The Role of *Chlamydia pneumoniae*-induced Platelet Activation in Cardiovascular Disease

*In vitro* and *In vivo* Studies

Hanna Kälvegren

During the course of the research underlying this thesis, Hanna Kälvegren was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.
The cover illustrates a manipulated scanning electron microscopy photograph of platelets activated by *Chlamydia pneumoniae* taken by the author.
“Acute infectious diseases may cause some pretty general lesions throughout the arterial system, either from diffuse action of toxins or from a widespread invasion of the arterial system by the infecting organism. The exact nature of these lesions in human cases and their final result has not been so well worked out.” (Frothingham, 1911)

Nearly 100 years later, this issue has still not been completely solved…

With love to

Isak and Joakim
CONTENTS........................................................................................................ 5

ABSTRACT........................................................................................................ 7

POPULÄRVETENSKAPLIG SAMMANFATTNING........................................... 9

PAPERS........................................................................................................... 11

TACK! .............................................................................................................. 13

ABBREVIATIONS............................................................................................ 16

BACKGROUND ............................................................................................... 17

A historical view ................................................................................................................................. 17
- Atherosclerosis ................................................................................................................................. 17
- Infections and atherosclerosis........................................................................................................... 18
- Chlamydia pneumoniae ..................................................................................................................... 19

Atherosclerosis................................................................................................................................. 21
- The risk factors of atherosclerosis ....................................................................................................... 21
- Atherosclerotic lesion initiation and formation of fatty streaks ............................................................ 22
- Formation of the fibrous atherosclerotic plaque .................................................................................. 27
- The vulnerable plaque .......................................................................................................................... 29
- Plaque rupture and thrombosis ............................................................................................................ 30

Platelets ................................................................................................................................................. 32
- Platelet aggregation ............................................................................................................................... 35
- Platelet ROS production ......................................................................................................................... 36
- Platelets and atherosclerosis ................................................................................................................. 37
- Platelet-bacteria interaction .................................................................................................................... 40

Chlamydia (Chlamydophila) pneumoniae ............................................................................................. 43
- Morphology .......................................................................................................................................... 43
- The life cycle ......................................................................................................................................... 44
- The attachment to the host cell ............................................................................................................... 45
- The life in the inclusion ........................................................................................................................... 46
- Activation of the host cell ......................................................................................................................... 47
- Persistence ............................................................................................................................................. 47

The connection between Chlamydia pneumoniae and atherosclerosis .................................................. 48
- Infections and cardiovascular disease ................................................................................................. 48
- Epidemiological studies ......................................................................................................................... 49
- The presence of C. pneumoniae in atherosclerotic plaques .................................................................... 50
- The presence of C. pneumoniae in circulating blood cells .................................................................... 51
- Animal models ....................................................................................................................................... 52
- Antibiotic trials ......................................................................................................................................... 55
Abstract

The common risk factors for atherosclerosis, such as obesity, high cholesterol levels, sedentary lifestyle, diabetes and high alcohol intake, only explain approximately 50% of cardiovascular disease events. It is thereby important to identify new mechanisms that can stimulate the process of atherosclerosis. During the past decades, a wide range of investigations have demonstrated connections between infections by the respiratory bacterium *Chlamydia pneumoniae* and atherosclerosis. Earlier studies have focused on the interaction between *C. pneumoniae* and monocytes/macrophages, T-lymphocytes, smooth muscle cells and endothelial cells, which are present in the atherosclerotic plaque. However, another important player in atherosclerosis and which is also present in the plaques is the platelet. Activation of platelets can stimulate both initiation and progression of atherosclerosis and thrombosis, which is the ultimate endpoint of the disease. The aim of the present thesis was to investigate the capacity of *C. pneumoniae* to activate platelets and its role in atherosclerosis.

The results show that *C. pneumoniae* at low concentrations binds to platelets and stimulates platelet aggregation, secretion, reactive oxygen species (ROS) production and oxidation of low-density lipoproteins (LDL), and that these effects are mediated by lipopolysaccharide (LPS). Activation of protein kinase C, nitric oxide synthase and 12-lipoxygenase (12-LOX) was required for platelet ROS production, whereas platelet aggregation was dependent on activation of GpIIb/IIIa. Pharmacological studies showed that the *C. pneumoniae*-induced platelet activation is prevented by inhibitors against 12-LOX, platelet activating factor (PAF) and the purinergic P2Y1 and P2Y12 receptors, but not against cyclooxygenase (COX). These findings were completely opposite to the effects of these inhibitors on collagen-stimulated platelets. We also present data from a clinical study indicating that percutaneous coronary intervention (PCI or balloon dilatation) leads to release of *C. pneumoniae* into the circulation, which causes platelet activation and LDL oxidation.

In conclusion, these data support a role for *C. pneumoniae*-induced platelet activation in the process of atherosclerosis. Stimulation of platelets by *C. pneumoniae* leads to release of growth factors and cytokines, oxidation of LDL and platelet aggregation, which are processes that can stimulate both atherosclerosis and thrombosis. Development of novel drugs that prevent *C. pneumoniae*-platelet interaction by inhibiting 12-LOX and/or PAF, may be important in the future treatment of cardiovascular disease.
Populärvetenskaplig sammanfattning


Resultaten visar att *C. pneumoniae* binder till trombocyter och aktiverar dessa till att klumpa ihop sig och bilda aggregat. Dessutom frisätter trombocyterna olika substanser som är centrala vid åderförkalkning. Klamydiabakterien stimulerar även en produktion av reaktiva syremetaboliter från trombocyten, vilket leder till en oxidering av LDL, den skadliga typen av kolesterol. Oxidering av LDL är den mekanism som tros påbörja åderförkalkningsprocessen. Dessutom visar vi i en klinisk studie att interaktionen mellan *C. pneumoniae* och trombocyter spelar en roll hos patienter med kranskärlssjukdom (åderförkalkning i hjärtats blodvägar). I denna studie togs prover från patienter som genomgick ballongutvidgning (PCI) av kranskärlen. Resultaten visar att ballongutvidgningen leder till en frisättning av *C. pneumoniae* bakterier till blodcirkulationen som i sin tur stimulerar trombocyter och oxiderar LDL. Det sista arbetet i avhandlingen presenterar hur man farmakologiskt kan förhindra en aktivering av trombocyter orsakad av *C. pneumoniae*. Det visade sig att helt andra farmakologiska substanser än de som
traditionellt används vid behandling av hjärtkärlsjukdom (t.ex. aspirin) krävs för att förhindra en sådan typ av trombocytstimulering.

Resultaten som presenteras i denna avhandling är av betydelse för att ytterligare förklara de bakomliggande orsakerna till att vi drabbas av hjärtkärlsjukdomar. En trombocytaktivering som beror på *C. pneumoniae* infektion kan medverka till både åderförkalkning och trombos. Utveckling av nya läkemedel som förhindrar en *C. pneumoniae*-orskad stimulering av trombocyter kan bli en framgångsrik strategi vid behandling av hjärtkärlsjukdomar.
Papers

This thesis is based on the following Papers, which will be referred to by their Roman numerals:


Tack!

Under doktorandtiden har jag fått möjligheten att arbeta med många underbara människor som varit betydelsefulla på olika sätt. Förutom handledare och samarbetspartners så finns det även många utanför ”forskarvärlden” som betytt mycket för att avhandlingen nu har blivit slutförd.

Jag vill rikta ett särskilt tack till:

Min huvudhandledare **Torbjörn Bengtsson** och mina biträdande handledare **Arina Richter** och **Erik Kihlström**:

**Torbjörn**, för allt du har lärt mig och sättet du har stöttat mig på under de här åren. Ditt handledarskap har passat mig perfekt!! Du finns alltid närvarade när man behöver diskutera forskning och andra viktiga frågor, och kommer med bra synpunkter och råd. Samtidigt har jag haft ditt förtröende att få arbeta relativt självständigt, vilket har varit väldigt utvecklande.

**Arina**, min handledare i den kliniska delen av avhandlingen, för att du har stöttat mig och visat ett stort intresse under min doktorandtid. Man blir alltid på bra humör när vi haft möten, du utstrålar verkligen en smittande glädje och kommer med många uppmuntrande ord!! Tack för alla trevliga middagar i Ekängen!

**Erik**, för stöttning, givande möten, goda råd och för att du delat med dig av dina kunskaper om klamydia forskning.

**Övriga samarbetspartners:**

**Magnus Grenegård** för ett väl fungerande samarbete och för allt du lärt mig om farmakologi och olika dataprogram… Du är väldigt inspirerande att arbeta med!

**Lena Wind** för ditt alltid strålande humör och för att du tar så väl hand om patienterna i BAPCAD studien! **Micha Milovanovic** för hjälp med provinsamling. **Marie Högdahl** för att du delat med dig av dina kunskaper om cell- och klamydia-odling, och även för allt trevligt småprat om annat än forskning.
Per Leanderson för ett väl fungerande samarbete och för att du lärde mig TBARS metoden. Peter Garvin och Margaretha Kristenson för ett bra samarbete och för att ni bidrog med provmaterial till kontrollgruppen i BAPCAD projektet.

Kristina Orselius för din lysande praktiska handledning under den första tiden, bra samarbete på labb och stöd i mycket annat. Margaretha Lindroth för hjälp med att ta de vackra elektronmikroskopibilderna.

Rolf Andersson för visat intresse av min forskning och för att jag fått möjlighet att genomföra min forskarutbildning på farmakologen! Anita Thunberg för att du alltid är så hjälpsam och trevlig!

mina vänner på labb, speciellt Ann-charlotte Svensson och Caroline Skoglund. Utan er hade den här avhandlingen inte varit lika rolig att genomföra!! Tack Carro för hjälp med provinsamling i BAPCAD projektet!

övriga i trombocytgruppen: Eva Lindström, Anna Asplund, Peter Pålsson, Peter Gunnarson och Louise Levander för alla lärorika forskningsmöten och för bra konstruktiv kritik till min forskning!

alla andra på avdelningen för farmakologi för den goda stämningen och trevliga arbetsmiljön.

alla andra på avdelningen för medicinsk mikrobiologi för den trevliga första perioden av min doktorandtid (trots allt buller från ombyggnationen, men det var ju inte ert fel!). Speciellt tack till Olle Stendahl för stöd under den första delen av doktorandtiden.

Carin Starkhammar och Nils Ravald för ett bra samarbete i BAPCAD projektet!

alla examens/projektarbetare och stipendiater som jag har fått möjligheten att handleda. Ett speciellt tack till Jonna Fridfeldt, Johanna Andersson och Helena Bylin.

forskarsskolan Forum Scientium och Stefan Klinstström för alla givande forskningsträffar och seminarier.
Lillemor Fransson och Torbjörn Bengtsson för det fantastiska året med Biomedicinska forskarskolan. Jag vill också tacka alla övriga medlemmar under året 00/01 speciellt Susanne, Nahreen och Camilla för att vi fortfarande håller så god kontakt. Susanne, du är verkligen en inspirationskälla!!

Alla mina övriga vänner, ni vet vilka ni är, för att ni finns där när man behöver prata och hitta på något kul. Speciellt tack till mina ”barndomsvänner” Maria och Linn! Jag uppskattar verkligen att ha så goda vänner som man alltid vet att man kan lita på, även om vi har lite olika uppfattning om vetenskap emellanåt… Jag vill även tacka Pia för alla givande samtal om livet med barn och för att du ställer upp när det verkligen behövs.

Min älskade familj:

min farmor som tyvärr inte finns hos oss längre, men som alltid var mycket intresserad av allt som jag företog mig (även min forskning)…

mamma och pappa för att ni har gett mig så goda förutsättningar i livet, och för allt ni gör för att jag ska ha det bra! Mina syskon Stefan, Tobias och Viktor, ni är såå betydelsefulla!

de viktigaste personerna in mitt liv, Isak och Joakim, ni är en stor del av att jag har lyckats med det här! Till min lilla Isak för att du är en sådan glädjespridare och för att du ser till att din mamma kommer ned på jorden ibland. Min älskade Jocke, för att du alltid finns hos mig, ställer upp och orkar lyssna!!

Finansiärerna till denna avhandling:

- Strategiområdena ”Inflammation” och ”Cardiovascular Inflammation Research Centre (CIRC)” vid Linköpings Universitet
- Forsknings-och Forskarutbildningsnämnden (FUN), Hälsouniversitet i Linköping
- Vetenskapsrådet
- Trygg Hansas forskningsfond
- Landstinget i Östergötland (kommittén för medicinsk forskning och utveckling)
- Hjärtfonden vid Linköpings Universitet
- Lions forskningsfond mot folksjukdomar
- Fonden för forskning utan djurförsök
- Forskningsområdet i sydöstra sverige (FORSS)
Abbreviations

CAD  coronary artery disease
LDL  low-density lipoprotein
oxLDL oxidized LDL
HDL high-density lipoprotein
apo apolipoprotein
LOX-1 lectin-like oxidized receptor-1
TNF-α tumor necrosis factor-α
TGF-β transforming growth factor-β
MCP-1 monocyte chemoattractant protein 1
ICAM-1 intracellular adhesion molecule-1
VCAM-1 vascular adhesion molecule-1
NO nitric oxide
IL interleukin
IFN interferon
MHC major histocompatibility complex
PDGF platelet derived growth factor
ROS reactive oxygen species
MMP matrix metalloproteinase
vWF von Willebrand factor
GP glycoprotein
ADP adenosine diphosphate
TxA₂ thromboxane A₂
PF 4 platelet factor 4
PSGL-1 P-selectin glycoprotein ligand-1
ATP adenosine triphosphate
NOS nitric oxide synthase
COX cyclooxygenase
LOX lipoxygenase
MIP macrophage inflammatory protein
PMP platelet microbiocidal protein
CMV cytomegalovirus
LPS lipopolysaccharide
TLR toll-like receptor
HSP heat shock protein
COMP *Chlamydia pneumoniae* outer membrane complex
Pmp polymorphic proteins
MOMP major outer membrane protein
Omp outer membrane protein
CRP cystein rich protein
EB elementary body
RB reticulate body
ELISA enzyme-linked immunosorbent assay
MIF microimmunofluorescence
EIA enzyme-linked immuno assay
PBMC peripheral blood mononuclear cells
FBS fetal bovine serum
IFU inclusion forming units
PRP platelet rich plasma
PCI percutaneous coronary intervention
CABG coronary artery bypass graft
MDA malondialdehyde
TBARS thiobarbituric acid reactive substances
PAF platelet activating factor
Background

A historical view

Atherosclerosis

Atherosclerosis contributes to diseases such as coronary artery disease (CAD) and stroke, which are the major causes of death in the western world. However, atherosclerosis and its complications is not a new problem. In 1911, Marc Ruffer identified degenerative arterial changes in an Egyptian mummy, which in 1962 were confirmed by another research group to be atherosclerotic plaques (Ruffer, 1911; Sandison, 1962). These findings show that atherosclerosis already existed in antiquity. The term atheroma was created by the Roman author Celsius two thousands years ago, at that time meaning fatty tumour (Cottet and Lenoir, 1992), but in 1755 the term was designated by Albrecht von Haller as the degenerative process observed in the intima of arteries (Haller, 1755). Interestingly, as early as 1815 the London surgeon Joseph Hodgson published the hypothesis that inflammation was the underlying cause of atheromateous arteries (Hodgson, 1815). However, most nineteenth-century pathologists followed Carl Rokitanski’s view that atherosclerosis was a degenerative process, with intimal proliferation of connective tissue and calcification (Tedgui and Mallat, 2006), a process that was assigned arteriosclerosis by the French pathologist Jean Lobstein (Lobstein, 1833). The inflammatory theory of atherosclerosis arose again in 1856 when the prominent German pathologist Rudolf Virchow designated atheroma as a chronic inflammatory disease of the intima (Virchow, 1856). Another important date in the history of this area was in 1904 when Marchand recognised the association of fatty acid degeneration and vessel stiffening and introduced the term atherosclerosis to indicate this combination (Blankenhorn and Kramsch, 1989).
A milestone was made in 1908 when the Russian scientist Alexander Ignatowski showed that atherosclerosis can be induced in rabbits by feeding them milk and egg yolk (Ignatowski, 1908). Shortly after this discovery, the ability of pure cholesterol to reproduce experimental atherosclerosis in rabbits was demonstrated (Chalatov, 1913). These findings revealed the importance of lipids and cholesterol in the atherosclerotic process. The next large step in the history of atherosclerosis was taken during the 1970s when Brown and Goldstein stated that acetylated low-density lipoprotein (LDL) and not native LDL induced foam cell formation of macrophages (Goldstein and Brown, 1977). A decade later, the ability of oxidized LDL (oxLDL) to induce foam cell formation was demonstrated by Daniel Steinberg and his group (Steinberg et al., 1989). Furthermore, in the late 1970s Russell Ross published the “response to injury hypothesis of atherosclerosis” (Ross et al., 1977). He viewed atherosclerosis as a fibroproliferative process that results from a chronic inflammatory response. He also revealed the additive contribution of the endothelium, mononuclear phagocytes, platelets and smooth muscle cells in atherosclerosis (Raines and Ross, 1995; Ross, 1979; 1985; 1990; Ross et al., 1982). His work has greatly influenced the research field of atherosclerosis during recent decades.

Infections and atherosclerosis

The concept that infectious agents have an impact on the process of atherosclerosis is not new, but was already proposed in the late 1800s and early 1900s. Huchard was the first to suggest the involvement of infectious agents in the process of atherosclerosis when he published the article “Infectious diseases of childhood as potential cause of inflammation” in 1891. Shortly thereafter, Weisel and Klotz found a relation between atherosclerosis and Streptococci infections, typhoid, scarlet fever and measles (Klotz, 1906; Weisel, 1906). In 1908, Osler wrote in his book “Modern Medicine: its practice and theory” about a potential link between
acute infection and atherosclerosis (Osler, 1908). In the late 1940s, a strong association between marek disease virus (MDV) and atherosclerosis was found, which was also demonstrated in the 1980s by Fabricant with co-workers (Cottral, 1950; Fabricant et al., 1981). Furthermore, in the 1960s Burch published a link between coxsackie B virus infection and atherosclerosis in chickens (Burch et al., 1966; Burch et al., 1967).

**Chlamydia pneumoniae**

*Chlamydia pneumoniae* was isolated for the first time in 1965 from the conjunctiva of a Taiwanese child participating in a trachoma vaccine trial (Grayston, 1965). This new strain was named TW-183. A role of this organism in human disease was revealed in 1983, when the first respiratory isolate (AR-39, AR=Acute Respiratory) was obtained in Seattle from a young student with pharyngitis (Grayston et al., 1986). Thereafter, this newly discovered bacteria species was named TWAR after a fusion of the names of the two first isolates (TW-183 and AR-39). TWAR was at this time thought to be “a human *Chlamydia psittaci* that is spread from human to human, without a bird or animal host” (Grayston et al., 1986). Further research on this bacterium characterised the TWAR organism as a member of the genus Chlamydia that was serologically distinguished from the two existing species, *C. trachomatis*, and *C. psittaci*. Furthermore, the extracellular form of this bacterium, the elementary body, differed morphologically from both *C. trachomatis* and *C. psittaci*, and there was less than 10% of DNA homology with the pre-existing species (Campbell et al., 1989; Grayston, 1989). These evidences led to the proposal of a new Chlamydia species in 1989, which was named *Chlamydia pneumoniae* (Grayston, 1989). The first study that demonstrated an association between *C. pneumoniae* infections and atherosclerosis was published by the Finnish scientist Pekka Saikku and his colleagues (Saikku et al., 1988). They found that significantly more subjects with coronary artery disease had elevated levels of IgA and IgG antibodies against *C. pneumoniae*, compared to
healthy matched controls. These observations started a new, expanding research field about the role of this newly discovered Chlamydia species in the process of atherosclerosis.
Atherosclerosis

The risk factors of atherosclerosis

Atherosclerosis is the underlying cause of CAD, peripheral vascular disease and stroke, which are the leading cause of morbidity and mortality in the Western world (Murray and Lopez, 1997). The process of atherosclerosis often starts very early in life, in fact already in the fetus state. In newborns accumulations of lipids, connective tissue and smooth muscle cells under the endothelium have been observed (Napoli et al., 1997). At the age of 10, streaks with smooth muscle cells are found in the intima. Furthermore, at the age of 20, fibromuscular plaques are present in 50% of the population. However, in most cases it takes about 30 more years before the fibromuscular plaque has grown so large that it leads to local enlargement under the intima. The atherosclerotic plaque in itself rarely causes clinical symptoms. However, in late stages of atherosclerosis the plaque may rupture, which leads to thrombosis and in some cases an infarction as a consequence (Ross, 1999).

The risk factors for atherosclerosis can be grouped into two types: modifiable and fixed. The modifiable are cholesterol and triglycerides (Abbott et al., 1988; Gardner et al., 1996; Lamarche et al., 2001), blood pressure (Hyman and Pavlik, 2001; Lewington et al., 2002; Vasan et al., 2001), cigarette smoking (Castelli et al., 1981; Howard et al., 1994), diabetes (Gaede et al., 2003; Haffner, 1998; Semenkovich, 2006), obesity (Wilson et al., 2002), sedentary lifestyle (Mittleman and Siscovick, 1996; Paffenbarger et al., 1986; Thompson et al., 2003) and alcohol intake (Kauhanen et al., 1999), whereas the fixed are age, gender (Lerner and Kannel, 1986) and genetics (Breslow, 2001; Dallongeville et al., 1992; Klerk et al., 2002; Luc et al., 1994). However, there are also other potential risk factors such as lipoprotein A (Scanu, 2003a; b; Schaefer et al., 1994), autoimmune elements (e.g. autoantibodies, autoantigens and autoreactive lymphocytes) (Abou-Rayya and Abou-Rayya, 2006) and infections (e.g.
Atherosclerotic lesion initiation and formation of fatty streaks

During the past decades, inflammation has been increasingly recognized as a key event in the formation of atherosclerotic plaques. Inflammatory processes participate both in the atherosclerotic initiation and progression and in thrombosis, which is the ultimate endpoint of the disease (Libby, 2006; Ross, 1999). A general theory of how the atherosclerotic process is initiated is by modification and accumulation of lipoprotein particles, particularly LDL, in the intima of the vessel wall (Berliner et al., 1997; Williams and Tabas, 1998). The fact that atherosclerosis does not develop in animal models with low levels of plasma lipoproteins strongly supports this theory. Lipid hydroperoxides, lysophospholipids, carbonyl compounds, and other biological active moieties of lipoproteins have been found in the lipid fraction of the atheroma (Witztum and Berliner, 1998). However, in addition to lipoproteins there are many other triggering factors that are capable of inducing endothelial dysfunction/activation and thereby trigger atheroma formation. Examples of such triggering factors are the products of glycooxidation associated with hyperglycemia, vasoconstrictor hormones inculpated in hypertension, proinflammatory cytokines derived from adipose tissue and certain viral and bacterial infections (Libby and Theroux, 2005). The role of infections in atherosclerosis, with focus on *Chlamydia pneumoniae*, will be described later in this thesis.

Plasma lipoproteins are spherical particles that transport various amounts of cholesterol and triglycerides in the circulation (Sacks, 2006). Already in 1913 it was discovered that high cholesterol levels can induce atherosclerosis in rabbits (Chalatov, 1913). The lipoproteins have traditionally been grouped into five major classes, in which the atherogenic
lipoproteins are the chylomicrones, the very low-density lipoproteins (VLDL), the intermediate-density lipoproteins (IDL) and the LDL, whereas the athero-protective are the high-density lipoproteins (HDL) (Wilson, 2005). Another atherogenic lipoprotein is Lipoprotein (a) (Lp(a)) that has properties similar to LDL (Discepolo et al., 2006). Apolipoprotein (apo) B is a protein found on the surface of the atherogenic lipoproteins, whereas apo A-I is mainly found on the anti-atherogenic HDL molecule (Sacks, 2006). There are now several studies that have demonstrated apo particle concentrations as a predictor of CAD. The Apo-related Mortality Risk Study (AMORIS) found that apo B-levels and the ratio between apo B/apo A-I are strongly and positively related to increased risk of fatal myocardial infarction in men and women, and they also found that apo B was a stronger predictor of risk for CAD than LDL-cholesterol (Walldius et al., 2001). The LDL particles are a very heterogenous group, i.e. some LDL-subtypes are more atherogenic than others. The small, dense LDL particles are more atherogenic than the large, buoyant ones (Carmena et al., 2004).

The earliest steps in the atherosclerotic process are characterized by endothelial activation and accumulation of cholesterol and triglycerides in macrophages, leading to foam cell formation. However, when studying the proposed trigger of foam cell formation *in vitro*, the LDL-particles, they were not found to be atherogenic. This discovery led to the conclusion that the LDL receptor is not responsible for foam cell formation (Goldstein and Brown, 1977). The mechanisms for foam cell formation were described in 1989 when Steinberg and co-workers proposed the original oxidative modification theory of atherosclerosis. This theory says that LDL needs to be oxidized to support foam cell formation (Quinn et al., 1987; Steinberg et al., 1989). There is by now much evidence for a fundamental role of LDL-oxidation in the initiation of atherosclerosis (Boyd et al., 1989; Haberland et al., 1988; Palinski et al., 1994; Palinski et al., 1989; Ross, 1995; Yla-Herttuala et al., 1989). However, LDL can also be modified to an atherogenic molecule in other ways than oxidation. Such modifications are
proteolytic modification of apo B in LDL and lipolysis and/or hydrolysis of the cholesterol esters of LDL (Pentikainen et al., 2000). However, this thesis will focus on oxLDL and its role in the initiation of atherosclerosis.

The tricky question that still not has been fully clarified is: Which is the most important location for atherogenic LDL oxidation, in the plasma or in the vessel wall? The best answer to this question based on current knowledge is probably at both sites, but it remains to be fully clarified. Several investigations have shown an association between oxLDL in plasma and CAD (Ehara et al., 2001; Hara et al., 2004; Holvoet et al., 1998; Toshima et al., 2000; Tsimikas et al., 2005). Oxidation of LDL in plasma is protected by antioxidants (Frei et al., 1988), which suggests that the detected oxLDL in plasma is due to LDL oxidation occurring in plasma and not due to re-entering of oxLDL from the subendothelial space to the plasma. It was discovered in 1997 that vascular endothelial cells express lectin-like oxidized receptor-1 (LOX-1), a vascular endothelial receptor for oxLDL (Sawamura et al., 1997). LOX-1 expression is not constitutive, but can be induced by proinflammatory stimuli, such as tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, C. pneumoniae (Yoshida et al., 2006), bacterial endotoxin, angiotensin II, oxLDL itself and fluid shear stress (Sawamura et al., 1997). In addition, LOX-1 expression is detectable on cultured macrophages and activated vascular smooth muscle cells (Kume and Kita, 2001). Apart from binding oxLDL, this receptor can also bind platelets, certain bacteria, aged red blood cells and apoptotic cells (Oka et al., 1998; Shimaoka et al., 2001). It has been demonstrated that the receptor is expressed on endothelial cells in the early stages of atherosclerosis, which suggests a role of this receptor in early atherogenesis (Kataoka et al., 1999). During more advanced stages, the receptor is also upregulated on macrophages and smooth muscle cells (Kataoka et al., 1999). OxLDL binding and uptake by LOX-1 in endothelial cells induces proinflammatory signals, such as monocyte chemoattractant protein 1 (MCP-1) expression (Cominacini et al., 2000; Li and Mehta, 2000), which further supports a role for the interaction between oxLDL and its
endothelial receptor in the triggering mechanisms of atherosclerosis (Adachi and Tsujimoto, 2006).

Apart from modification in plasma, LDL is also exposed to changes in the vessel wall. All cell types that are found in the atheroma are capable of oxidizing LDL. It has been suggested that LDL can be oxidized during transcytosis of lipoprotein particles from the circulation to the subendothelial area by the endothelium (Ross, 1995). OxLDL behaves as a potent proinflammatory agent and stimulates the synthesis of various cytokines such as MCP-1 from smooth muscle cells and endothelial cells, resulting in recruitment of monocytes and T-lymphocytes to the activated vessel wall (Liao et al., 1991; Quinn et al., 1987; Quinn et al., 1985). Furthermore, oxLDL by itself has chemoattractant activity on monocytes and stimulates differentiation of monocytes to macrophages. Napoli et al. found in 1997 that in very early lesions, present in human fetal aortas, native and oxLDL are frequently found in the absence of monocytes/macrophages, suggesting that intimal LDL accumulation and oxidation contributes to monocyte recruitment in vivo (Napoli et al., 1997). Furthermore, oxLDL also stimulates the expression of leukocyte adhesion molecules on endothelial cells, such as E-selectin, intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), leading to leukocyte binding to the vessel wall. Reduction in the synthesis of the atheroprotective molecule nitric oxide (NO) is also induced by oxLDL (Kugiyama et al., 1990).

The most important chemoattractant molecules in the migration of leukocytes to the activated endothelium are supposed to be MCP-1 and interleukin (IL) 8 for monocytes and interferon (IFN)-inducible protein 10 (IP-10), monokine induced by IFN-γ (Mig) and IFN-inducible T-cell α chemoattractant (I-TAC) for T-lymphocytes (Boisvert et al., 1998; Boring et al., 1998; Gu et al., 1998; Mach et al., 1999). The role for MCP-1 in atherogenesis was clearly demonstrated in 1998 when Boring et al. showed that apolipoprotein E knockout (apoE-/-) mice lacking the receptor for MCP-1, the CC2 receptor, had 83% less lipid deposition in plaques than
mice having the receptor (Boring et al., 1998). VCAM-1 has been proposed to be the most pivotal adhesion receptor for the recruitment of leukocytes to the early atheroma. This receptor binds the types of leukocytes found in early atheroma, the monocyte and the T-lymphocyte (Cybulsky et al., 2001). Furthermore, it has been shown that elevation in the expression of VCAM-1 occurs before leukocyte recruitment in both rabbit and mouse models of cholesterol-induced lesion formation, which supports a role of this adhesion molecule in the recruitment process (Li et al., 1993). When the monocytes enter the subendothelial space at the site of endothelial activation, they differentiate into macrophages and start to express scavenger receptors on their surface, under the influence of macrophage-colony stimulating factor (M-CSF) (Qiao et al., 1997). There are at least six types of scavenger receptors expressed on the macrophage cell surface that can bind to oxLDL. These are CD36, scavenger receptor class B (SR-BI), scavenger receptor class A (SR-A), CD68 and LOX-1 (Greaves and Gordon, 2005). Ingested modified apoB-containing lipoproteins, like LDL, are delivered to lysosomes in the macrophages and hydrolysed to release free cholesterol and fatty acids (Greaves and Gordon, 2005), which leads to the formation of foam cells. In parallel with this process, the macrophages multiply and release several growth factors and cytokines, which intensifies the inflammatory environment (Libby, 2006). Furthermore, T-lymphocytes appear in the atherosclerotic plaques, which was demonstrated in 1985. A few years later, major histocompatibility complex (MHC) class II antigen expression was detected on smooth muscle cells and interferon (IFN)-γ was found around T-lymphocytes, which supports the presence of activated T-lymphocytes in the atherosclerotic plaques (Jonasson et al., 1985; Hansson et al., 1989). Both CD4+ and CD8+ T-cells are found in the plaque, but the CD4+ generally dominate in number (Frostegard et al., 1999). Activation of T-lymphocytes is not thought to be important in the initiation of atherosclerosis but rather in the early progression of the disease (Khallou-Laschet et al., 2006). The lesion that contains foam cells and T-lymphocytes, situated under a monolayer of endothelial cells, is called a
fatty streak and is the first lesion in atherosclerosis (Kaperonis et al., 2006)(Fig. 1).

**Figure 1. The formation of a fatty streak**

The exact mechanisms involved in the formation of the fatty streak are not fully understood. However, the most commonly used theory is the accumulation and modification/oxidation of low-density lipoproteins (LDL) in the intima. This leads to release of cytokines and expression of leukocyte adhesion receptors on the endothelium, which stimulates the recruitment of monocytes and T-lymphocytes to the vessel wall. The monocytes are converted to macrophages in the intima and ingest modified/oxLDL, which subsequently leads to the formation of foam cells.

**Formation of the fibrous atherosclerotic plaque**

In the next step of the atherosclerotic process, inflammatory cytokines, produced by leukocytes, initiates migration and proliferation of smooth muscle cells through the internal elastic laminae to the subintimal area (Libby et al., 2002). The smooth muscle cells produce extracellular matrix proteins, such as collagen and fibronectin, which are characteristic for the advanced lesion (Ross, 1999). Furthermore, the smooth muscle cell matrix proteins contain proteoglycans that can bind to lipoproteins. This binding retains the lipoproteins in the subendothelial space, which in turn increases their availability to undergo modification such as oxidation (Srinivasan et al., 1991; Williams and Tabas, 1995; 1998). The LDL modification further
stimulates inflammation and accumulation of lipid-loaded macrophages in the subendothelial space. As the atherosclerotic lesion develops, the central core, which consists of deposited lipids and both viable and necrotic leukocytes, is covered by a thick fibrous cap composed of smooth muscle cells and extracellular matrix.

It has been observed from clinical angiographic studies that the growth of a lesion occurs in bursts (Bruschke et al., 1989; Yokoya et al., 1999). This discontinuous progression of atherosclerosis is suggested to occur because of physical disruption of the plaque followed by accumulation of mural thrombus in the plaque (Davies, 1996). Thrombosis can occur as a consequence of three kinds of physical disruptions (Libby, 2002). The first type is superficial erosion of the endothelium that covers the plaque. This process may happen as a result of inflammation-induced production of extracellular matrix degrading enzymes by endothelial cells, which leads to loss of endothelium, and thereby superficial erosion (Libby, 2002).

Secondly, in atherosclerotic plaques fragile microvessels are formed by neo-angiogenesis which have a function in serving the growing plaque with nutrients (de Boer et al., 1999; Kolodgie et al., 2003). There is evidence that thrombosis can occur as a result of intra-plaque haemorrhage of these micro-vessels (Kolodgie et al., 2003). These intra-plaque thromboses may subsequently lead to thrombin generation and secretion of platelet-derived growth factor (PDGF) and TGF-β from activated platelets. PDGF and TGF-β in turn mediates stimulation, proliferation and collagen synthesis of smooth muscle cells, which stimulates the growth of the plaque. The third and most common mechanism of plaque disruption is fracture of the fibrous cap. The lipid core and the fibrous cap consist of pro-thrombotic and platelet-activating factors, such as tissue factor and collagen. If the cap ruptures, these factors stimulate the coagulation system and platelets in the circulation which eventually lead to thrombosis.

Most of the cap ruptures do not lead to clinical symptoms. Instead of total occlusion of the vessel lumen, a limited mural thrombus frequently forms. This, in turn, provokes a healing response that leads to smooth muscle cell
proliferation, collagen secretion and formation of a more fibrous plaque (Libby, 2002). Thrombus incorporation and platelet activation are thereby fundamental events in the growth and development of the atherosclerotic plaque. In addition to secreting growth factors, platelets release inflammatory cytokines and reactive oxygen species (ROS) that have important roles in atherosclerotic progression, which will be described later in this thesis.

The vulnerable plaque

The mechanisms that change the characteristics of a stable plaque with a thick fibrous cap to an unstable vulnerable plaque involve the production of various cap-degrading enzymes. These includes collagenases such as the matrix metalloproteinases (MMPs) -1, -8 and -13 (Galis et al., 1994; Herman et al., 2001; Sukhova et al., 1999), which are enzymes produced by a wide range of cell types including mononuclear phagocytes, endothelial cells and smooth muscle cells in response to certain inflammatory mediators found in the atherosclerotic plaque, e.g. IL-1β and TNF-α (Visse and Nagase, 2003). Furthermore, the cytokine IFN-γ, secreted from leukocytes in the plaque, inhibits the production of collagen by smooth muscle cells, which further promotes the fragility of the plaque (Gupta et al., 1997b). Moreover, the surface proteins CD40 and CD40 ligand are expressed on macrophages, activated T-lymphocytes, endothelial cells and smooth muscle cells, whereas platelets express only the CD40 ligand. Engagement of CD40 with its ligand induces the production of IFN-γ, MMPs and tissue factors, which further stimulates the dissolution of the cap and promotes thrombosis (Mach et al., 1998; Schonbeck and Libby, 2001; Mach et al., 1998; Schonbeck and Libby, 2001). One potential trigger of CD40 ligand expression is oxLDL (Schonbeck et al., 2002).

In addition to collagenases, certain gelatinases (MMP-2 and -9) are found in their active forms in the plaque and have a central role in the progression
of atherosclerotic lesions and vascular remodelling (Newby, 2005). The composition of the cap is very important for the stability of the plaque. Early in atherogenesis, smooth muscle cells are present in the cap, but as the lesion progresses they decline in numbers (Hangartner et al., 1986; Stary, 1989). When the smooth muscle cells disappear, possibly due to apoptosis, the cap becomes less stiff in its character (Lee et al., 1991) and the risk of rupture increases.

Plaque rupture and thrombosis

Clinical manifestations of atherosclerosis are most often a consequence of rupture of the fibrous cap that covers the lipid core of the plaque, which leads to thrombosis. In some cases, the thrombus occupies a large part of the arterial lumen, leading to infarction of the tissue that the blood vessel supplies. Pathologic and angiographic studies have suggested that the lesions that are most prone to rupture are characterised by a large core of extracellular lipids, a high density of lipid-containing macrophages, and a reduced number of smooth muscle cells and collagen in the cap (Falk, 1991). Such plaques are soft in consistence and it is therefore not surprising that they easily rupture. A lipid core that occupies more than 40% of the plaque has been established as a threshold, above which the plaque is considered to be at particularly high risk of rupture (Davies et al., 1993).

The rupture occurs when strain within the fibrous cap exceeds the deformability of its components, and it is thought to happen as a consequence of shear stress or pressure changes transpiring in an artery (Loscalzo, 2005). The plaque rupture leads to exposure of many extracellular matrix structures to the cells in the circulation such as collagen, laminin and fibronectin. The main inducers of platelet activation are collagen and the plasma protein von Willebrand factor (vWF) associated with collagen on the surface of the subendothelium (Blockmans et al., 1995). The platelet has the capacity to bind directly to collagen via the glycoprotein (GP) Ia-IIa (integrin α2β1) and GPVI or indirectly via collagen-immobilized vWF by the receptor GPIb-V-IX (CD42b-CD42d-
CD42a), leading to platelet activation, spreading and granule secretion. Additional platelets are recruited after secretion of platelet activators, such as serotonin, adenosine diphosphate (ADP) and thromboxane A2 (TxA2). Furthermore, platelet activation leads to surface exposure and activation of GPIIb/IIIa (CD41/CD61, integrin αIIb/β3), which then binds to both vWF and fibrinogen. Fibrinogen is a molecule with a dimeric structure that has two binding sites for GPIIb/IIIa on each molecule, thus mediating adhesion of platelets to each other and thereby platelet aggregation (Badimon 2002). Thrombosis is further stimulated by exposure of tissue factor, which is a small molecular weight GP that is present in large amount in the plaque. After plaque rupture, tissue factor initiates the extrinsic clotting cascade, leading to activation of factor IX and X and subsequently to thrombin generation (Zaman et al., 2000). Thrombin is a key enzyme in the formation of the thrombus. It cleaves fibropeptides A and B from fibrinogen and thereby insoluble fibrin is formed, which stabilizes the growing thrombus (Badimon 2002). In addition, thrombin acts by cleaving the proteas-activated receptors PAR1 and PAR 4 on platelets, thereby causing very potent platelet activation (Fig. 2).
Figure 2. Plaque rupture and thrombosis
Degradation of the fibrous cap results in an atherosclerotic plaque that is fragile and thus more vulnerable to rupture. If the plaque ruptures, the circulating platelets are exposed to matrix proteins such as collagen in the plaque, which leads to platelet adhesion and subsequent aggregation and thrombosis. A thrombus that occupies the arterial lumen causes infarction of the tissue that the blood vessel supplies.

Platelets
Platelets, observed in 1842 by Donné, were the last of the circulating blood cells to be recognised (Donné, 1842). However, the ability of platelets to form arterial thrombi was not discovered until the late 1940s (Zucker, 1947). The platelets are small cell fragments, about 3.6 x 0.7 µm in size, and without nucleus. They are formed in the bone marrow and in the pulmonary vasculature by cytoplasmic fragmentation of a giant precursor
cell, the megakaryocyte (Cramer, 2002; Levine et al., 1993). Platelets are present in the circulation at a concentration of 150-400x10^9 cells/L and have a lifespan of approximately 10 days, after which they are degraded in the liver or spleen (Cramer, 2002). Despite the fact that nucleus is absent, the platelet contains mRNA and is able to translate mRNA into proteins after activation (Lindemann et al., 2001; Weyrich et al., 1998).

**Figure 3.** Scanning electron microscopy (SEM) photograph of a solid platelet adhered to a surface.

The main function of platelets in the circulation is to participate in haemostasis. However, platelets also possess properties related to inflammation and thereby participate in inflammatory responses (Klinger, 1997). In the circulating, inactivated state the platelet has a typical discoid shape, whereas after activation it swells and forms pseudopods. After a blood vessel is damaged and the subendothelial surface is exposed, platelets adhere to the injured vessel wall within milliseconds. Thereafter platelet spreading and aggregation is induced in order to prevent blood loss and restore the integrity of the circulation (Blockmans et al., 1995). The platelets contain three types of cytoplasmatic granules that are formed before they are budded off from the megakaryocyte: the dense granule, the α-granule and the lysosome. The granules fuse with the plasma membrane
after platelet activation via exocytosis, leading to release of granule contents and expression of surface molecules.

The α-granule is the largest and most abundant secretory granule in platelets and contains adhesive proteins (vWF, vitronectin, fibronectin, thrombospondin), growth factors (PDGF, endothelial growth factor, TGFβ), cytokines (IL-1β, CD40 ligand, platelet factor (PF) 4, β thromboglobulin), coagulation factors (fibrinogen, plasminogen, protein S, factors V, VII, VIII, XI, XII, XIII) and protease inhibitors (protein C, plasminogen activator inhibitor-1) (King and Reed, 2002). P-selectin is a transmembrane protein stored in the membrane of the α-granule of resting platelets and is expressed on the surface upon activation. Surface expression of P-selectin has frequently been used as a marker of α-granule secretion and platelet activation. Furthermore, P-selectin is considered to have an important role in atherosclerosis and thrombosis (Kupatt et al., 2002; Massberg et al., 1998). P-selectin mediates rolling of platelets and leukocytes on activated endothelial cells by interacting with its ligand P-selectin glycoprotein ligand-1 (PSGL-1). Moreover, it also interacts with the GPIb/V/IX on platelets, thereby stabilizing platelet aggregates, and activates leukocytes though binding to PSGL-1 (Bengtsson and Grenegard, 2002; Furie and Furie, 2004; Furie et al., 2001; Merten and Thiagarajan, 2004; Tsuji et al., 1994).

Of the three types of granules lysosome secretion requires the strongest degree of activation. However, there is less evidence for lysosomal granule secretion in vivo compared to the secretion of the other types of granules (Ciferri et al., 2000). The dense granule contains high concentrations of small molecules, such as ADP, adenosine triphosphate (ATP), calcium, magnesium and serotonin. After exocytosis, ADP acts as a platelet agonist via the two purinergic receptors P2Y₁ and P2Y₁₂. The P2Y₁ receptor mediates mobilization of Ca²⁺, shape change and transient aggregation, whereas ligation of the the P2Y₁₂ receptor potentiates platelet secretion and induces a more sustained, irreversible platelet aggregation (Gachet, 2006). ADP is predicted to be the prominent platelet activator of initial platelet
activation (Jurk and Kehrel, 2005). Furthermore, ADP secretion is important in the recruitment and activation of circulating platelets to adherent platelets at sites of wound healing and thrombosis (McNicol and Israels, 1999). Serotonin released from the dense granule is relatively stable and functions as a weak platelet agonist via 5HT2 receptor activation and amplifies together with ADP, the platelet response (De Clerck et al., 1984). It has been shown that serotonin promotes vasoconstriction, thrombosis, and proliferation of vascular cells. Furthermore, increase in the total level of serotonin in blood has been connected to CAD (Hara et al., 2004; Vikenes et al., 1999).

Platelet aggregation

The GPIIb/IIIa receptor is a heterodimeric transmembrane integrin molecule composed of two subunits, and plays a central role in platelet aggregation by linking activated platelets to dimeric fibrinogen molecules. The resting platelet expresses about 40 000–50 000 of GPIIb/IIIa receptors on its surface; however, in this state it is unable to bind to its ligands (such as fibrinogen, vWF, thrombospondin, fibronectin or vitronectin) (Jurk and Kehrel, 2005). After platelet activation and α-granule secretion, the number of GPIIb/IIIa receptors increases on the platelet surface, and they also become activated whereupon they bind to their ligands (Jurk and Kehrel, 2005). The binding of fibrinogen results in conformational changes of the receptor, which leads to phosphorylation of tyrosines of the cytoplasmatic GPIIIa chain, a process that is suggested to be important in the outside-in signalling of the receptor (Payrastre et al., 2000).
Platelet ROS production
The release of ROS from platelets was demonstrated for the first time by Marcus in 1977 (Marcus et al., 1977). Since then it has been reported that platelets produce ROS, including $\text{O}_2^-$, OH', $\text{H}_2\text{O}_2$ and ONOO', upon activation with different agonists, such as collagen and thrombin. However, platelets have also been found to generate ROS in the inactivated state (Caccese et al., 2000; Finazzi-Agro et al., 1982; Wachowicz et al., 2002). In platelets, several types of enzymatic sources for ROS have been implicated. However, the most attention has been given to the platelet isoform of NAD(P)H-oxidase (Iuliano et al., 1997; Krotz et al., 2002). Inhibition of the NAD(P)H-oxidase has been demonstrated to prevent aggregation (Salvemini et al., 1991), which suggests a role for oxygen radicals in platelet activation. Furthermore, ROS inhibits the anti-aggregatory effects of NO due to the NO-scavengering effect of $\text{O}_2^-$ (Tajima and Sakagami, 2000) and there is evidence that the GPIIb/IIIa receptor is regulated by oxidants, both at extra- and intracellular sites (Irani et al., 1998; Walsh et al., 2004).

The magnitude of NAD(P)H-oxidase dependent $\text{O}_2^-$ production is in the nanomolar range, which is similar to the production in endothelial cells but
less than 1% of the amount of ROS formed in neutrophils (Krotz et al., 2002; Lassegue and Clempus, 2003). Besides the NAD(P)H-oxidase, other sources for ROS have also been suggested in platelets. Endothelial NO synthase (eNOS) is found in platelets and inhibition of this enzyme in mice results in reduced $O_2^-$ production from platelets which implicates a role for this enzyme in platelet ROS production (Wolin et al., 2002). Furthermore, xanthine oxidase in platelets has been found to contribute to thrombin-induced ROS production (Wachowicz et al., 2002). Cyclooxygenase (COX) and 12-lipoxygenase (12-LOX), that catalyze the metabolization of arachidonic acid, have also been suggested to have a role in the formation of ROS in platelets (Jahn and Hansch, 1990; Niwa et al., 2001; Singh et al., 1981).

Platelets and atherosclerosis

Platelet activation is a key event in thrombotic occlusion of vessels and tissue infarction following rupture of an atherosclerotic plaque. Besides being the main player in thrombosis, the platelet has also been found to be an important actor in early stages of atherosclerosis, both in the initiation and progression of the disease. A pivotal role for platelets in the process of atherosclerosis was demonstrated by the finding that in vivo inhibition of platelet COX-1 in mice, leading to decreased TxA$_2$ production, caused a significant reduction in atherosclerotic lesions (Pratico et al., 2001; Ruggeri, 2002).

The recruitment of platelets to the site of atherosclerosis may be induced in several ways and during several stages of atherosclerosis. Interestingly, it has been discovered that platelets are the first cells to arrive at the site of endothelial dysfunction, and they interact directly with the intact monolayer (Massberg et al., 2002). It was shown that the interaction between the activated endothelial cells and platelets occurred as a result of binding of the platelet receptor GPIbα to vWF and P-selectin on the endothelium. Activation of platelet GPIIb/IIIa was thereafter required to
obtain firm platelet adhesion (Massberg et al., 2002). Furthermore, as mentioned earlier in this thesis, several investigations have shown that mural thromboses are accumulated in the plaque during the different stages of atherosclerosis (Libby, 2002). These thromboses contain activated platelets that can influence the plaque development in several ways. Another mechanism that can lead to platelet recruitment is the secretion of vWF from the vessel wall in response to inflammatory stimulation, a process that is favoured by hypercholesterolemia (Theilmieier et al., 2002). It has been shown that deficiency of vWF affords some level of protection against atherosclerotic diseases (Methia et al., 2001). Furthermore, platelets interact with different bacteria species and bacterial products, and it is also known that antibody-bacteria complexes may activate platelets through the immunoglobulin G receptor FcγRIIa expressed on platelets. The presence of bacteria in atherosclerotic plaques is common, thus these mechanisms may also be involved in platelet recruitment and activation at sites of atherosclerosis (Ruggeri, 2002).

The platelet can, after activation, affect the plaque progression in many different aspects. Platelet granule secretion leads to the release and surface expression of pro-inflammatory mediators and growth factors that can promote atherosclerosis. Chemoattractant molecules, such as the chemokines PF-4 and macrophage inflammatory protein (MIP)-1α, may induce leukocyte migration to the vessel wall (Brydon 2006). Furthermore, secretion of mitogenic factors such as PDGF and serotonin stimulates chemotaxis, mitogenesis and proliferation of monocytes, fibroblasts and vascular smooth muscle cells (Cirillo et al., 1999). Leukocyte binding to the vessel wall is promoted by the release of IL-1β and PF-4 from platelets, which induces the expression of the adhesion molecules VCAM-1 and E-selectin on endothelial cells (Brydon et al., 2006). As mentioned earlier, P-selectin is expressed on the activated platelet surface and has been found to stimulate a number of inflammatory responses. The interaction between P-selectin and its ligand PSGL-1 leads to the formation of platelet-leukocyte aggregates, a process that has been found to promote leukocyte recruitment
to the vessel wall (Huo et al., 2003; Merten et al., 2005; Merten and Thiagarajan, 2004). Interestingly, inhibition of platelet-endothelium interaction in an apo E<sup>−/−</sup> mouse model delayed the onset of atherosclerotic disease (Massberg et al., 2002). A connection between P-selectin and atherosclerosis has also been demonstrated in human studies where patients with stable coronary disease have elevated levels of activated platelets expressing P-selectin and platelet-leukocyte aggregates, compared to healthy controls (Furman et al., 1998; Nijm et al., 2005). Furthermore, exposure of monocytes to platelet P-selectin and chemokines promotes the production of inflammatory cytokines and ROS from these cells. The uptake and esterification of oxLDL in monocytes is also stimulated by P-selectin (Huo and Ley, 2004). Besides stimulating the ROS production in other cell types, the platelets themselves produce oxygen metabolites, and it has recently been found that platelets induce oxidation of LDL (Carnevale et al., 2006; Krotz et al., 2004).
Figure 3. The role of platelets in atherosclerosis
Platelets are found in the atherosclerotic plaque and may influence the progress of atherosclerosis in several ways. Platelets secrete different components from their granules after activation such as macrophage inflammatory protein-1α (MIP-1α), platelet factor 4 (PF-4) and interleukin 1β (IL-1β) that can induce leukocyte migration and binding to the atherosclerotic plaque. Furthermore, secreted growth factors (e.g. platelet derived growth factor (PDGF)) and serotonin can stimulate the proliferation of smooth muscle cells. P-selectin is expressed on the surface of activated platelets and binds to its ligand P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, thereby initiating leukocyte activation. Furthermore, platelets produce reactive oxygen species (ROS) and oxidize low-density lipoproteins (LDL), which stimulates the formation of foam cells.

Platelet-bacteria interaction
Platelets have been found to interact with several bacteria and virus species in the circulation. Certain bacteria and viruses activate platelets, which can lead to serious complications in vivo, such as infective endocarditis (Petti
and Fowler, 2003), disseminated intravascular coagulation (Levi et al., 2003) and immune thrombocytopenia purpura (Franchini and Veneri, 2004; Veneri et al., 2004). Infective endocarditis is the consequence of bacteria-induced platelet activation at the surface of a heart valve. Thrombocytopenia can be a consequence of a small degree of bacteria-induced platelet activation, which may lead to an increased consumption of platelets. Bacteria that have colonised inside a thrombi can be very difficult to treat by antibiotics and may also be isolated from the immune system (Schierholz et al., 2000). On the contrary, platelets have the capacity to secrete antimicrobial peptides, which protect the body against microbial infections (Yeaman, 2002). The platelet microbicidal proteins (PMP) or thrombin induced PMP (tPMP) are chemokines like PF-4, CTAP-3, RANTES, fibrinopeptide B and thymosin β-4 (Cole et al., 2001; Krijgsvedel et al., 2000; Tang et al., 2002). Different strains of bacteria are more or less susceptible to these PMP and tPMP, which may affect the ability of these bacteria to cause vascular infections (Bayer et al., 1998; Fowler et al., 2000; Fowler et al., 2004). The bacteria species that have been found to interact with platelets in vitro and/or in vivo include Staphylococcus aureus (Bayer et al., 1995), S. epidermidis (Usui et al., 1991), Streptococcus sanguis (Douglas et al., 1990), S. pyogenes (Kurpiewski et al., 1983), S. gordonii (Bensing et al., 2004), Porphyromonas gingivalis (Lourbakos et al., 2001), Helicobacter pylori (Byrne et al., 2003) and Borrelia burgorferi (Coburn et al., 1993). Furthermore, cytomegalovirus (CMV), which is connected to atherosclerosis, has been demonstrated to activate platelets (Agbanyo and Wasi, 1994). A very common instrument to study platelet activation in vitro is light transmission aggregometry. By using this technique certain bacteria, such as Staphylococcus and Streptococcus species, have been found to stimulate platelet aggregation. However, stimulation of platelet aggregation in vitro normally requires very high, nonphysiological concentrations of the bacteria, thus the actual ability of some of the bacteria to induce aggregation in vivo can be discussed (Fitzgerald et al., 2006).
Interestingly, there are several evidences that platelets engulf bacteria, such as *S. aureus*, which could be a strategy for the bacteria to avoid the immune system in the blood (Pawar et al., 2004; Youssefian et al., 2002). The adhesion of bacteria to platelets can occur in several ways depending on the bacteria species involved. The initial adhesion of bacteria to platelets may either involve a direct contact between the cells or be mediated by a plasma-protein bridge between a platelet receptor, usually GPIIb/IIIa or GPIb, and a receptor on the bacteria. However, this type of interaction is often itself insufficient for platelet activation, but requires a circulating antibody specific for the bacteria surface protein to engage the platelet FcγRIIa receptor (Fitzgerald et al., 2006). Interestingly, the surface structure lipopolysaccharide (LPS) of gram-negative bacteria has been shown to directly bind to and thereby activate platelets (Stahl et al., 2006; Zielinski et al., 2001). The proposed platelet surface structure that LPS interacts with, is the toll-like receptor (TLR) 4 (Andonegui et al., 2005). Several TLRs have been demonstrated on platelets such as TLR1, 2, 4, 6 and 9 (Cognasse et al., 2005; Shiraki et al., 2004). These receptors have a crucial role in innate immunity and are “pathogen-associated molecular pattern recognition molecules” binding to microbial antigens, such as LPS and bacterial heat shock proteins (HSPs). The binding of LPS to TLR4 on platelets depend on soluble CD14 (Stahl et al., 2006).
Chlamydia (Chlamydophila) pneumoniae

*Chlamydia pneumoniae* is one of the nine members in the family *Chlamydiaceae*. The others that cause human disease are *C. trachomatis* (causes trachoma and sexually transmitted diseases) and *C. psittaci* (causes atypical pneumonia in humans). In 1999 the family *Chlamydiaceae* was divided into the *Chlamydia* and *Chlamydophila* genus, based on the genetic distinction between the species (before that year *Chlamydia* was the only genus). *C. pneumoniae* and *C. psittaci* belongs to the *Chlamydophila* genus, whereas *C. trachomatis* belongs to the *Chlamydia* genus (Everett et al., 1999).

*C. pneumoniae* is a gram-negative obligate intracellular bacterium that infects the upper and lower respiratory pathways and thereby causes respiratory diseases in human. The diseases range from sinusitis, bronchitis, and pharyngitis to severe pneumonia. However, most of the infections by this bacterium show little or no clinical symptoms. Infections by *C. pneumoniae* are very widespread among humans and about 50% of the population is infected at the age of 20, and about 80% of men and 70% of women are infected at the age of 65 (Krull et al., 2005; Kuo et al., 1995a). Furthermore, reinfections with the bacteria are common.

**Morphology**

*C. pneumoniae* has a lifecycle that consists of two distinct phases: the extracellular elementary body (EB) and the intracellular reticulate body (RB). These two forms differ both functionally and morphologically from each other. The EB is very small (0.3 to 0.35 μm), whereas RB is larger (0.5-2 μm). Furthermore, EB is the infective but metabolically inactive phenotype, whereas RB is the reproductive and metabolically active one (Krull et al., 2005; Kuo et al., 1995a). The EB of some strains of *C. pneumoniae* has been found to be pear-shaped, whereas others are round and “fried egg”-shaped (Matsumoto, 2004; Miyashita et al., 1993). The
outer membrane of EB is made rigid by a network of disulphide bonds. The cell wall of *C. pneumoniae* lacks the common bacteria cell wall constituent peptidoglycan, but several investigations have found that *C. pneumoniae* is sensitive to antibiotics that inhibit peptidoglycan synthesis. Furthermore, the genes for peptidoglycans are found in the chlamydia genome (Krull et al., 2005). The structures that build up the *C. pneumoniae* outer cell membrane complex (COMC) is composed of a wide range of structures including polymorphic proteins (Pmps), LPS, major outer membrane protein (MOMP) (Hatch et al., 1981), HSPs, outer membrane protein (Omp)2, Omp3, Omp4 and Omp5 (Allen et al., 1990; Clarke et al., 1988; Knudsen et al., 1999), cystein rich proteins (CRPs) (Watson et al., 1994) and a type III secretion system (Muschiol et al., 2006). There are 21 Pmps transcribed in *C. pneumoniae* but only a few of them are presented on the surface (Grimwood and Stephens, 1999; Henderson and Lam, 2001). However, the actual roles of these Pmps and the other structures in the COMC are not completely understood.

**The life cycle**

The reproduction and spreading of *C. pneumoniae* begins with EB attachment to host cell surface. Thereafter EB is phagocytosed into a phagosome that is called an inclusion via a mechanism that is proposed to involve receptor-mediated endocytosis. After infection, EB transforms into the intracellular state of its lifecycle, the RB, which uses energy sources from the host cell for replication and multiplies by binary fission. After about 48-72 hours most of the RB have been converted back to EB and the bacteria is eventually released extracellularly, with or without lysis of the host cell. Thereafter EBs are ready to infect new cells in their surroundings and thereby spread the infection (Miyashita et al., 1993) (Fig. 5).
Figure 5. The lifecycle of Chlamydia pneumoniae.

1) The extracellular form of *C. pneumoniae*, the elementary body (EB), binds to the host cell and is endocytosed into inclusions, 2) EB differentiates to the larger and replicative form of *C. pneumoniae*, the reticulate body (RB), 3) RB undergoes replication and multiplies, 4) and 5) RB increases in number and starts to redifferentiate to EB, 6) After 48-72h the new formed EBs are released into the extracellular space by lysis of the cell.

The attachment to the host cell

The structures and mechanisms involved in the attachment of EB to the host cell surface and the following receptor-mediated endocytosis are incompletely described. However, one suggested cellular receptor for the attachment of *C. pneumoniae* is heparin sulphate-like glycosaminoglycan. Electrostatic interactions between host cell and *C. pneumoniae* have also been proposed to have a role (Wuppermann et al., 2001). The chlamydial components and structures that have been suggested to be involved in the attachment are HSP70 (Raulston et al., 1993), MOMP (Su et al., 1990), glycosaminoglycans (Beswick et al., 2003), a high mannose type oligosaccharide that comprises the glycan moiety of MOMP (Campbell et al., 2006; Kuo et al., 1996; Kuo et al., 2004), PmpD (Pmp21) (Wehrl et al., 2004) and type III secretion system (Muschiol et al., 2006). By using blockers against these structures the attachment and/or infection of
chlamydia species (most research has been performed on *C. trachomatis*) into cells can be prevented. However, more specific mechanisms leading to ligation of *C. pneumoniae* to cells still need to be discovered.

The type III secretion system was recently demonstrated to have a role in the early stages of infection when Muschiol et al. found that blocking of the type III secretion system inhibited the entry of *C. trachomatis* into host cells (Muschiol et al., 2006). The type III secretion is a well-recognised virulence factor in gram-negative bacteria that functions as a protein injector into the host cell cytosol. By this injector the bacteria can deliver different components into the host cell and thereby create an environment that is favourable for the infection and replication of the bacteria (Blocker et al., 2003).

**The life in the inclusion**

When *C. pneumoniae* has infected the cell via receptor-mediated endocytosis, the EB converts into RB after a few hours and as the RB multiplies the inclusion increases in volume. One important feature of the chlamydia inclusion is that it escapes from lysosome fusion. Instead of fusing with lysosomes, the inclusion fuses with vesicles from the Golgi apparatus, which supplies the bacteria with components that are necessary for replication and growth (Wolf and Hackstadt, 2001). Furthermore, chlamydiae are “energy parasites” because they depend on ATP and other high energy metabolites generated by the host cell (Moulder, 1991). The mechanisms by which the chlamydiae inclusions manipulate with the host cell are not yet known. However, it is known that chlamydia secretes proteins into the host cell cytosol and into the inclusion membrane, such as the so-called Inc proteins, probably via the type III secretion system. Some of these proteins are supposed to be involved in preventing phagolysosome fusion (Fields and Hackstadt, 2000; Fling et al., 2001; Subtil et al., 2005; Subtil et al., 2001). Interestingly, chlamydia also secretes a protease-like activity factor called CPAF into the host cell cytoplasm, and this protein
has been found to degrade host cell transcription factors required for expression of the MHC complex. This mechanism protects chlamydia against host immune recognition, and is thereby important in the establishment of chronic infections (Fan et al., 2002; Zhong et al., 2001).

**Activation of the host cell**

The most important components of *C. pneumoniae* that mediate the stimulatory effects on cells are supposed to be LPS and HSPs, in particular HSP60 (Costa et al., 2002; Da Costa et al., 2004; Kalayoglu, 2002; Kalayoglu et al., 2000; Netea et al., 2002; Sasu et al., 2001). Chlamydial LPS has a unique structure that contains pentaacyl-1,4′-diphosphoryl lipid A moiety instead of the classical hexacyl-1,4′-diphosphoryl lipid A moiety of enterobacteria (Kalayoglu et al., 2000). The chlamydia LPS is supposed by be a weaker inducer of cell activation than other types of LPS, which is due to its unique structure. This more low-grade cellular activation characterizes chronic inflammatory diseases, such as atherosclerosis. Furthermore, the targets on host cells that mediate the stimulatory effects of *C. pneumoniae* are mainly found to be TLR-2 and -4 (Bulut et al., 2002; Cao et al., 2006; Da Costa et al., 2004; Netea et al., 2002; Prebeck et al., 2001; Sasu et al., 2001; Yang et al., 2005).

**Persistence**

When *C. pneumoniae* is exposed to different stress factors such as cytokines (e.g. IFN-γ) (Mehta et al., 1998; Summersgill et al., 1995), antibiotics (Gieffers et al., 2004a), nutrient depletion (Harper et al., 2000) and tobacco smoke (Wiedeman et al., 2004), it undergo a phase in its lifecycle called the persistent state. In this state, the ability of RB to divide and differentiate to EB is obstructed. Instead, the inclusions contain atypical, aberrant bodies (ABs) which are larger than the typical RB. Once the stress factors are removed, these ABs can differentiate back to normal RBs again and thereafter to infectious EBs (Matsumoto, 2004). The
capacity of *C. pneumoniae* to develop into a persistent state is proposed to be an important mechanism for the ability of the bacteria to induce chronic infections that do not respond to antibiotic treatment, for example in atherosclerotic plaques.

The connection between *Chlamydia pneumoniae* and atherosclerosis

Infections and cardiovascular disease

It is by now clearly established that atherosclerosis is an inflammatory disease, but the actual mechanisms of how the disease is initiated and progressed are not fully understood. The classical risk factors for atherosclerosis such as high cholesterol levels, hypertension, smoking and sedentary lifestyle can only explain about 40-50% of cardiovascular disease events (Ross, 1999). The identification of new risk factors for atherosclerosis is thus very important. The concept of the involvement of infectious agents in atherosclerosis is far from new as can be read at the beginning of this thesis. During the past century several bacteria and viruses have been suggested to be involved in the initiation and/or the development of atherosclerotic plaques and subsequent cardiovascular events. By now, however, the main infectious agents that are associated with cardiovascular disease are *C. pneumoniae* (Campbell et al., 1995; Fryer et al., 1997; Grayston et al., 1995; Kuo et al., 1995a; Saikku et al., 1988; Thom et al., 1992), *Porphyromonas gingivalis* (DeStefano et al., 1993; Haraszthy et al., 2000; Hujoel et al., 2000; Mattila et al., 1989), cytomegalovirus (Adam et al., 1987; Hendrix et al., 1989; Melnick et al., 1990; Zhou et al., 1996), *Helicobacter pylori* (Danesh et al., 1997; Hoffmeister et al., 2001; Videm et al., 2006), *Mycoplasma pneumoniae* (Gurfinkel, 1998; Higuchi Mde et al., 2002; Higuchi Mde et al., 2003) and
Epidemiological studies

As described in the beginning of this thesis, *C. pneumoniae* was first identified as a chlamydial respiratory pathogen in 1986 (Grayston et al., 1986). Two years later Siakku et al. showed a connection between *C. pneumoniae* infections and increased risk for cardiovascular disease (Saikku et al., 1988). Since then a large number of studies have repeated the finding of an association between previous or ongoing infections with *C. pneumoniae* and atherosclerotic diseases (Mussa et al., 2006). These studies include serological studies (measuring specific IgA, IgG and IgM antibodies against *C. pneumoniae*) that show that previous or ongoing infection with *C. pneumoniae* is a risk factor for cardiovascular disease (Danesh et al., 2000; Huittinen et al., 2003; Saikku et al., 1988; Saikku et al., 1992). Danesh et al. reviewed 18 epidemiological studies in 1997 and found an ORs ratio of 2 for the association between *C. pneumoniae* infection and cardiovascular disease or stroke (Danesh et al., 1997). Furthermore, Fong reviewed in 2003 that 38 of 62 (63.3%) retrospective case-control or cross-sectional studies up to that year showed a positive relationship between *C. pneumoniae* and atherosclerosis. However, the same review found only a relationship between positive *C. pneumoniae* serology and vascular disease (cardiovascular endpoints) in 6 of 26 (26%) longitudinal, prospective studies (Fong, 2003). Furthermore, no association between *C. pneumoniae* antibodies and increased risk for restenosis after percutaneous coronary intervention has been found (Mattila et al., 2001). However, in patients with abdominal aortic aneurysm, subjects that are serological positive for *C. pneumoniae* have a greater expansion of the atherosclerotic plaques after a period of four years (Lindholt et al., 2001). Interestingly, there are also several studies that recently have shown a positive correlation between the presence of *C. pneumoniae* antibodies, inflammation markers such as IL-6, and cardiovascular disease.
al., 2006; Yavuz et al., 2006). In a meta-analysis by Gutiérrez et al. in 2005 the association between *C. pneumoniae* and cardiovascular disease was investigated. Comparing different techniques used to diagnose *C. pneumoniae* infection, they found that a connection between *C. pneumoniae* infection and atherosclerosis can be detected by enzyme-linked immunosorbent assay (ELISA) and microimmunofluorescence (MIF) tests to detect high IgG levels (OR=2) and IgA levels (OR=1.9) (Gutierrez et al., 2005).

There is a high diversity regarding the serological evidence for a connection between *C. pneumoniae* and atherosclerosis, which can be explained in several ways. First the diagnostic tools to detect *C. pneumoniae* antibodies vary between different studies. Some use MIF tests, whereas others use enzyme linked immunoassay (EIA) or ELISA in determining the presence and levels of antibodies (Persson and Boman, 2000). The cut-off value for serological positive respective negative samples varies and the designs of the studies are very heterogeneous. However, the most important disadvantage is the lack of methods to detect persistent, chronic *C. pneumoniae* infections. Many studies have used *C. pneumoniae* IgA values, despite the fact that IgA has not clearly been proven to associate with chronic *C. pneumoniae* infections.

**The presence of *C. pneumoniae* in atherosclerotic plaques**

Another interesting finding was done in 1992 when Shor et al. demonstrated the presence of *C. pneumoniae* antigens in atherosclerotic plaques (Shor et al., 1992). Since then numerous investigations have repeated this finding and showed that both *C. pneumoniae* DNA, antigen and viable *C. pneumoniae* are commonly detected in atherosclerotic plaques, by using PCR, immunohistochemistry, Southern hybridization, in situ hybridization and electron microscopy (Grayston et al., 1995; Kuo et al., 1993a; Kuo et al., 1995a; Kuo et al., 1993b; Ramirez, 1996; Shi and Tokunaga, 2002). It has been shown that *C. pneumoniae* is present already
in the fatty streaks of young adults, which indicates a role for the bacterium in early atherogenesis (Kuo et al., 1995a; Shor et al., 1992). In healthy vessels, the presence of *C. pneumoniae* is very uncommon (Gutierrez et al., 2005). In a review by Boman and Hammerschlag from 2000, the results regarding presence of *C. pneumoniae* in atherosclerotic plaques were summarized from 43 studies. They found that *C. pneumoniae* could be detected in 336 of 676 (49.7%) atherosclerotic lesions by immunohistochemistry with *Chlamydia* genus-specific antibodies and 202 (45.6%) of 443 plaques by using *C. pneumoniae* specific antibodies. By using PCR techniques, the DNA from *C. pneumoniae* could be detected in 558 (24.3%) of 2294 plaques and electron microscopy found *C. pneumoniae* in 38 (39.2%) of 97 samples. In one of these studies, *C. pneumoniae* antigen was detected in 71 of 90 (79%) specimens of atherosclerotic plaques from symptomatic patients undergoing atherectomy. In contrast, *C. pneumoniae* was only detected in 4% of non-atherosclerotic plaques (Muhlestein et al., 1996). Furthermore, another study showed that 18 of 48 samples from atherosclerotic plaques contained *C. pneumoniae* mRNA, which indicates the presence of viable bacteria (Johnston et al., 2001). The presence of *C. pneumoniae* mRNA in plaques has also been demonstrated by more recent investigations (Nystrom-Rosander et al., 2006). *C. pneumoniae* has been cultured from the plaques in seven reports from six different centers, which further reveals the presence of viable bacteria (Fong, 2003). Interestingly, it has been found that *C. pneumoniae* is most likely to be detected in plaques with fresh thrombi, which suggests that the bacterium has a role in thrombosis (Chiu et al., 1997).

The presence of *C. pneumoniae* in circulating blood cells

In addition to atherosclerotic plaques, *C. pneumoniae* is also found in circulating blood cells such as monocytes, T-lymphocytes and neutrophils (Cirino et al., 2006; Kaul et al., 2000; Smieja et al., 2002). Boman et al. were the first to demonstrate the presence of *C. pneumoniae* in peripheral
blood mononuclear cells (PBMC) in 1998 (Boman et al., 1998). They detected *C. pneumoniae* in 59% of patients undergoing coronary angiography and in 46% of matched controls. However, in a review from 2002 the overall detection rate of *C. pneumoniae* in PBMC from nine studies was 14.3% in patients with cardiovascular disease versus 8.5% in controls (Smieja et al., 2002). An interesting recent investigation found that circulating DNA in whole blood was associated with the severity and extent of coronary artery disease. Indeed, the presence of *C. pneumoniae* DNA was correlated with multi-vessel disease with an ODs ratio of 5.1 (Wang et al., 2006).

These detection rates in blood samples are much lower than the detection rates in atherosclerotic plaques, suggesting that the presence of the bacteria in PBMC does not reflect its presence in atherosclerotic plaques. However, there are studies that have shown a strong correlation between the presence of *C. pneumoniae* in atherosclerotic plaques and in circulating PBMC (Prager et al., 2002). *C. pneumoniae* infection of PBMC is supposed to be the most important spreading mechanism for the bacteria from infected lung epithelium to the atherosclerotic plaques.

**Animal models**

Many interesting findings regarding the ability of *C. pneumoniae* to stimulate atherosclerosis have been performed by using experimental animal models. The first studies in this area used C57BL7/6J mice that need high fat/high cholesterol diet to induce atherosclerosis and apoE/ apoE/ mice that develop hypercholesterolemia and atherosclerosis spontaneously (Campbell et al., 2000; Hu et al., 1999). The studies with the C57BL7/6J mice showed that intranasal inoculation with *C. pneumoniae* induced pneumonia and the bacteria were detected in monocytes/macrophages and in the arteries after infection. Furthermore, after inoculation with *C. pneumoniae*, a persistent infection in the aorta was established in the apoE/ apoE/ mice and the bacteria were found in foam cells in the lesions. Both
studies also found that *C. trachomatis* had no effects on atheroma formation, thereby suggesting atherogenic-specific properties of *C. pneumoniae*. Blessing et al. found that *C. pneumoniae* infection of normocholesterolemic C57BL7/6J mice induced inflammatory changes of the heart and aorta, but did not influence the formation of atherosclerotic lesions (Blessing et al., 2000). Recent investigations have demonstrated that *C. pneumoniae*-infection of LDLR/apoE<sup>−/−</sup> mice leads to increased production of MMP-2 and MMP-9 and reduced fibrous cap area, which indicates a capacity of the bacterium to affect destabilization of the plaque (Ezzahiri et al., 2003). Treatment of mice with anti-*C. pneumoniae* antibiotics, such as azithromycin, eradicated the cultivatable form of the bacteria in the lung tissue, but the bacteria could still be detected by PCR (Malinverni et al., 1995). This suggests the presence of a non-cultivable, persistent form of the bacteria even after antibiotic treatment. Furthermore, another study found that in apoE<sup>−/−</sup> mice, antibiotic treatment two weeks after infection had no effect on *C. pneumoniae* accelerated atheroma formation (Rothstein et al., 2001).

Another animal model that has been used to study the capacity of *C. pneumoniae* to stimulate atheroma formation is the New Zealand white rabbit, which is fed with a high cholesterol diet. Infection of these animals with *C. pneumoniae* leads to atherosclerotic changes in the aorta, and antibiotic treatment (azithromycin) prevents this process. However, despite antibiotic treatment the bacteria are still found in the tissues (Muhlestein et al., 1998). In addition, also normolipidaemic rabbits develop inflammatory changes in the aorta after *C. pneumoniae* infections, such as smooth muscle cell growth factor production and proliferation and aortic intimal thickening through increased PDGF B mRNA expression (Coombes et al., 2002). Fong et al. found that antibiotic treatment (azithromycin, clarithromycin, moxifloxacin and doxycyclin) of *C. pneumoniae*-infected rabbits can prevent atherosclerotic lesions if administered within five days after infection. However, if treatment is delayed to six weeks after infection, azithromycin was uneffective whereas clarithromycin reduced
the lesion size by 62.5%. These findings suggest early antibiotic treatment
to prevent the stimulatory effects of *C. pneumoniae* on atherosclerosis.
Furthermore, they also found that *Mycoplasma pneumoniae*, another
respiratory pathogen, did not affect atheroma formation in rabbits (Fong,
2000).

More recent studies have also used pig models, which are supposed to be
the animal model most similar to human atherosclerosis. It was found that
after balloon dilatation-induced vessel injury, direct inoculation of *C.
pneumoniae* into the pulmonary and coronary arteries resulted in increased
maximal intimal thickening of the coronary artery, but not in the
pulmonary artery (Pislaru et al., 2003). Furthermore, *C. pneumoniae* was
found in a porcine model to induce endothelial dysfunction by promoting a
procoagulant status through impaired vasodilatation, induced vasospasm
and elevated fibrinogen (Chesebro et al., 2003; Liuba et al., 2003).

In summary, the results obtained from the animal models strongly indicate
an ability of *C. pneumoniae* to accelerate atherosclerosis. Furthermore,
some studies also indicate the capacity of this bacterium to initiate
atherosclerosis. However, most investigations show that the bacterium is a
cofactor to high cholesterol levels in triggering atherosclerosis. The
antibiotic studies also show that *C. pneumoniae* needs to be eradicated very
shortly after exposure to the host if the stimulatory effects on
atherosclerosis are to be prevented. To prevent *C. pneumoniae* from
reaching the circulation and creating a chronic infection by antibiotics is,
however, very difficult, since *C. pneumoniae* infections are rarely
diagnosed and discovered. Instead, the development of new anti-
chlamydial vaccines is probably required to prevent the bacterium from
colonizing the vessels.
Antibiotic trials

The first antibiotic trials that were performed gave promising results. Gupta et al. were the first to study the effect of antibiotic treatment on cardiovascular clinical endpoints and found a 68% reduction in cardiovascular events (Gupta et al., 1997a). These data greatly increased the interest regarding the ability of antibiotics to prevent cardiovascular diseases. However, a Meta analysis from 2005 summarized 11 randomized, placebo-controlled trials and found no impact of antibiotics on mortality, myocardial infarction or on the combined endpoint of myocardial infarction and unstable angina (Andraws et al., 2005). Furthermore, in a recent investigation by Grayston et al., 4012 patients with documented stable coronary disease received either 600 mg of azithromycin or placebo weekly for one year. There were no risk reductions regarding death due to heart disease, non-fatal myocardial infarction, coronary revascularization or hospitalization for unstable angina after one year of antibiotic treatment (Grayston et al., 2005). Furthermore, in another large trial by Cannon et al. no beneficial effects with fluoroquinolone (gatifloxacin) were found after two years of treatment (Cannon et al., 2005).

The conclusion drawn from the antimicrobial studies is that no beneficial effects on cardiovascular disease are obtained by the antibiotics used so far. However, this cannot exclude the involvement of *C. pneumoniae* in atherosclerosis. Chronic infections of *C. pneumoniae* in atherosclerotic plaques and circulating blood cells are very difficult, if not impossible, to treat with the antibiotics available at the present time. Instead, antibiotics have *in vitro* been found to induce a persistent *C. pneumoniae* infection in different cell types. Furthermore, the ability of antibiotics to penetrate the atherosclerotic plaques has not been studied. The results obtained from the animal models clearly show that antibiotics need to be administered very shortly after the first infection by the bacterium to have a beneficial effect. In humans this is not possible, due to the fact that *C. pneumoniae* infections are rarely recognized and diagnosed in the clinic. However, if new anti-
chlamydial vaccines are discovered it could be possible to prevent the effects of *C. pneumoniae* on atherosclerosis.

**In vitro studies and a model for *C. pneumoniae*-induced atherosclerosis**

There are by now numerous *in vitro* investigations that have demonstrated the capacity of *C. pneumoniae* to infect the different cell types that are present in the atherosclerotic plaque and alter their activation in ways that can stimulate atherosclerotic processes. Animal studies have shown that *C. pneumoniae* may be transmitted from the infected lung epithelium to the vessel wall via infected PBMC. The cell that is supposed to be most important in this transfer is the monocyte/macrophage; however, other cells may also be involved (Gieffers et al., 2004b; Moazed et al., 1998).

Recently, Gieffers et al. showed in New Zealand rabbits that alveolar macrophages infected with *C. pneumoniae*, transmigrated through the mucosal barrier, which gave the pathogen access to the lymphatic system and the systemic circulation. They found that granulocytes were the first to arrive to the infected lung tissue and that living *C. pneumoniae* were present in these cells whereas macrophages were involved during later infection. They suggested granulocytes as reservoirs for *C. pneumoniae* that can transmit the infection to macrophages during later stages of infection (Gieffers et al., 2004b). However, whether these macrophages transmit the infection directly to the vascular wall is not yet stated. The *C. pneumoniae*-infected macrophages may be trapped in the spleen where *C. pneumoniae* can infect other cell types such as monocytes and lymphocytes and by these cells be translocated to the vessel wall. It has indeed been found *in vitro* that PBMCs are able to spread the infection of *C. pneumoniae* to vascular cells, such as smooth muscle cells.

*C. pneumoniae* have *in vitro* been demonstrated to infect and replicate in the major cell types that are present in the atherosclerotic plaque, such as endothelial cells, macrophages and smooth muscle cells (Gaydos, 2000;
Kaukoranta-Tolvanen et al., 1996b). The infected cells have been shown to upregulate the expression of adhesion molecules such as E-selectin, VCAM-1 and ICAM-1 that can lead to an increased leukocyte infiltration (Kaukoranta-Tolvanen et al., 1996a). The infection of endothelial cells by \textit{C. pneumoniae} has indeed been found to stimulate transendothelial migration of both neutrophils and monocytes (Molestina et al., 1999). The infected endothelial cells also produce and secrete increased amounts of IL-1, IL-2, IL-6, IL-8, TNF-α, MCP-1, PDGF, basic fibroblast growth factor (bFGF), tissue factor and plasminogen activator inhibitor-1 (PAI-1) (Dechend et al., 1999; Fryer et al., 1997; Gaydos, 2000; Gibbs et al., 1998; Summersgill et al., 2000; Coombes et al., 2002; Gois et al., 2006). These factors may alter the inflammation of the intima, cause a procoagulant surface on the endothelium, stimulate the infiltration of leukocytes and induce proliferation of smooth muscle cells, processes that are important in atherogenesis. \textit{C. pneumoniae}-infected macrophages secrete inflammatory cytokines, such as IL-1β, IL-6, IL-12, MCP-1, MIP-1α and TNF-α, which may further promote lesion formation (Miller et al., 2000; Netea et al., 2000).

Infected macrophages and smooth muscle cells also produce MMPs, which may increase the degradation and instability of the plaque (Arno et al., 2005; Choi et al., 2002; Kim et al., 2005; Rodel et al., 2003; Rupp et al., 2004; Stintzing et al., 2005). There are conflicting data regarding the effects of \textit{C. pneumoniae} on cellular apoptosis and necrosis. The bacterium has been found to inhibit apoptosis during growth in phagocytes and during the persistent phase of its life cycle, but induces the apoptosis of T-cells (Miyairi and Byrne, 2006). Interestingly, stimulating effects of \textit{C. pneumoniae} on smooth muscle cell apoptosis have been demonstrated, which may promote the formation of an unstable plaque. Furthermore, there are studies showing both stimulating and inhibiting effects respectively on smooth muscle cell proliferation (Hirono et al., 2003; Rodel et al., 2004; Rupp et al., 2005). However, this may be explained by different experimental designs in the studies. In 2004 Rodel et al. used
medium from infected smooth muscle cells and found anti-proliferative properties of this medium on uninfected smooth muscle cells, probably due to PGE$_2$. In 2003, however, Hirono et al. reported increased proliferation in C. pneumoniae-infected smooth muscle cells. The formation of atherosclerotic plaques and the pathogenesis of cardiovascular disease is dependent on proliferation of smooth muscle cells and during later stages smooth muscle cells actually stabilize the plaque and make it less fragile for rupture. Thus, the findings that C. pneumoniae influences the proliferation of these cells in different ways may be of importance in the pathogenesis of atherosclerosis.

Another crucial event in atherosclerosis is the formation of foam cells, a process that is supported by C. pneumoniae in vitro. The bacterium has been found to induce ROS production in macrophages and stimulate the oxidation and uptake of LDL into macrophages, thereby increasing the formation of foam cells (Azenabor et al., 2005; Kalayoglu and Byrne, 1998a; b; Kalayoglu et al., 1999a; Kalayoglu et al., 1999b). Furthermore, it was recently found that C. pneumoniae-infected endothelial cells enhance their expression of LOX-1 (Yoshida et al., 2006) and promote the oxidation of LDL (Dittrich et al., 2004). These processes may have a pivotal role in the initiation and development of an atherosclerotic plaque (Fig. 7).

Chlamydia pneumoniae and thrombosis

There are some investigations that have shown associations between C. pneumoniae infection and increased risk for platelet activation and thrombosis. In a study from 1997 it was found in patients with carotid artery stenosis, that atherosclerotic plaques with thrombosis more likely contained C. pneumoniae than plaques without thrombosis (Chiu et al., 1997). An association between C. pneumoniae serology and thrombosis-related embolization in patients with symptomatic carotid artery disease has also been demonstrated (Vainas et al., 2002). Furthermore, Lozínguez et al.
found a connection between high *C. pneumoniae* IgG titers and venous thromboembolism (Lozinguez et al., 2000). In addition, in patients with myocardial infarction and ST-segment elevations, an association between infection with *C. pneumoniae* and platelet activation was found (Jaremo, 2001).

**Figure 7** The role of *Chlamydia pneumoniae* in atherosclerosis.

1: *C. pneumoniae* is spread to the vessel wall by infected monocytes and/or T-lymphocytes. In the atherosclerotic plaque, the bacterium infects endothelial cells, smooth muscle cells (SMC) and macrophages. 2, 3: Endothelial infection results in an increased expression of adhesion receptors and production of monocyte chemoattractant protein 1 (MCP-1) and cytokines, which in turn stimulates the recruitment and accumulation of leukocytes into the vessel wall. 4: *C. pneumoniae* stimulates the oxidation and accumulation of LDL in macrophages, thereby increasing the amount of foam cells in the lesion. 5: *C. pneumoniae* stimulates the release of growth factors e.g. platelet derived growth factor (PDGF), which induces proliferation of SMC. 6: Infected macrophages and SMC secrete matrix metalloproteinases (MMPs) thereby making the plaque more fragile for rupture. 7: The infected endothelial cells also produce plasminogen activator inhibitor-1 (PAI-1) and tissue factor, which promotes the formation of a procoagulative surface.
Aims

The overall aim of the present thesis was to investigate the effects of *Chlamydia pneumoniae* on platelet activation and the impact of *C. pneumoniae*-platelet interaction on atherosclerosis.

The specific aims were to elucidate:

- whether *C. pneumoniae* activates platelets *in vitro*
- the mechanisms involved in *C. pneumoniae*-platelet interaction and the capacity of *C. pneumoniae*-activated platelets to oxidize LDL
- the effects of *C. pneumoniae*, released into the circulation after percutaneous coronary intervention (PCI), on platelet activation and LDL-oxidation
- how to pharmacologically prevent *C. pneumoniae*-induced platelet activation
Methods

In vitro studies

Cell culture

*C. pneumoniae* is an intracellular bacterium that needs to be cultured in cells for replication. For this purpose the epithelial HEp2 cell line (ATCC: LGC Promochem AB, Borås, Sweden) was used. The cells were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mg/L gentamicin, and 2 mmol/L L-glutamine (Gibco, BRL, Life Technologies, Paisley, Scotland). The cells were incubated at 37°C and 5% CO₂ in 75 cm² culture flasks, and then subcultured in 6-well plates at a density of 0.7 x10^6 cells/well prior to infection with *C. pneumoniae*.

Culture of *Chlamydia pneumoniae*

Strain T45 of *C. pneumoniae* is a clinical isolate derived from a nine-year-old boy suffering from respiratory *C. pneumoniae* infection in Umeå in 1994 (the so-called “Byske epidemic”). The isolate is PCR- and culture-positive for *C. pneumoniae*, and the variable regions 1, 2, 3 and 4 on the MOMP-gene are well-matched to the subsequent gene regions on other well-known *C. pneumoniae* isolates. The bacteria were cultured in HEp2 cells essentially as described by Redecke et al. (Redecke et al., 1998). *C. pneumoniae* was added to subconfluent monolayers of HEp2 cells in 6-well plates. The plates were centrifuged at 480 x g for 45 minutes at 25°C, and incubated for 2 h at 37°C and 5% CO₂. Nonadherent bacteria were removed and infected cells were incubated in fresh RPMI 1640, supplemented with 1 μg/mL cyclohexamide (ICN Biomedicals Inc, Aurora, OH, USA). The infected cells were incubated for 72 h under the same conditions as above to allow development of characteristic chlamydial inclusions. The bacteria
were harvested by disrupting the HEp2 cells with glass beads followed by sonication and centrifugation at 900 x g for 10 minutes at 4°C to remove cellular debris. Supernatants were centrifuged at 12 000 x g for 30 minutes at 4°C, and the bacteria were suspended in sucrose-phosphate buffer supplemented with FBS (10%, heat inactivated) and stored at -70°C until use. Uninfected HEp2 cells (HEp2 cell debris) were handled exactly as chlamydia-infected cells and used as a control.

The concentration of *C. pneumoniae* inclusion-forming units (IFU) were determined by diluting the bacteria suspension 10³, 10⁴, 10⁵, 10⁶, 10⁷ and 10⁸ times and thereafter adding the suspension to a subconfluent layer of HEp2 cells cultured in a 24-well plate. The plates were then centrifuged at 480 x g for 45 minutes at 25°C, and incubated for 2 h at 37°C and 5% CO₂. Nonadherent bacteria were removed and infected cells were incubated in fresh RPMI 1640, supplemented with 1 μg/mL cyclohexamide. After incubation for 72 h at 37°C and 5% CO₂ to allow the bacteria to form inclusions, the cells and bacteria were fixed with methanol. Thereafter the cells were stained with propidium iodide and *C. pneumoniae* with Phadebact Chlamydia culture confirmation test (Boule diagnostics, Huddinge, Sweden). The number of IFU in each well was counted with an inverted fluorescence microscope and the concentration could then be calculated.

**Mycoplasma PCR**

Mycoplasma contamination of cell lines is common, and has therefore been a problem when culturing *C. pneumoniae* (Timenetsky et al., 2006). The cells and bacteria were tested for mycoplasma contamination by using a mycoplasma-specific PCR, essentially according to van Kuppeveld et al. (van Kuppeveld et al., 1992). The nucleotide sequences of primers used in the PCR assay were as follows: upstream primer GPO-3; 5´-GGGAGCAAAACAGGATTAGATACCC T-3´ and downstream primer
MGSO; 5’-TGCACCATCTGC ACTCTGT TAACTC-3’ (SGS AB, Köping, Sweden). In short, the DNA of the Hep2 cells and C. pneumoniae cultures were extracted by using genomed jetquick tissue DNA spin kit (Genomed, Löhne, Germany). The DNA (1 μL) was added to 25 μL of the following PCR mixture: 1.5 units of Taq DNA polymerase, 10 mmol/L Tris-HCL (pH 9.0 at room temperature), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 200 μmol/L of each dNTP and stabilizers, including BSA (Amersham Biosciences, Uppsala, Sweden), 0.8 μmol/L upstream primer GPO-3, and 0.8 μmol/L downstream primer MGSO. The PCR protocol used in a PTC-100™ was as follows: denaturation at 94 °C for 40 seconds, annealing temperature at 55 °C for 40 seconds, extension at 72 °C for 1 minute at 25 cycles.

Separation of platelets

Platelets were isolated in room temperature from freshly drawn heparinised human peripheral blood, donated by apparently healthy and drug-free adult volunteers as previously described (Bengtsson et al., 1996). Five parts of blood were mixed with one part of an acid-citrate-dextrose solution (85 mmol/L sodium citrate, 71 mmol/L citric acid and 111 mmol/L glucose), followed by centrifugation for 20 minutes at 220 x g to obtain platelet-rich plasma (PRP). The PRP was then centrifuged for 20 minutes at 480 x g, and the platelets were gently washed and resuspended in KRG without Ca²⁺ and stored in plastic tubes until use. To obtain functional but non-activated platelets, the isolation was performed without any specific platelet inhibitors, and, due to this, extra care was taken when handling the cells. The platelet concentration was determined in a Bürker chamber. Morphological studies showed discoid, solitary platelets displaying no signs of activation due to the preparation procedure. The contamination of other blood cells was negligible. The cells were recalcified immediately before each experiment.
Platelet aggregation and ATP secretion

Platelet aggregation and ATP secretion were analyzed by using a two-sample, Lumi-Aggregometer model 560 (ChronoLog Corporation, Haverton, PA, USA). With this instrument, aggregation was measured as change in light transmission by using KRG buffer as a reference. An unstimulated platelet suspension was set to 0% light transmission and KRG buffer to 100% light transmission. In parallel, ATP secretion was measured as a change in bioluminescence using a mixture of luciferin/luciferase. In the presence of ATP, luciferin interacts with the enzyme luciferase and light is emitted that can be detected by the instrument.

Before each experiment, the cells were incubated for five minutes at 37°C under stirring (700 rpm) conditions. The cells were then stimulated (i.e. with *C. pneumoniae*) and the platelet aggregation and ATP secretion was registered under stirring conditions (700rpm) for a period of 30-45 minutes. By adding a known amount of ATP at the end of each measurement, the ATP release in each sample was calculated.

P-selectin expression and binding of *C. pneumoniae* to platelets

Platelet P-selectin expression and binding of *C. pneumoniae* to platelets were studied with flow cytometry. Before measurement, platelets (2 x 10⁸/mL) were preincubated for 10 min at 37°C under stirring conditions in a 24-well plate (Nunc, Roskilde, Denmark) and then mixed with various concentrations of *C. pneumoniae*. Samples were taken immediately before, and 1, 5, 10 and 20 minutes after co-incubation. Immunofluorescence staining of platelets and *C. pneumoniae* was performed by incubation with saturating concentrations of monoclonal FITC-conjugated anti-P-selectin (CD62p) (BD Biosciences, Pharmigen, San Diego, CA) or phycoerythrin-conjugated anti-GpIb (CD42b) (Dakopatts, Glostrup, Denmark), and
monoclonal FITC-labelled anti-chlamydia LPS (Boule Diagnostics, Huddinge, Sweden), respectively, at room temperature for 10 minutes in the dark. The samples were then fixed with Optilyse (with 2.5% formaldehyde; Immunotech, Marseille, France) under the same conditions and diluted in distilled H₂O. Phycoerythrin- or FITC-labelled irrelevant isotype-matched monoclonal antibodies were used as controls for non-specific staining. After staining, the samples were analysed in a Becton Dickinson FACS Calibur. The platelet population was identified by means of its light-scatter characteristics, and by confirming that more than 99% of analysed particles in each sample were GpIb-positive. Events stained positive for both platelet and C. pneumoniae antigens (GpIb and LPS, respectively) were considered to represent platelet-chlamydia complexes and were distinguishable from events staining positive for GpIb alone. The extent of platelet activation was assessed by analysing anti-P-selectin FITC-fluorescence in the platelet gate. The mean fluorescence value of each sample was determined from cells counted during a time period of 20 seconds or from 500 000 counted cells at most.

ROS production

The intracellular production of ROS in platelets was measured with flow cytometry by using the fluorescent dye, 2’,7’-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is a nonpolar and uncoloured molecule that passes though cellular membranes. Inside the cell cytosol, DCFH-DA is converted to the membrane-impermeable, non-fluorescent polar derivative 2’,7’-dichlorofluorescin (DCFH) by cellular esterases. DCFH rapidly oxidizes to the highly fluorescent 2’,7’-dichlorofluorescin (DCF) in the presence of intracellular hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻), peroxidases and hydroxyl radicals (•OH) (Myhre et al., 2003).

Before flow cytometry measurement, platelets (2 x 10⁸/mL) were loaded with 5 μmol/L DCFH-DA for 15 minutes at 37°C in a 24-well plate, and then mixed with C. pneumoniae. Samples were taken immediately before,
and 5, 10 and 20 minutes after adding *C. pneumoniae* to the platelet suspension. The samples were fixed with Optilyse (with 2.5% formaldehyde) in the dark and diluted in distilled H₂O. The platelet population was identified by means of its light-scatter characteristics, and by confirming that more than 99% of analysed particles in each sample were GpIb-positive. The mean fluorescence value of each sample was determined from 7000 cells with flow cytometry in a Becton Dickinson FACS Calibur.

**Preparation of LDL**

LDL was isolated from fresh human plasma by sequential density-gradient ultracentrifugation. In short, EDTA-treated blood was centrifuged at 1200 x g (3500 rpm) for 10 minutes at room temperature, after which the plasma was pooled and mixed with EDTA (final concentration 1 mg/mL) and sucrose (final concentration 0.5%) to prevent LDL oxidation and aggregation, respectively. Thereafter the density of the plasma was adjusted to 1.22 g/mL by addition of KBr (0.3264 g/mL) and the plasma was centrifuged together with ice-cold phosphate buffered saline (without mixing the two phases) at 290 000 x g for 2 h at 4°C. LDL was collected by careful aspiration of the yellow band in the middle of the tube and centrifuged in a tube covered with KBr solution with a final density of 1.10 g/mL at 290 000 x g for 2 h at 4°C. The albumin-free LDL in the top of the tube was aspirated and desalted with a PD10 desalting column (Amersham Pharmacia Biotech, USA) with PBS (154 mM, pH 7.3) as desalting buffer. Protein concentration was determined by a protein assay (Bio-Rad no. 500-0006, Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as standard. Isolated LDL was used within two weeks.
The clinical project

Patient selection and medication

160 patients who underwent coronary angiography between February 2003 and December 2006 due to chest pain were enrolled in the study and 73 of these were included in Paper III. Exclusion was based on the following criteria: age above 75 years, ongoing cortisone medication, ongoing treated infection and malignity. The patients were divided into three groups as follows: i) the angiography group, comprising 13 patients without any visible lesions and subjected only to angiography; ii) the percutaneous coronary intervention (PCI) group, including 40 patients who had lesions and underwent angiography followed by PCI; and iii) the coronary artery bypass graft (CABG) group, consisting of 14 patients who had lesions and underwent angiography followed by CABG within two months. An age and gender matched control group of 60 individuals was also enrolled in the study.

All patients were treated once daily with 75 mg aspirin, both before and after the procedures. Furthermore, they received a loading dose of 300 mg clopidogrel approximately 12 hours before the angiography. Patients on warfarin received low molecular heparin, and warfarin was excluded 3-4 days before the angiography. After PCI or CABG procedures, treatment with low molecular heparin was stopped, and the patients were put on warfarin again. Those with unstable angina were given aspirin, clopidogrel and low molecular heparin until the time of the revascularization. Clopidogrel was also given for three months after PCI.

Sampling of blood

Blood samples were drawn from the patients before and then 24 hours, 1 month (PCI group only), 6 months and 1 year after angiography. 10 mL EDTA-treated, 7 mL heparin-treated and 5 mL untreated blood was
collected each time. The following samples were fractionated from the blood and stored in -70°C until use: whole blood, plasma (EDTA and heparin), buffy coat, serum and red blood cells.

**Chlamydia pneumoniae** serology

The levels of *C. pneumoniae* IgG and IgA in plasma were determined by using a commercially available ELISA test (Medac, Wedel, Germany). According to the company, this test is based on a highly purified native *C. pneumoniae* antigen from which LPS has been eliminated. In short, 50 μL of serum samples (diluted 1:50) was added to wells in a microplate coated with a *C. pneumoniae*-specific antigen for 60 minutes at 37°C to allow *C. pneumoniae*-specific antibodies to bind. Thereafter wells were washed and then treated with peroxidase-conjugated anti-human IgG antibodies for 60 minutes at 37°C. This was followed by washing steps, addition of 50 μL of TMB-substrate and incubation for 30 minutes at 37°C. The reaction was stopped by the addition of a stop solution and the microplate was read in a Spectra MAX microplate reader (Molecular Devices, Sunnyvale, California, USA).

**Lipid peroxidation in plasma - marker for oxidative stress**

The level of lipid peroxidation in plasma was analyzed by measuring plasma concentrations of malondialdehyde (MDA), which is one of several byproducts of lipid peroxidation processes. MDA was quantified by using thiobarbituric acid (TBA) reactive substances (TBARS) (Nielsen et al., 1997). In this method, TBA forms a red adduct with two molecules of MDA, by which the fluorescence can be measured and the amount of MDA can be calculated. MDA was released from proteins by adding 50 μL of EDTA plasma to a mixture of 20 μL 2.5 mol/L NaOH and 20 μL 0.1% butylated hydroxytoluene (antioxidant), followed by incubation in a shaking water-bath for 30 minutes at 60°C. The proteins were then
denaturated by treating the samples with 300 μL 7.2% (0.44 mol/L) trichloroacetic acid (TCA), containing 1% potassium iodide. The samples were then centrifuged (9000 x g, 10 minutes) and 200 μL of supernatants and 50 μL of TBA were placed in each well of a 96-well microplate (Corning Incorporated, NY, USA). The plate was sealed with a heat-resistant adhesive PCR film (Abgene, Surrey, UK), incubated at 90°C for 45 minutes, and then chilled on ice. Fluorescence at 590 nm was detected using a FLUOStar plate reader (BMG Labtechnologies, Offenburg, Germany) at an excitation wavelength of 544 nm. MDA standards prepared from 1,1,3,3-tetraethoxypropane (Sigma Aldrich, Stockholm, Sweden), which transforms into one molecule of MDA upon heating, were analysed in parallel.

**Serotonin level in plasma - marker for platelet activation**

The concentration of serotonin in EDTA plasma was measured by using a commercially available enzyme immunoassay (EIA; Labor Diagnostika Nord, Nordhorn, Germany). This is a competitive EIA in which solid phase-bound and acetylated plasma serotonin compete for a fixed number of antiserum binding sites. In short, the plasma samples were diluted 1:25 and added to a microplate for acylation. After acylation the plasma was transferred to another microplate coated with serotonin. Serotonin antiserum was then added to each well, which was followed by shaking incubation for 60 minutes at room temperature. Thereafter the plate was washed and enzyme conjugate was added for 30 minutes under the same conditions as before, followed by washing steps and then addition of substrate. After incubation for 25 minutes with the substrate, stop solution was added and the absorbace was read in a Spectra MAX microplate reader (Molecular Devices, Sunnyvale, California, USA) set to 450 nm and a reference wavelength of 635 nm.
Ethics

The clinical study of this thesis was approved by the Human Research Ethics Committee at the Faculty of Health Sciences, Linköping University, Sweden.
Results and Discussion

There are by now many investigations that have confirmed a role for *C. pneumoniae* in atherosclerosis. Both *in vivo* and *in vitro* models have demonstrated the capacity of *C. pneumoniae* to infect and stimulate leukocytes in the circulation and cells in the atherosclerotic plaque. Furthermore, epidemiological studies have established a connection between *C. pneumoniae* infections and cardiovascular disease, and animal studies have shown that *C. pneumoniae* can trigger atherosclerosis (Mussa et al., 2006).

The platelet is another important player in the atherosclerotic process. In addition to being an essential cell in haemostasis and thrombosis, platelets have a role in various inflammatory processes, including atherosclerosis. Activated platelets are accumulated in the plaques at different stages of atherosclerosis. Interestingly, it has been demonstrated that platelets are the first cells to arrive at a site of endothelial dysfunction in early atherogenesis. By releasing granule components, such as cytokines and growth factors, activated platelets have the capacity to promote the development of atherosclerotic lesions (Huo and Ley, 2004; Ruggeri, 2002).

Earlier investigations have focused on the ability of *C. pneumoniae* to infect and stimulate cells involved in atherosclerosis, such as monocytes/macrophages, T-cells, endothelial cells and smooth muscle cells. However, the effects of *C. pneumoniae* on platelet activation have been sparsely investigated. In this regard, the present thesis has examined the capacity of *C. pneumoniae* to interact with platelets, both *in vitro* and *in vivo*, and elucidated its role in atherosclerosis.
Paper I

In the first paper we investigated the capacity of *C. pneumoniae* to bind to platelets and induce platelet aggregation and secretion. Furthermore, some mechanisms involved in this interaction were elucidated.

By using flow cytometry, we found that stirring incubation of *C. pneumoniae* with platelets resulted in a rapid binding of *C. pneumoniae* to the platelet surface (Fig. 1A). On the contrary, the binding of the bacterium to neutrophils was markedly lower and required a higher bacteria: cell ratio (Fig. 1B).

Next we found that *C. pneumoniae* stimulated an extensive, irreversible aggregation of platelets at relative low ratios (*C. pneumoniae*: platelet ratio of 1:30). The extent of this response was comparable to the aggregation triggered by collagen, which is a strong platelet activator. However, in contrast to collagen-induced platelet aggregation, the aggregatory response to *C. pneumoniae* was preceded by a surprisingly long lag phase. A lower amount of *C. pneumoniae* increased the lag phase, however, the extent of aggregation was unaffected (Fig. 2). The long lag phase can probably be explained by the low *C. pneumoniae*: platelet ratio used in these experiments. Initially, the bacteria bind to few platelets, which leads to autocrine and paracrine signalling that eventually causes extensive platelet activation and aggregation.
Figure 1.

Binding of *C. pneumoniae* to platelets and neutrophils. Separate samples of platelets and neutrophils were incubated with *C. pneumoniae* at a bacteria: cell ratio of 1:10 and 1:5, respectively. Before mixing and after 1, 5, 10, and 20 minutes of stirring incubation, the platelets were stained with phycoerythrin conjugated anti-GpIb and the *C. pneumoniae* with monoclonal FITC labelled anti-chlamydia LPS. Neutrophils were stained with phycoerythrin-conjugated CD11b. The samples were thereafter fixed and diluted, and the fluorescence intensity of the cells was analyzed with flow cytometry. Events stained positive for both *C. pneumoniae* and platelets or neutrophils, respectively, were considered as *C. pneumoniae*–cell complexes. Figure B shows the binding of *C. pneumoniae* to platelets and neutrophils, respectively, after 5 minutes of incubation.

Platelet aggregation was not induced by *Staphylococcus aureus*, *Salmonella typhimurium* or *Escherichia coli* at similar bacteria: platelet ratios as used in the *C. pneumoniae* experiments (1:30-1:5). However, at considerably higher ratios (2:1) an incomplete platelet aggregation was triggered by *S. aureus*. Other studies have also demonstrated the capacity of different bacteria and viruses to induce activation and aggregation of platelets, however, most investigations have used very high, non-physiological bacteria concentrations (Fitzgerald et al., 2006). In contrast, we show that *C. pneumoniae* causes platelet activation at a relatively low and probably more physiological concentration, which implies a role of specific mechanisms in *C. pneumoniae*-induced platelet activation.
Figure 2.
Representative tracings of *C. pneumoniae*-induced platelet aggregation. *C. pneumoniae* was added to a platelet suspension (2 x 10^8/mL) in different *C. pneumoniae*: platelet ratios (1:5-1:20). The aggregation was thereafter registered in an aggregometer for 30 minutes.

The *C. pneumoniae*-induced platelet activation also involved an extensive α- and dense-granule secretion from platelets. *C. pneumoniae* stimulated a surface expression of P-selectin on platelets already after one minute of incubation and a release of ATP simultaneously with the aggregatory response. In contrast, no P-selectin expression, ATP secretion or platelet aggregation was observed after adding the negative control consisting of Hep-2 cell debris.

Dense- and α-granule secretion leads to release of molecules that activate neighbouring platelets and other cell types in the surroundings (King and Reed, 2002; McNicol and Israels, 1999). P-selectin is stored in the α-granule of platelets and is exposed on the surface of the activated platelet. Through binding to its ligand PSGL-1, P-selectin mediates the recruitment and activation of leukocytes and thereby initiates an inflammatory response (Furie and Furie, 2004; Furie et al., 2001). Several investigations have
indeed demonstrated a role for P-selectin in cardiovascular disease (Furman et al., 1998; Huo and Ley, 2004; Merten and Thiagarajan, 2004; Nijm et al., 2005). Besides P-selectin, the α-granule releases cytokines and growth factors, which can stimulate inflammation and atherosclerosis (King and Reed, 2002).

Next we elucidated the involvement of some surface components in the cell-bacteria interaction. LPS is considered to be an important component of C. pneumoniae in cellular activation (Costa et al., 2002; Da Costa et al., 2004; Kalayoglu, 2002; Kalayoglu et al., 2000; Netea et al., 2002; Sasu et al., 2001). In the present study, we found that the C. pneumoniae-induced P-selectin expression was unaffected by heat treatment (70°C, 30 min), but prevented by the LPS-inhibitor polymyxin B, which supports a crucial role for LPS in the interaction. Chlamydia LPS has a unique structure that contains a pentaacyl-1,4´-diphosphoryl lipid A moiety instead of the classical hexacyl-1,4´-diphosphoryl lipid A moiety and exposes a highly immunogenic epitope on the polysaccharide core (Rund et al., 1999). Interestingly, other bacteria with modified LPS, such as Porphyromonas gingivalis, have been found to cause a more potent activation of platelets than more classical gram-negative bacteria (Endo et al., 1997; Grabarek et al., 1988). A recent study showed that LPS induces P-selectin-mediated formation of platelet-leukocyte aggregation (Montrucchio et al., 2003), which further supports a role for LPS in the stimulation of platelets in inflammatory processes and atherosclerosis.

Earlier studies on bacteria-platelet interaction have identified a number of potential structures on platelets to which bacteria bind. A common mechanism involves indirect binding via a plasma protein bridge to a platelet receptor, usually GpIIb/IIIa or GpIb, or direct ligation of the bacteria to these receptors (Fitzgerald et al., 2006). When exploring the role of different platelet surface structures, we found that blocking of GpIb or P-selectin had no antagonizing effects on platelet activation induced by C. pneumoniae. However, inhibition of the GpIIb/IIIa receptor with abciximab
(40 µg/mL) or Arg-Gly-Asp-Ser (RGDS, 1mg/mL) significantly reduced platelet aggregation. This supports a contribution of dimeric binding of fibrinogen molecules to activated GpIIb/IIIa receptors in the platelet aggregation induced by *C. pneumoniae*. However, whether GpIIb/IIIa is involved in the ligation of the bacteria to the platelet surface still needs to be clarified. One potential receptor type on platelets involved in *C. pneumoniae* binding and activation is the toll like receptor, mainly TLR 2 and 4, which have been found to be involved in *C. pneumoniae*-induced activation of other cells (Bulut et al., 2002; Cao et al., 2006; Da Costa et al., 2004; Netea et al., 2002; Prebeck et al., 2001; Sasu et al., 2001; Yang et al., 2005) and which are also expressed on the surface of platelets (Cognasse et al., 2005; Shiraki et al., 2004).

Our findings that *C. pneumoniae* binds more effectively to platelets than to neutrophils and possibly resides in platelet aggregates, may represent a strategy by which the bacteria avoid phagocytes of the innate immune system. Another explanation is that highly reactive platelets assist inflammatory reactions by specific and rapid binding of *C. pneumoniae*, followed by expression of P-selectin, release of inflammatory mediators and presentation of the bacteria for recruited phagocytes.

The monocyte is considered to be the most important cell responsible for spreading *C. pneumoniae* from the infected lung epithelium to the vessel wall. Binding of *C. pneumoniae* to platelets in the circulation may represent an alternative spreading mechanism of the bacteria. Megakaryocytes that fragment platelets into the circulation are also present in the lungs (Levine et al., 1993). It is possible that *C. pneumoniae* infects or binds to megakaryocytes and then are pinched off into the circulation associated to platelets.

In conclusion, the results in this study imply a role for *C. pneumoniae*-induced platelet activation in atherosclerosis. Platelet dense and α-granule secretion stimulated by *C. pneumoniae* causes release of several factors that
can stimulate atherogenesis, such as growth factors, cytokines and P-selectin. This in turn can contribute to leukocyte activation and smooth muscle cell proliferation.

**Figure 3. Summary of the results in Paper I**

*Chlamydia pneumoniae* binds to platelets and induces dense and α-granule secretion, GpIIb/IIIa activation and aggregation. LPS on *C. pneumoniae* is important in this interaction.

**Paper II**

In paper II we explored the capacity of *C. pneumoniae* to induce ROS production in platelets and its subsequent impact on LDL oxidation. Furthermore, the role of different enzyme systems in ROS generation was investigated.

Oxidation of LDL due to high oxidative stress is an important event both in the initiation and progression of atherosclerosis (Stocker and Keaney, 2004). By using flow cytometry and the ROS-sensitive fluorescence dye DCFH, we found that *C. pneumoniae* significantly stimulated ROS generation in platelets after 10 minutes of co-incubation (Fig. 4). The ROS production was counteracted by the intracellular ROS scavenger N-acetyl-L-cysteine (NAC, 0.5-5 µmol/L). However, blocking of the fibrinogen binding site on GpIIb/IIIa with Arg-Gly-Asp-Ser (RGDS, 1mg/mL)
resulted in even more pronounced ROS production. Furthermore, polymyxin B inhibited, whereas heat treatment did not affect, the chlamydia-induced ROS production in platelets. These results show that *C. pneumoniae* stimulates platelet ROS production, a process that is dependent on chlamydial LPS but not mediated by GpIIb/IIIa activation.

The origin of ROS in platelets is incompletely understood. However, several enzyme systems have been implicated to have a role, such as NADH/NADPH oxidase, COX, LOX and NOS (Chamulitrat and Mason, 1989; Iuliano et al., 1997; Jahn and Hansch, 1990; Muruganandam and Mutus, 1994; Seno et al., 2001; Zielinski et al., 2001). In the present study, we found that incubation of platelets with nitro-L-arginine, a competitive inhibitor of NOS, significantly lowered the ROS production induced by *C. pneumoniae*. Furthermore, inhibition of LOX and COX with 5,8,11,14-eicosatetraynoic acid (ETYA) and LOX with 5,8,11-eicosatriyoic acid (ETI) antagonized the *C. pneumoniae*-induced ROS production. However, inhibition of the NADH/NADPH oxidase did not affect the ROS production. LPS has been shown to trigger ROS production in platelets through activation of platelet protein kinase C (PKC) and phosphoinositide 3-kinase (Saluk-Juszczak et al., 2000; Zielinski et al., 2001). We found that inhibition of PKC, but not of phosphoinositide 3-kinase, lowered the ROS production in *C. pneumoniae*-stimulated platelets.
Figure 4.
*C. pneumoniae* stimulates ROS production in platelets. Platelets were loaded with the fluorescent ROS dye DCFH for 15 min and then mixed with *C. pneumoniae* (bacteria: platelet ratio of 1:15) or Hep-2 cell debris. Samples were fixed and diluted immediately before and 5 and 10 minutes after addition of *C. pneumoniae* to a platelet suspension. The DCF fluorescence was measured with flow cytometry.

These results indicate that activation of the NOS, LOX and PKC pathways in platelets are important for the ROS generation stimulated by *C. pneumoniae*. The species of oxygen radicals that are registered by the DCFH/DCF method are unclear. However, recent studies have found that DCFH is sensitive towards oxidation by ONOO⁻, H₂O₂ and •OH, while it is not suitable for detecting NO, NOCl or O²⁻ (Myhre et al., 2003). Lufrano and Balazy (2003) reported that the fluorescence of DCF-loaded platelets is specific for ONOO⁻. These findings thus suggest that *C. pneumoniae* stimulates production of ONOO⁻ (Lufrano and Balazy, 2003). Furthermore, we propose NOS as the source for ONOO⁻, since this enzyme has the capacity to generate both NO and O²⁻ (Pou et al., 1992). However, increased activity of 12-LOX probably also has a role in *C. pneumoniae*-induced platelet ROS production (Figure 6). The ONOO⁻ formed is most
likely released extracellularly, since it is well established that this radical can pass through the lipid part of biological membranes or be transported through anion transporters (Denicola et al., 1998; Khairutdinov et al., 2000; Lufrano and Balazy, 2003; Romero et al., 1999).

As already mentioned, oxidation of LDL is a crucial event in the initiation of atherosclerosis and in the progress of the disease. Interestingly, co-incubation of *C. pneumoniae* and platelets induced an extensive oxidation of LDL. Both platelets and *C. pneumoniae* separately caused an LDL oxidation, although not to the same degree as the mixture.

*C. pneumoniae* and platelets are present in the circulation and in atherosclerotic plaques. The findings that *C. pneumoniae* stimulates platelet ROS production and a subsequent LDL oxidation may therefore have an impact on atherogenesis. The results from the present study together with the findings in the first paper suggest that the interaction between *C. pneumoniae* and platelets may have a role in several steps of the atherosclerotic process: i) initiates atherogenesis and inflammation by promoting LDL-oxidation; ii) stimulates the growth of the plaque by releasing of platelet-derived growth factors and cytokines; and iii) stimulates platelet aggregation, thrombosis and vascular occlusion.

![Diagram of the interaction between *C. pneumoniae* and platelets](image)

**Figure 6. Summary of Paper II**

*C. pneumoniae* binds to platelets and induces activation of PKC, NOS and LOX via LPS. The activity of both NOS and LOX leads to generation of ROS, reflected as ONOO⁻ by the DCF technique. This generation of ROS in turn leads to oxidation of LDL.
Paper III

In paper III we used a human model to study the ability of *C. pneumoniae* to alter platelet activity and LDL-oxidation *in vivo*. It is well known that *C. pneumoniae* is often present in human atherosclerotic plaques. In this study, we recruited patients that underwent coronary angiography and a following percutaneous coronary intervention (PCI) or coronary artery bypass graft (CABG). Thereafter we investigated if *C. pneumoniae* is released into the circulation after the interventions and if this causes increased platelet activation and lipid peroxidation.

Peripheral blood samples were collected from patients that underwent angiography with or without following PCI or CABG; before intervention; and at several points in time up to a year later. The concentrations of *C. pneumoniae* IgA and IgG antibodies were determined before the procedure and then one month and six months after (the one-year sample had not been collected in all of the patients at the time of this study), and in a healthy gender- and age-matched control group. The results showed that there was a significantly higher amount of *C. pneumoniae* IgA positive individuals in the patients than in the control group (Fig. 7).
However, the amount of individuals positive for *C. pneumoniae* IgG was high in all three groups and there was no significant difference between the controls and the patients. In contrast to *C. pneumoniae* IgA, however, the concentrations of IgG increased in 38% of the *C. pneumoniae* IgG positive individuals one month after PCI. If *C. pneumoniae* is exposed to immune cells in the circulation after PCI, a rise in chlamydial IgG would be expected after approximately one month (Fig. 8) (Kuo et al., 1995b). We therefore propose that the increase in IgG after one month reflects a release of *C. pneumoniae* during or after the PCI procedure. A few other investigations have also demonstrated a rise in serum *C. pneumoniae* IgG after PCI (Kaehler et al., 2005; Tiran et al., 1999; Yetkin et al., 2004). Interestingly, Kaehler *et al* found that the PCI procedure increases serum *C. pneumoniae* IgG, but not IgG directed towards *Helicobacter pylori*, *Herpes simplex* or cytomegalovirus (Kaehler et al., 2005), which implies that the

**Figure 7.** *Chlamydia pneumoniae* IgA levels in controls, angiography, PCI and CABG patients before intervention. The dots above the broken line represent *C. pneumoniae* IgA-positive samples.
rise in *C. pneumoniae* IgG is not due to unspecific stimulation of immune cells as a consequence of the PCI procedure.

Figure 8
Hypothetical model of how released *C. pneumoniae* affects the kinetics of serum *C. pneumoniae* IgG levels after PCI (Kuo et al., 1995b).

Another aim of this project was to investigate whether an expected exposure of *C. pneumoniae* to the circulating blood during the PCI procedure causes activation of platelets and oxidation of LDL. In order to determine platelet activation, we measured the plasma levels of serotonin (5-HT). Plasma serotonin is released from the dense granule during platelet activation and is relatively stable in the circulation (De Clerck et al., 1984; McNicol and Israels, 1999; Vikenes et al., 1999). High levels of serotonin in plasma have been considered a risk factor for cardiovascular disease (Vikenes et al., 1999). Serotonin activates 5-HT$_2$ receptors, expressed on several cell types, thereby causing vasoconstriction, platelet aggregation, proliferation of vascular endothelial cells and smooth muscle cells and endothelial injury (Ashton et al., 1987; Kishi and Numano, 1989; Pakala et al., 1994; 1997; Seuwen and Pouyssegur, 1990). We found that in several patients there was an increase in the plasma-serotonin level 24 hours after PCI. Interestingly, this rise in serotonin correlated to the increase in *C. pneumoniae* IgG levels one month after PCI (Fig. 9a). In conclusion, these
data suggest that a release of *C. pneumoniae* during PCI causes platelet activation.

Instead of analyzing LDL oxidation, which requires a large amount of blood for isolation, lipid peroxidation in plasma was measured in this study. Lipid peroxidation has frequently been used as a marker for oxidative stress, and has been proposed as a risk factor for restenosis after PCI (Davi and Falco, 2005; Imai et al., 2001; Kovacs et al., 1997; Nielsen et al., 1997). A possible mechanism by which oxidized lipids in plasma induce atherosclerosis is through oxLDL binding to the LOX-1 receptor, expressed on endothelial cells, leading to a proinflammatory response of these cells (Adachi and Tsujimoto, 2006). We found that the lipid peroxidation in plasma increased 24h after PCI in about 40% of the patients, an observation which correlates with several other studies (Coghlan et al., 1994; Oostenbrug et al., 1997; Roberts et al., 1990; Tsimikas et al., 2004). The most interesting finding of this study, however, was that the elevation of lipid peroxidation after 24 hours correlated significantly to the rise in *C. pneumoniae* IgG after one month (Fig. 9b). In fact all the patients that showed an increase in the IgG level also demonstrated an elevated lipid peroxidation. These findings suggest that, in addition to platelet activation, the release of *C. pneumoniae* after PCI contributes to oxidation of lipids, including LDL.

Next we wanted to elucidate whether the increased lipid peroxidation in the patient groups was due to a chlamydia-induced platelet activation. We found no significant correlation between increase in serotonin level and lipid peroxidation 24 hours after PCI, although there was a tendency for a correlation.
Figure 9. Correlation between increase in *C. pneumoniae* IgG level one month after PCI and increase in plasma level of serotonin (A; $r=0.62$, $P=0.002$) and lipid peroxidation (B; $r=0.91$, $P<0.0001$), respectively, 24 hours after PCI.

In conclusion, this paper suggests that *C. pneumoniae* is released from atherosclerotic plaques during PCI, which contributes to platelet activation and lipid peroxidation. The subsequent consequences of these processes in the patients regarding future cardiovascular events remain to be studied.
Figure 10. *Summary of the results from Paper III*

During percutaneous coronary intervention (PCI), the atherosclerotic plaque is compressed leading to a release of *C. pneumoniae* into the circulation and a subsequent platelet activation and lipid oxidation.

**Paper IV**

The findings in papers I-III strongly suggest a role for *C. pneumoniae*-induced platelet activation in atherosclerosis. Earlier investigations have found that *C. pneumoniae* infections in the cardiovascular system are very difficult, if not impossible, to combat by antibiotics. In this paper, we have focused on how to pharmacologically prevent *C. pneumoniae*-induced platelet activation. More specifically, the inhibitory effects of a variety of pharmacological agents on platelet aggregation and secretion induced by *C. pneumoniae* were investigated. For comparison, the effects of the drugs
were also tested on the responses triggered by collagen and thrombin, respectively.

The most interesting findings in this study were that *C. pneumoniae*-induced platelet aggregation and ATP secretion were totally blocked by the 12-LOX inhibitors baicalein and CDC, whereas the COX inhibitors aspirin and piroxicam had no effects. On the contrary, the COX inhibitors effectively inhibited collagen-induced platelet activation, whereas the 12-LOX inhibitors showed no effects on this response (Fig. 11). Platelets also synthesise from arachidonic acid the lipid mediator platelet activating factor (PAF), which activates platelets and other cells by binding to specific receptors (Nunez et al., 1986). We found that *C. pneumoniae*-induced platelet activation was totally inhibited by the PAF antagonist Ginkgolide B. In contrast, collagen-induced activation was unaffected by Ginkgolide B. Furthermore, the P2Y₁ and P2Y₁₂ purinergic receptor antagonists MRS2179 and cangrelor significantly inhibited the *C. pneumoniae*-induced platelet activation when administered together, but not separately, whereas the collagen-induced platelet responses were inhibited by both MRS2179 and cangrelor (Fig. 11).

Activation of COX and subsequent production of TxA₂ is supposed to have an important role in platelet activation. TxA₂ binds to TP-receptors on the surface of platelets, which potentiates stimulatory pathways in platelets including activation of phospholipase C (PLC), and subsequent cytosolic liberation of Ca²⁺ (Jurk and Kehrel, 2005). In contrast, agonist-induced stimulation of platelets mediated by activation of 12-LOX has rarely been reported. However, it was recently demonstrated that stimulation of platelet aggregation by PAF requires activation of 12-LOX (Michibayashi, 2005). 12-LOX in platelets metabolizes arachidonic acid to 12-hydroperoxyeicosatetraenoic acid (12-HPETE). 12-HPETE can either be reduced to 12-hydroxyeicosatetraenoic acid (12-HETE) or metabolised to lipoxins. The intracellular mechanisms induced by 12-HETE and lipoxins leading to platelet activation are unknown.
Figure 11. The effects of different pharmacological agents on *C. pneumoniae*- and collagen-induced platelet aggregation. The figure shows representative traces of aggregometry measurements on platelets, preincubated with or without aspirin (100 µmol/L), CDC (1 µmol/L), baicalein (1 µmol/L), cangrelor (10 nmol/L) and MRS2179 (10 µmol/L) and then stimulated with *C. pneumoniae* (*C. pneumoniae*:platelet ratio of 1:15) or collagen (1µg/mL).

The COX-inhibitor aspirin is commonly used in the treatment of cardiovascular disease, whereas 12-LOX or PAF inhibitors have not been used for this purpose. The findings that *C. pneumoniae*-induced platelet activation was unaffected by COX-inhibitors, but effectively prevented by inhibition of PAF and 12-LOX, may have a role in designing new therapies against cardiovascular diseases. It is well known that the efficacy of aspirin in preventing thrombosis in cardiovascular disease differs among patients,
despite the fact that activation of platelets with well-known platelet agonists, such as collagen, is strongly inhibited by aspirin (Horiuchi, 2006). The finding in this study that *C. pneumoniae*-induced activation of platelets was not affected by COX inhibition may explain why some patients do not respond to aspirin treatment.

This pharmacological paper also gives the opportunity to speculate about different signalling pathways that are activated in platelets stimulated by *C. pneumoniae*. As mentioned above, PAF-induced platelet aggregation has been found to be dependent on 12-LOX activity (Michibayashi, 2005). One possible scenario therefore is that *C. pneumoniae* binds to platelets and triggers the formation of PAF, which is expressed on the platelet surface or released, leading to activation of PAF-receptors on neighbouring platelets. This activation induces signalling cascades in the platelet that eventually causes 12-LOX activation. The subsequent production of 12-HETE and/or lipoxins stimulates granule secretion, GpIIb/IIIa activation and thereby platelet aggregation. Another hypothesis is that *C. pneumoniae* causes a simultaneous production of PAF and 12-LOX metabolites, which synergistically activate the platelets.
Summary

- These studies show that *Chlamydia pneumoniae* binds to platelets via LPS and triggers secretion, aggregation and ROS production at low *C. pneumoniae*: platelet ratios. The binding of *C. pneumoniae* to platelets was significantly higher than the binding to neutrophils. The extent of the chlamydia-induced platelet aggregation was comparable to the aggregatory response triggered by collagen.

- The chlamydia-induced platelet aggregation was dependent on activation of GpIIb/IIIa and was not mimicked by other tested bacteria despite considerably higher bacteria: platelet ratios. The platelet ROS production induced by *C. pneumoniae* was dependent on activation of PKC, NOS and 12-LOX, and the interaction between *C. pneumoniae* and platelets caused oxidation of LDL.

- The clinical study suggests that *C. pneumoniae* is released into the circulation after PCI and causes platelet activation and lipid peroxidation, which implies a role for *C. pneumoniae*-induced platelet activation and oxidative stress in patients suffering from coronary artery disease.

- Pharmacological studies showed that inhibition of the P2Y₁ and P2Y₁₂ purinergic receptors, PAF and 12-LOX, but not by COX, counteracted the *C. pneumoniae*-induced platelet activation.
Figure 12. Hypothetical model of how the Chlamydia pneumoniae-induced platelet activation stimulates growth of an atherosclerotic plaque

1: In the circulation, *C. pneumoniae* binds to platelets and triggers reactive oxygen species (ROS) production, which in turn causes oxidation of low-density lipoprotein (LDL). *C. pneumoniae*-induced P-selectin expression causes activation of leukocytes via P-selectin glycoprotein ligand-1 (PSGL-1), which leads to further oxidative stress and LDL-oxidation. The oxidized LDL binds to the lectin-like oxidized receptor 1 (LOX-1) on endothelial cells and triggers the production of monocyte chemoattractant protein 1 (MCP-1). This leads to recruitment of leukocytes to the vessel wall which increases the inflammation in the intima and accelerates atherogenesis. Oxidized LDL is accumulated in the intima and internalized by macrophages, which forms foam cells.

2: In the arterial wall, *C. pneumoniae*-activated platelets adhere to the endothelium and accumulate in the atherosclerotic plaque. The release of ROS and cytokines from the *C. pneumoniae*-activated platelets further stimulates leukocyte recruitment and inflammation. Platelet-released growth factors trigger proliferation of smooth muscle cells (SMCs). *C. pneumoniae*-infected platelets also spread the infection to other atherosclerotic cell types.
Figure 13. Hypothetical model of how Chlamydia pneumoniae stimulates atherothrombosis and vascular occlusion

*Chlamydia pneumoniae* elementary bodies (EB) are released from endothelial cells during the *C. pneumoniae* development cycle, and bind to circulating platelets leading to platelet secretion and aggregation. An extensive platelet secretion and aggregation leads to the formation of a thrombus, which causes death of a tissue (such as the cardiac tissue).
Importance

Only about half of the cardiovascular disease events can be explained by the pre-existing risk factors for atherosclerosis. The present thesis suggests novel mechanisms for atherosclerotic vascular disease involving *Chlamydia pneumoniae*-induced platelet activation. A strategy for how to pharmacologically prevent such activation is also presented. Thus, the findings in this thesis could represent a new central mechanism that contributes to atherosclerotic changes in the vessel wall and following thrombosis. In the future it might be important to prevent a *C. pneumoniae*-induced activation of platelets in order to circumvent the atherosclerotic process.
Andraws, R., J.S. Berger and D.L. Brown, 2005, Effects of antibiotic therapy on outcomes of patients with coronary artery disease: a meta-analysis of randomized controlled trials, Jama 293, 2641.


Breslow, J.L., 2001, Genetic markers for coronary heart disease, Clin Cardiol 24, II.


Byrne, M.F., S.W. Kerrigan, P.A. Corcoran, J.C. Atherton, F.E. Murray, D.J. Fitzgerald and D.M. Cox, 2003, Helicobacter pylori binds von Willebrand factor and interacts with GPIIb to induce platelet aggregation, Gastroenterology 124, 1846.


Cirino, F., W.C. Webley, C. West, N.L. Croteau, C. Andrzejewski, Jr. and E.S. Stuart, 2006, Detection of Chlamydia in the peripheral blood cells of normal donors using in vitro culture, immunofluorescence microscopy and flow cytometry techniques, BMC Infect Dis 6, 23.


Coombes, B.K., B. Chiu, I.W. Fong and J.B. Mahony, 2002, Chlamydia pneumoniae infection of endothelial cells induces transcriptional activation of platelet-derived growth factor-B: a potential link to intimal thickening in a rabbit model of atherosclerosis, J Infect Dis 185, 1621.


Cottet, J. and M. Lenoir, 1992, [Two thousand years of historical study on the words atheroma, atheromatosis, atherosclerosis, arteriosclerosis], Bull Acad Natl Med 176, 1385.


Discepolo, W., T. Wun and L. Berglund, 2006, Lipoprotein(a) and thrombocytes: potential mechanisms underlying cardiovascular risk, Pathophysiol Haemost Thromb 35, 314.


Everett, K.D., R.M. Bush and A.A. Andersen, 1999, Emended description of the order Chlamydiaceae, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms, Int J Syst Bacteriol 49 Pt 2, 415.


Falk, E., 1991, Coronary thrombosis: pathogenesis and clinical manifestations, Am J Cardiol 68, 28B.


Gutierrez, J., J. de Dios Luna, J. Linares, M. del Rosario Montes, E. Quesada, A. Rojas, M.J. Soto and A. Sorlozano, 2005, Relationship between peripheral arterial occlusive disease (PAOD) and chronic Chlamydophila (Chlamydia) pneumoniae infection. A meta-analysis, Thromb Haemost 93, 1153.


Haller, A.v., 1755, Opuscula pathologica, Observatio LI.


Harper, A., C.I. Pogson, M.L. Jones and J.H. Pearce, 2000, Chlamydial development is adversely affected by minor changes in amino acid supply, blood plasma amino acid levels, and glucose deprivation, Infect Immun 68, 1457.


Kalayoglu, M.V. and G.I. Byrne, 1998b, Induction of macrophage foam cell formation by Chlamydia pneumoniae, J Infect Dis 177, 725.


Kalayoglu, M.V., G.S. Miranpuri, D.T. Golenbock and G.I. Byrne, 1999b, Characterization of low-density lipoprotein uptake by murine macrophages exposed to Chlamydia pneumoniae, Microbes Infect 1, 409.


Klotz, 1906, Atherosclerosis correlation with typhoid, streptococci.


Li, D. and J.L. Mehta, 2000, Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells, Circulation 101, 2889.


Melnick, J.L., E. Adam and M.E. DeBakey, 1990, Possible role of cytomegalovirus in atherogenesis, Jama 263, 2204.
inhibitor of type III secretion inhibits different stages of the infectious cycle of Chlamydia trachomatis, Proc Natl Acad Sci U S A 103, 14566.


Myhre, O., J.M. Andersen, H. Aarnes and F. Fonnum, 2003, Evaluation of the probes 2',7'-dichlorofluorescin diacetate, luminol, and lucigenin as indicators of reactive species formation, Biochemical Pharmacology 65, 1575.


Newby, A.C., 2005, Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture, Physiol Rev 85, 1.


Quinn, M.T., S. Parthasarathy and D. Steinberg, 1985, Endothelial cell-derived chemotactic activity for mouse peritoneal macrophages and the effects of modified forms of low density lipoprotein, Proc Natl Acad Sci U S A 82, 5949.


Ross, R., 1979, Platelets: cell proliferation and atherosclerosis, Metabolism 28, 410.


Ruffer, M., 1911, On arterial lesions found in Egyptian mummies, J Pathol Bact 15, 453.


Scanu, A.M., 2003a, Lipoprotein(a) and the atherothrombotic process: mechanistic insights and clinical implications, Curr Atheroscler Rep 5, 106.


Watson, M.W., P.R. Lambden, J.S. Everson and I.N. Clarke, 1994, Immunoreactivity of the 60 kDa cysteine-rich proteins of Chlamydia trachomatis, Chlamydia psittaci and Chlamydia pneumoniae expressed in Escherichia coli, Microbiology 140 ( Pt 8), 2003.


Weisel, 1906, Relation to typhoid, scarlet fever, measles to atherosclerosis.


