Mess, it’s amazing how much can be learnt from little ‘bleeps’. Every time I thought I understood the significance of micro-embolic signals you showed me how limited my understanding was. Our discussions about TCD monitoring, micro-embolic signals and carotid plaques were most inspiring and I hope we can continue them after completion of my PhD.

Dr Morré, we joined forces quite late in the course of my studies. We, in Maastricht, had the patient material and you, in Amsterdam, had the SNP’s and thousands of ideas. Our meetings used to be very fruitful and -I have to confess- sometimes dazzling. Occasionally I would get lost in the plethora of hypotheses, ideas for new studies etc. I hope we will continue our collaboration in the future.

Professor van Dieijen-Visser, thank you for your faith in my ideas and the hundreds of high sensitive CRP measurements carried out in your laboratory. I look forward to working with you on aortic proteomics.

I spent countless hours in the microbiology lab. In contrast to my expectations, I grew to like the lab-work, not in the least due to the people there. In Gert I found a surrogate mother. She always knew where I misplaced my samples and made sure I cleaned up my mess after a day’s work. Without her help, I probably would still be staining countless plaques. The molecular work would have not been possible without the commitment and hard work of Selma. It is said that shared happiness is double happiness and shared pain is half the pain. I have to thank the other PhD students (Inge, Manuela, Tania and Rajaa) for doubling the fun and reducing the misery all these years.

Although it never felt like it at the time, it actually was a privilege to work with the many keen students. To have the ability to motivate young minds to enter the world of research and to be inspired by them was an amazing experience. I would like to thank, Marianne, Remy, Roger, Tim and Steef for their contribution to my work. Eric, I’m sorry that you didn’t join the club earlier. Good luck with your surgical training and I hope you will find time to continue your scientific efforts. Rick and Linda, thank you for all the work and the long days in the lab. It was a privilege to supervise you in your first steps in science. I am sure you both have a brilliant career ahead of you. I am happy to be able to pass the ‘torch’ on to Femke. I wish you the best of luck.

Keeping track of 509 patients would not be possible without the relentless work of the outpatient clinic nurses of Maastricht University Hospital, Atrium Medical
Centre Heerlen and Maasland Hospital Sittard. It’s amazing how much can be achieved on one strawberry pie. Many thanks are due to the vascular technicians of Maastricht University Hospital, Atrium Medical Centre Heerlen and Maasland Hospital Sittard, for countless ankle-brachial pressure measurements and duplex ultrasound scans.

I would like to thank the attending surgeons of the department of Surgery of Maastricht University Hospital for letting me loose on their patients. Many thanks also to my fellow residents, for supporting me in my initial endeavours as a clinician.

Ruben, we practically lived together in that bloody room in the hospital. I am ashamed to admit that often we spent more time with each other than with our partners. I would like to especially thank you and Diana for making these 4 years as inspiring and memorable as they were.

I am proud that my brother, Damian, will support me during the defence of my thesis. I cannot thank my family enough for their unconditional support and love. Μάνα και πατέρα, Όλγα, Μπριγγίτε, Αντώνη, Δάμιαν, Δέσποινα, Σέβη, Ηλέκτρα και Χριστίνα, σας ευχαριστώ για την συγκίνη, βοήθεια και κατανόησή σας. I will always be grateful to my parents for encouraging me to pursue my dreams and granting me the freedom to do so on my own terms.

Saïqa, you probably will not believe this but I really finished that bloody PhD finally. Thank you for listening to my boring presentations, correcting my dull manuscripts, spending time with me in the lab, and above all never losing faith in me. ‘In six years the young adventurer had travelled half the world and had seen some of the amazing wonders of nature and man. He had tried new tastes and talked to many wise men. But only now, back at home, he realised that the ultimate wisdom lay in the depths of the eyes of the woman who, during the entire journey, accompanied him in his thoughts’.

Τρύφωνας
List of publications
List of publications

Original Papers


Peeters MCE, Vainas T, Pietersen AM, Hekking JWM, van Straaten HWM. The curvature of embryos as measured at each somite level. Eur J Morphol 1997;35:42.


Vainas T, Stassen FRM, Kurvers HA, Grauls GE, Bruggeman CA, Kitslaar PJ. Chlamydia pneumoniae in atherosclerotic disease: association between serum antibody titers and presence of antigens/DNA in vascular tissue. Submitted.

Vainas T, Stassen FRM, de Graaf R, Bruggeman CA, Kitslaar PJEH. Presence of inflammatory cells in veins implanted as bypass grafts is associated with subsequent graft failure. Submitted.
Vainas T, Stassen FRM, Bruggeman CA, Kitslaar PJEHM, Pena AS, Morre SA. Synergistic effect of Toll like receptor 4- and CD-14 polymorphisms on total atherosclerosis burden in patients with peripheral arterial disease. Submitted.


Book Chapters

Curriculum Vitae

The author was born on October 26, 1969 in Heinsberg, Germany, into a family of Greek emigrants. In 1980 he followed his family back to Greece where he graduated from the 1st Lyceum of Thessaloniki in 1988. After a period of soulsearching, the author settled in Maastricht in 1992 where he studied at the medical faculty of Maastricht University (formerly known as Rijksuniversiteit Limburg) from September 1992 until November 1998. During his studies, the author served as student research assistant for the departments of Anatomy and Embryology (1995, Dr M Peeters & Dr H van Straaten), Obstetrics and Gynaecology (1996-97, Dr H Keunen & Dr T Hasaart) and General Surgery (1997-98, Dr K Hulsewe & Prof Soeters). In 1998 he followed an elective clinical and research attachment for 3 months at the department of vascular surgery, Leicester Royal Infirmary, Leicester, UK (Sir Prof PE Bell & Prof AR Naylor). After graduation the author accepted a position as PhD-student at the department of Surgery, Maastricht University Hospital and Cardiovascular Research Institute of Maastricht. During that period the work described in this thesis was performed. In May 2003 he started his training in General Surgery at Maastricht University Hospital (Supervisor Prof M.J. Jacobs and later Prof J.W. Greve) which will be completed at the department of Surgery of Catharina Ziekenhuis Eindhoven (Supervisor Dr G. Nieuwenhuizen). Together with Dr Schurink (Dept of Surgery, Maastricht University Hospital) he has been awarded a CARIM/Maastricht University grant in 2005 which will enable him to continue his scientific endeavors. The author has lost his heart to and found his inspiration in Miss Saiqa Sayed.
Printing of this thesis and the activities related to the defense of the PhD has financially been supported by:

AstraZeneca BV, Nycomed Nedenand BV, Sanofi-Synthelabo BV, Johnson & Johnson Medical BV, B.Braun Medical BV, Wyeth Pharmaceuticals BV, Bristol-Myers Squibb BV, Cook Nederland BV, Pie Medical Equipment BV.