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Flow-mediated dilation

The link between *Chlamydia pneumoniae* antibodies and ischemic heart disease. The effect of clarithromycin on endothelial function in men with acute coronary syndrome.

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1. **" *Chlamydia Pneumoniae* Antibodies and Endothelial Function as Assessed by Flow-Mediated Dilation in Patients With Chest Pain With and Without Ischemic Heart Disease."**
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2. **" Effect of Clarithromycin versus Placebo on Endothelial Function expressed as Flow-Mediated Dilation in Male Patients with Ischemic Heart Disease. Randomized, double-blind, parallel-group trial."**
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* Members of the IAMA Group are listed in the section: Abbreviations

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Abbreviations

A	azithromycin
ACADEMIC	The Azithromycin in Coronary Artery Disease: Elimination of Myocardial Infection with *Chlamydia*
ACE	angiotensin-converting enzyme
ACES	The Azithromycin and Coronary Events Study
ACS	acute coronary syndrome
AMI	acute myocardial infarction
ANTIBIO	The Antibiotic Therapy After Acute Myocardial Infarction
ATL	Advanced Technology Laboratories
AZACS	Azithromycin in Acute Coronary Syndrome
BD	Birgitte Diness
BM	Beata Malmqvist
BP	blood pressure
C	clarithromycin
CABG	coronary artery bypass graft surgery
CAD	coronary artery disease
CAG	coronary angiography
CI	confidence intervals
CK	creatinine kinase
CK-MB	creatinine kinase-myocardial band
CLARICOR	Clarithromycin in Patients with Stable Coronary Heart Disease
CLARIFY	Clarithromycin in Acute Coronary Syndrome Patients in Finland
C. pneumoniae	*Chlamydia pneumoniae*
CROAATS	Croatian Azithromycin in Atherosclerosis
CRP	C- reactive protein
CV	coefficient of variation
D	doxycycline
D1	brachial artery diameter at baseline
D2	brachial artery diameter after cuff release
D3	brachial artery diameter at re-baseline
D4	brachial artery diameter after sublingual administration of a 0.5 mg glyceryl trinitrate tablet
D1O1	baseline diameter at occasion 1
D1O2	baseline diameter at occasion 2
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial NO synthase
FMD	flow-mediated dilation
G	gatifloxacin
HDL	high-density lipoprotein
hsCRP	high sensitive C-reactive protein
ICAM	intercellular cell adhesion molecule
IgA	immunoglobulin A
IgG	immunoglobulin G
IHD	ischemic heart disease

IL-6	interleukin-6
ISAR-3	The Intracoronary Stenting and Antibiotic Regimen
LA	limits of agreement
LDL	low-density lipoprotein
M	metronidazole
MARBLE	Might Azithromycin Reduce Bypass List Events
MI	myocardial infarction
Mm	millimeter
NMD	nitroglycerin-mediated dilation
NO	nitric oxide
O	omeprazole
OR	odds ratio
PAD	peripheral arterial disease
PAI-1	plasminogen activator-inhibitor-1
PCI	percutaneous coronary intervention
PROVE-IT	Pravastatin or Atorvastatin Evaluation and Infection Therapy
PTCA	percutaneous transluminal coronary angioplasty
R	roxithromycin
RECIFE	The Reduction of Cholesterol in Ischemia and Function of the Endothelium
ROXIS	Randomized Trial of Roxithromycin in Non-Q-Wave Coronary Syndromes
RR	relative risk
SAS	Statistical Analysis System
SD	standard deviation
SMC	smooth muscle cells
SPSS	Statistical Package for the Social Sciences
STAMINA	The South Thames trial of Antibiotics in Myocardial Infarction and uNstable Angina
STEMI	ST elevation myocardial infarction
TNF-α	tumor necrotizing factor-α
t-PA	plasminogen activator
UAP	unstable angina pectoris
VCAM	vascular cell adhesion molecule
WIZARD	Weekly Intervention with Zithromax for Atherosclerosis and its Related Disorders
X	amoxicillin
AKS	akut koronar syndrom
IHS	iskæmisk hjertesygdom

The IAMA Group Infections, Atherosclerosis and Macrolide Antibiotics

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English Summary

There is an increasing body of evidence suggesting that infection and inflammation are involved in the pathogenesis of arteriosclerosis and myocardial infarction. Infection with the bacteria *Chlamydia pneumoniae* (*C. pneumoniae*) has been implicated in the pathogenesis of ischemic heart disease (IHD). *C. pneumoniae* antibodies have a weak association with IHD and with known risk factors for arteriosclerosis. *C. pneumoniae* has been found in atherosclerotic plaques in coronary and carotid arteries in humans. *C. pneumoniae* accelerates the development of arteriosclerosis in animal models. Intervention with macrolide antibiotics in *C. pneumoniae* infected experimental animals delays or inhibits development of arteriosclerotic lesions. The hypothesis, that infection with *C. pneumoniae* may be a possible etiologic and/or pathogenetic factor for arteriosclerosis and IHD, was substantiated by two small interventional studies, demonstrating a reduction in future cardiovascular events in patients with IHD, who were treated with macrolide antibiotics.

Endothelial function plays a crucial role in atherogenesis. A number of important physiologic functions depends on an intact endothelium, i.e. regulation of vascular tone via the production of vasoactive substances, such as nitrous oxide. A repeated, continuous influence on the endothelium by various noxes results in gradual loss of these essential functions, thus leading to endothelial dysfunction. Endothelial function is measured non-invasively by ultrasonography of the brachial artery. It has been demonstrated that endothelial function of the brachial artery is well correlated to endothelial function of the coronary vessels, and is associated with known risk factors for IHD. The ultrasonographic method for determining endothelial function of the brachial artery exploits the vasodilator response of the endothelium to an increased blood perfusion, i.e. flow-mediated dilation (FMD). Endothelial dysfunction occurs very early in atherogenesis, before any macroscopic anatomical changes are observed. Known risk factors for IHD, such as age, hypercholesterolemia, diabetes mellitus and smoking, are associated with endothelial dysfunction and reduced FMD. It has been extensively documented that FMD improves, when the classical cardiovascular risk factors are reduced. FMD correlates with prognosis and can predict cardiovascular events. Consequently, FMD may be a possible proxy for coronary arteriosclerosis and IHD.

The aim of this thesis was to examine whether presence of C. *pneumoniae* Immunoglobulin A (IgA) and IgG antibodies might be associated with reduced FMD, and whether intervention with macrolide antibiotics (clarithromycin) in patients with IHD might improve FMD.

We have done 2 studies. The first is an observational cross-section study of 225 patients, referred with chest pain indicating acute coronary syndrome (ACS). The study population was divided in 2 groups: patients with IHD, and patients who had a normal exercise test and had never been diagnosed with IHD. FMD and occurrence of positive *C. pneumoniae* IgA and IgG antibodies in the two groups were compared. Mean value of FMD in patients with versus without IHD was 4.2% vs. 5.6%. Students t-test for unpaired data showed that FMD in the two groups was significantly different ($p < 0.001$). Seropositivity for *C. pneumoniae* IgA antibodies was defined as IgA titer $\geq$ 32. Seropositivity for *C. pneumoniae* IgG antibodies was defined as IgG titer $\geq$ 64. The occurrence of positive *C. pneumoniae* IgA and IgG (each one separately) antibodies in the two groups of patients (with and without IHD) was compared. The chi-square test showed that there were no differences in prevalence of seropositivity for *C. pneumoniae* IgA and IgG between patients with versus without IHD ($p = 0.404$ for IgA and $p = 0.488$ for IgG). FMD in patients with positive *C. pneumoniae* IgA and IgG antibody titers was compared with FMD in patients with negative *C. pneumoniae* IgA and IgG antibody titers. There was no difference in FMD between *C. pneumoniae* seropositive and seronegative patients. This applied to IgA and IgG *C. pneumoniae* antibodies. These findings were observed in males as well as females with and without IHD.

We demonstrated that, in patients with IHD, FMD was impaired when compared to patients without IHD. Age and prevalence of IHD was significantly associated with FMD in a multiple linear regression analysis, where other risk factors for arteriosclerosis (smoking, cholesterol, sex and *C. pneumoniae* IgA and IgG antibodies) were included. Novel risk factors for IHD, such as C. *pneumoniae* IgA and IgG seropositivity, are not associated with FMD or IHD. Only patients with very high titers of *C. pneumoniae* IgA and IgG antibodies had a higher frequency of IHD than patients with titers < 256. However, the number of patients with the highest titers was very small, and so the results should be viewed with reservations. Especially, there was no significant difference in FMD in patients with *C. pneumoniae* IgA and IgG antibody titers ≥ 256, and in patients with *C. pneumoniae* IgA and IgG antibody titers < 256. Based on the first study, it is concluded that positive *C. pneumoniae* antibody titers were not associated with impaired FMD. There was no association between *C. pneumoniae* titers and FMD, either. There was no correlation between *C. pneumoniae* IgA and IgG on one side and FMD on the other. There were no differences in prevalence of seropositivity for *C. pneumoniae* IgA and IgG in patients with versus without IHD. A possible weak association between high *C. pneumoniae* antibodies (titer ≥ 256) and IHD may have been mediated via a potentially detrimental effect on the endothelium of infection with *C. pneumoniae*. On the other hand, we were not able to rule out that *C. pneumoniae* infection may play a role in plaque instability and rupture.

The second study is a randomized, double-blinded, interventional study comparing the effect of a macrolide antibiotic (clarithromycin) with placebo in 40 patients with ACS. After stabilization, the patients were treated with 500 mg of clarithromycin or placebo once daily for 2 weeks. FMD and *C. pneumoniae* IgA and IgG titers were measured 3 times: before intervention, and 2 and 6 weeks after commencement of intervention. Before trial drug administration, FMD did not differ significantly between groups. After two weeks of treatment, FMD was not significantly different in the 2 groups. Six weeks after randomization (4 weeks after completion of intervention with clarithromycin), FMD was higher in the clarithromycin group compared to the placebo group (6.2% vs. 4.9%, $p = 0.07$). The potentially beneficial effect could be observed 4 weeks after conclusion of the intervention. The effect of clarithromycin intervention was independent of *C. pneumoniae* IgA and IgG seropositivity at the outset of the intervention. The *C. pneumoniae* IgA and IgG antibody titers neither changed in the clarithromycin, nor in the placebo group during the course of the study.

The results of the second study suggested that FMD was reversible after intervention with macrolide antibiotics. The effect did not depend on the presence of positive *C. pneumoniae* IgA and IgG titers. However, the long-term effect of macrolide antibiotics on FMD has not yet been elucidated. Whether these results might eventually have clinical implications, has to be determined through intervention with macrolide antibiotics in a larger number of patients during a long-time follow-up of FMD, while keeping in focus the development of clinical arteriosclerotic manifestations. Furthermore, there is a need to document more extensively, whether FMD may be of prognostic importance. These studies are underway.

Chapter 1: Background

Chlamydia pneumoniae antibodies and IHD

Atherosclerosis has a multifactorial etiology (genetic predisposition, hypercholesterolemia, male gender, diabetes mellitus, hypertension, smoking, adiposity). However, many patients, who develop atherosclerosis, do not have any of these risk factors.

There is an increasing body of evidence suggesting that infection and inflammation are involved in the pathogenesis of arteriosclerosis and myocardial infarction. Infection with the bacteria *Chlamydia pneumoniae* (*C. pneumoniae*) has been implicated in the pathogenesis of ischemic heart disease (IHD). *C. pneumoniae* may be a risk factor for atherosclerosis *per se* in parallel with other coronary risk factors. It is well known that " influenza-like illness" with upper respiratory tract symptoms may precede acute myocardial infarction (AMI). An acute respiratory infection during two preceding weeks was a risk factor for AMI in persons without a history of classical cardiovascular risk factors (1). Various infectious agents have been postulated to contribute to atherosclerotic manifestations. Some studies suggest the existence of an association between the total infectious burden (the total number of different pathogens a person has been exposed to) and the extent of atherosclerosis. Moreover, the risk of future death increases with the number of different infectious pathogens, especially in patients with advanced atherosclerosis (2;3). Of the various organisms studied as possible causes of IHD, evidence indicates *C. pneumoniae* to be the most likely:

- A seroepidemiological association exists between the presence of *C. pneumoniae* antibodies and AMI and IHD
- *C. pneumoniae* is more frequently found in the atherosclerotic plaque than in normal arterial tissue
- *C. pneumoniae* can infect endothelial and smooth muscle cells in vitro
- Macrophages infected with *C. pneumoniae* are transformed into foam cells, which represent the earliest stage in development of atherosclerosis
- *C. pneumoniae* is able to both induce and accelerate the development of atherosclerosis in animals
- Intervention with macrolide antibiotics in *C. pneumoniae* infected animals delays or inhibits development of atherosclerotic lesions
- Some interventional studies demonstrate a reduction in future cardiovascular events in patients with IHD treated with macrolide antibiotics effective in *C. pneumoniae* infection

C. pneumoniae, an obligatory intracellular, Gram-negative bacterium, is 1 of 4 chlamydia species causing human disease. *C. pneumoniae* is transmitted by aerosol droplets and can lead to pharyngitis, sometimes followed by laryngitis, sinusitis, bronchitis, or interstitial pneumonia. Generally, the symptoms are mild to moderately severe, and most cases do not require hospitalization. *C. pneumoniae* is a significant cause of community-acquired respiratory infection, occurring worldwide and without seasonal variation of incidence. Overall prevalence of chlamydial IgG seropositivity in the general population has been estimated at 40% to 50% (4). *C. pneumoniae* has a tendency to cause chronic, persistent infections (5).

Seroepidemiological studies

The first paper that suggested an association between *C. pneumoniae* seropositivity and cardiovascular disease, i.e. chronic coronary artery disease (CAD) and AMI, was published by Saikku et al. in 1988 (6). They found that 68% of men younger than 50 years of age with AMI had elevated IgA and IgG titers, compared with only 17% of control subjects. Subsequently, several groups have studied this association. In a meta-analysis, involving more than 2700 patients, Danesh et al. found an association between *C. pneumoniae* seropositivity and vascular disease. They reported > 2-fold odds ratios (OR) (7). However, only 2 of 18 studies were prospective. Subsequent meta-analysis of 15 prospective studies completed by 2000 revealed an OR 1.15 (95% CI: 0.97-1.36) and suggested only a modest association between *C. pneumoniae* IgG titers and CAD (8). Most recently Bloemenkamp et al. (9) performed a meta-analysis of seroepidemiologic studies of the relation between *C. pneumoniae* and atherosclerosis. Their findings agreed with previous reports, in the sense that cross-sectional studies generally report a significant association between *C. pneumoniae* IgG seropositivity and atherosclerosis with an OR around 2.0. Prospective studies, however, fail to support a causal relation between *C. pneumoniae* seropositivity and atherogenesis, yielding an OR around 1 (9). However, this apparent lack of a relation does not entirely rule out, that a relation between the infection and atherosclerosis might exist. While it is possible that infection with *C. pneumoniae* relates to atherogenesis, the infection might not be reflected as seropositivity in the blood of patients.

The discrepancy between the results from cross-sectional case control studies and prospective studies might be caused by the following factors: Prospective studies are, by far, the most reliable, investigate temporal relations and are best suited for the study of a potential causal relation between 2 factors. Prospective studies have the advantage that cases and controls subjects are both part of the same population. However, the disadvantage of prospective studies is that they might underestimate the relation between the 2 studied factors. This situation might occur if, for instance, a control subject, who is seronegative to *C. pneumoniae* at the start of the study, is infected with *C. pneumoniae* during the follow-up period. Unlike prospective studies, the cross-sectional case control studies register the potentially causal exposure variable and the outcome variable simultaneously. This makes it impossible to decide, whether a given association is caused by the fact that the exposition was a consequence of the disease, or whether the disease was a consequence of the exposition. Hence, cross-sectional case control studies are not particularly informative and suited for the study of etiologic/causal connexions. Furthermore, it is the vulnerable point of the cross-sectional case control studies that it is difficult to make sure that the group of cases and the group of controls are comparable. Frequently, the 2 groups come from very different populations. An additional disadvantage of the cross-sectional case control studies is the inherent large risk of recall bias.

Histopathological studies, animal experiments and cell biology studies

Several studies provide evidence that *C. pneumoniae* was more frequently found in atherosclerotic tissue samples than in noncardiovascular tissues (10) or normal arteries (11). In coronary plaques, *C. pneumoniae* seemed to be present more frequently in patients with acute coronary syndromes than in patients with stable angina (12). Atherosclerotic plaques of the carotid arteries with thrombosis were more likely to contain *C. pneumoniae* than plaques without thrombosis (13). In some studies it was possible to cultivate the *C. pneumoniae* bacteriae, thus confirming that the infectious agent was, indeed, alive (14). *C. pneumoniae* was detected in early atherosclerotic lesions, such as fatty streaks, and this fact opens up the possibility of a primary etiologic role (15). A majority of the histopathological studies did not find that a positive *C. pneumoniae* titer was

obligatorily associated to a detection of *C. pneumoniae* organisms in atherosclerotic tissues by a positive polymerase chain reaction or immunocytochemistry method (11;13;16).
In animals, experimental infection with *C. pneumoniae* was able to both induce and accelerate the development of atherosclerosis, and intervention with macrolide antibiotics in *C. pneumoniae* infected animals delayed or inhibited development of atherosclerotic lesions (17;18). Liuba et al. reported that *C. pneumoniae* infection impaired the endothelial function in apolipoprotein E-knockout mice (19).
In vitro, *C. pneumoniae* could infect macrophages as well as endothelial and smooth muscle cells, i.e. the cells considered important in the development of atherosclerosis (20). In vitro, *C. pneumoniae* induced and accelerated the transformation of monocytes and macrophages to foam cells, an important step in atherogenesis (21).
These studies support the hypothesis that *C. pneumoniae* is capable of causing infection and inflammation within the vessel wall. The direct presence of the pathogen in the macrophages in the atherosclerotic plaques could possibly induce a local inflammatory reaction, increased production of inflammatory cytokines and adhesion molecules that stimulate atherogenesis (22).
However, as these data do not establish a causal relationship between *C. pneumoniae* and onset or progression of atherosclerosis, it is possible, that *C. pneumoniae* is no more than an innocent bystander within atherosclerotic plaques.

Antibiotics studies
Entusiasm concerning the hypothesis, that infection with *C. pneumoniae* might be a possible etiologic and/or pathogenetic factor for arteriosclerosis and IHD, grew substantially after 2 independent publications of 2 small interventional studies, demonstrating a reduction in new cardiovascular events in patients with IHD, treated with macrolide antibiotics (23;24). Macrolides were effective in *C. pneumoniae* infection (25;26) and, apart from an antibacterial effect, also had an antiinflammatory effect (27;28).
Subsequently, larger intervention studies with antibiotics in patients with CAD have been designed and undertaken. In these trials the hypothesis, that future cardiovascular events might be reduced through antibiotic treatment directed against the bacteria, was tested. Some trials have already been finished without disclosing any beneficial effect of antibiotics on reinfarction, revascularization, and death (ACADEMIC, ANTIBIO, AZACS, WIZARD), others have demonstrated a beneficial effect on clinical outcome (STAMINA, CLARIFY, Wiesli et al.). A number of studies have reported a decrease in inflammatory markers (ACADEMIC, ROXIS, STAMINA), others have only demonstrated an effect in patients with high *C. pneumoniae* titers (ISAR-3). A series of studies has not, yet, been completed, but is underway and includes, among others, CLARICOR and PROVE-IT (Table 1A, Appendix A).
These intervention studies did not clarify the mode of action of macrolides. It has not been resolved whether the effects, if there are any, of macrolide antibiotics are due to an antimicrobial effect, their anti-inflammatory properties, or a combination of the two. None of the quoted studies, however, have proven a causal relationship between *C. pneumoniae* and atherosclerosis. The studies diverge from each other both with respect to inclusion criteria, type of antibiotic treatment and duration of treatment, measured effect, and are not comparable. No studies have, yet, yielded a clear answer, and many have still not been completed.
There are only a few published, randomized clinical trials that have evaluated the effect of antibiotics on endothelial function and flow-mediated dilation (FMD) (Table 3A, Appendix A).

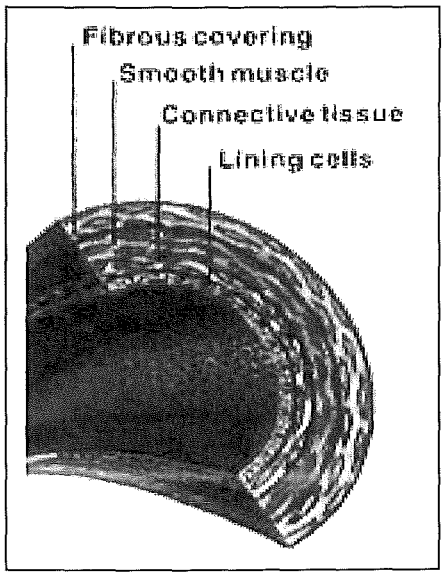
Endothelium and atherogenesis

The endothelium and the endothelial function play the key role in atherogenesis.
Atherosclerosis is an inflammatory process, that involves the endothelium (29). The process begins, when the vascular endothelium, in response to the repeated or continuous effects of mechanical stress, infections, and immunological reactines, recruits inflammatory cells into the arterial intima, leading to the formation of foam cells (the predominant cells of the early atherosclerotic lesion) and fatty streaks (the earliest histological lesions in atherosclerosis) (30). Physiologically, the earliest detectable manifestation of atherosclerosis is endothelial dysfunction, defined as a state of reduced production or bioavailability of nitric oxide (NO) in response to pharmacological or haemodynamic stimuli (29;31). Endothelial dysfunction appears long before the macroscopic structural atherosclerotic changes (32;33).

Functions of and substances released by the endothelium

The endothelium is a single-layered cell lining, covering the internal surface of blood vessels, cardiac valves and numerous body cavities. Endothelial cells, together with the underlying basement membrane, constitute the tunica intima. The tunica media, consisting of smooth muscle cells (SMC), constitutes the intermediate layer of the blood vessels, while the tunica adventitia, consisting of fibroblasts, represents the outermost vessel layer.

Figure 1. Cross-section of an artery, visualizing the layers of the artery wall



The endothelium has a number of important regulatory functions. One of its primary functions is to maintain vascular tone (relaxation and contraction) of the underlying SMC, by releasing vasoactive substances (vasodilators and vasoconstrictors). One of the most potent vasodilators released by the endothelium is NO. A critical balance between endothelium-derived relaxing and contracting factors maintains vascular homeostasis. When this balance is disrupted, it predisposes the vasculature to vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, thrombosis, vascular inflammation, and atherosclerosis.

Normal Endothelial Function
• Maintains vascular tone and structure • Regulates leukocyte and platelet adhesion to its surface • Regulates thrombotic and fibrinolytic properties • Mediates inflammatory and immune mechanisms • Regulates vascular cell growth • Modulates lipid oxidation • Regulates vascular permeability

To maintain homeostasis, the endothelium synthesizes and releases other biologically active substances as mediators of inflammation, haemostasis and thrombosis, and growth mediators.

Substances Released by the Endothelium
Vasoactive Substances
Vasodilators
Nitric oxide (NO) / endothelium-derived relaxing factor (EDRF)
Endothelium-derived hyperpolarizing factor (EDHF)
Prostacyclin
Bradykinin
Vasoconstrictors
Endothelin-1 (ET-1)
Angiotensin II
Thromboxane A2
Prostaglandin H2, etc.
Inflammatory Mediators
P-and E-selectin
Intercellular adhesion molecule (ICAM)
Vascular cell adhesion molecule (VCAM)
Chemokines
Hemostasis and Thrombosis Mediators
Antithrombotic
NO
Prostacyclin
Plasminogen activator (t-PA)
Protein C
Tissue Factor Inhibitor
Von Willebrand factor
Prothrombotic
Endothelin-1
Plasminogen activator-inhibitor-1 (PAI-1)
Thromboxane A2
Fibrinogen
Tissue Factor
Growth Mediators
Vascular Endothelial Growth Factor
Endothelin-1

Nitric oxide = Endothelium-Derived Relaxing Factor

Nitric oxide (NO) is the key endothelium-derived relaxing factor (EDRF), that plays a pivotal role in the maintenance of vascular tone and reactivity. NO is the most potent endogenous vasodilator. NO is indispensable for normal functioning of the endothelium.

NO is synthetized inside the endothelial cell, from the amino-acid L-arginine via activity of the enzyme endothelial NO synthase (eNOS). Under normal physiological conditions, there exists a balance between endothelial production and secretion/release of NO, and release of endothelium contracting factors, and this balance guarantees normal physiological tonus of the vessels. Disruption of this balance leads to disturbances in the maintainance of normal vessel tone, and to disturbances of other functions of the endothelium - endothelial dysfunction.

In the normal endothelium, NO has a number of antiatherogenic effects. NO inhibits leukocyte (monocyte)-endothelial cell interactions, inhibits platelet aggregation, platelet-endothelial cell interactions, and SMC proliferation. When NO activity is reduced, these benefits may be lost, and significant alterations in arterial reactivity and abnormal interactions among the vessel wall, platelets and leukocytes will occur (34;35). All of these vascular changes are known to be important in atherogenesis and lead to endothelial dysfunction (29).

Endothelial dysfunction, definition

The term "endothelial dysfunction" refers to alterations in endothelial properties that may contribute to the development of atherosclerosis. Endothelial dysfunction, the first step in atherogenesis, is defined as the loss of one or more endothelial properties with ensuing disturbances in the maintainance of normal vessel tone. Endothelial dysfunction implies diminished production or bioavailability of NO and/or an imbalance in the relative contribution of endothelium-derived relaxing and contracting factors.

In clinical situations, endothelial dysfunction is defined as the loss of vasodilatory capacity (in response to a pharmacologic NO stimulus, like acetylcholine or in response to physical forces such as an increase of blood flow), as this response (endothelium-**dependent** dilation) is relatively easy to monitor. That NO is, in fact, responsible for the endothelium-**dependent** dilation, has been proved by demonstrating that pretreatment with eNOS inhibitors (36) blocks the endothelium-**dependent** dilation.

Vasodilation can also be elicited by infusion or an oral dose of nitroglycerine trinitrate, which has a direct vessel dilating effect by relaxing the smooth musculature of the vessel (an endothelium-**independent** response).

If the endothelium is dysfunctional, the endothelium-**dependent** dilation will be weakened, totally abolished or possibly even paradox, while the endothelium-**independent** response will be preserved.

In summary, endothelium and intact endothelial function play a crucial role in atherogenesis. According to Ross' hypothesis "Response to Injury", the repeated or continuous effects of mechanical stress, infections and immunological reactines may result in inflammation and endotelial damage.

Endothelial dysfunction is defined as the loss of one or more endothelial properties with ensuing disturbances in the maintainance of normal vessel tone. Endothelial dysfunction occurs very early in the atherogenesis. The earliest detectable manifestation of endothelial dysfunction and atherosclerosis is reduced production or bioavailability of NO (which is responsible for relaxation of SMC and protection against atherosclerosis). This has been applied in the development of methods to evaluate endothelial function.

Methods to evaluate endothelial function

Coronary circulation
Endothelial function in the coronary vasculature can be assessed by invasive tests. These methods include quantitative coronary angiography (37) and intracoronary Doppler flow velocity measurements.
Quantitative coronary angiography can be used to examine the change in diameter, and intracoronary Doppler techniques can be used to measure coronary blood flow, in response to intracoronary infusions of endothelium-dependent vasodilators, such as acetylcholine. In healthy vessels, acetylcholine evokes a NO-mediated (endothelium-dependent) vasodilatory response. In atherosclerotic vessels, this effect is blunted or paradoxical (vasoconstriction) (37).

Peripheral circulation
Invasive and non-invasive methods for the evaluation of endothelial function in the peripheral vasculature have been developed.

Invasive methods
The invasive methods apply the same principle as used for assessment of coronary vasculature, i.e. to measure the endothelium-dependent vasodilatory reaction in the peripheral vasculature in response to direct intra-arterial (brachial artery) administration of pharmacological stimuli, i.e. acetylcholine. Invasive techniques examine the change in blood flow by plethysmography (38).

Non-invasive methods
Non-invasive techniques include phase contrast magnetic resonance imaging (39;40) and high resolution ultrasound of the brachial, radial, popliteal and femoral artery. The brachial artery is most frequently used. Ultrasound is used to examine the change in artery diameter in response to an increase in the wall shear stress, due to increased blood flow, i.e. flow-mediated dilation (FMD). This method is more extensively covered in the Chapter 4.
The method is inexpensive, safer and faster than invasive methods. Furthermore, it is easily translatable to routine clinical practice, because the technology applied is already widely used to evaluate vascular disease. Finally, the equipment is portable.

Factors influencing FMD

Age, gender and smoking
Celermajer et al. examined 238 healthy persons between 15 and 72 years of age, without any known risk factors for atherosclerosis. On average, FMD was 7.9% ± 3.9%, but decreased with age. The age-dependent progressive decrease in FMD in men set in at the age of 41. In women, this decrease started approximately 13 years later, but seemed to progress with a greater decline than in men after the age of 50 years (41).
Premenopausal women had significantly higher FMD than age-matched men and postmenopausal women (42-47). Furthermore, FMD in premenopausal women varied with the phases of the menstrual cycle (48;49). In a study, 17 healthy young women and age–matched men were examined

3 times. The women were checked during three different phases of the menstruational cycle (menstruation phase, follicular phase, luteal phase). FMD was significantly higher during the follicular and luteal phases, where the plasma concentration of estradiol was higher. During the menstruation phase, where plasma estradiol values were low, FMD fell to its lowest level, corresponding to the value observed in men. FMD varied during pregnancy (50).
Both short-term (51), long-term (46) and passive smoking (52) had negative effects on endothelial function. And this effect was dose-dependent. An inverse association was found between pack-years and FMD (53). In the active smokers, there was an inverse association between the number of cigarets smoked daily and FMD, and, in the passive smokers, an inverse association between the intensity of exposure to tobacco smoke and FMD was found (52). Endothelial dysfunction, observed after cigarette smoking, was a phenomenon lasting at least 60 minutes (51). Nicotine alone caused acute endothelial dysfunction, although to a lesser extent than smoking a cigarette of the same nicotine yield (54).

Application of the FMD method - literature review

Results from studies, where the method has been applied, are reviewed.

Relation between FMD and cardiovascular risk factors
Several studies have shown an association between the classical and novel cardiovascular risk factors and abnormalities of endothelial function, measured as FMD.
Hypercholesterolemia (46;55), smoking (52;53), arterial hypertension (56), adiposity (57), diabetes mellitus (58;59) and other endocrine diseases (60;61), aging and male gender (41), were all associated with impaired FMD.
Novel cardiovascular risk factors, such as hyperhomocysteinemia (62-64) and neurophysiologic conditions, such as depression (65) and mental stress (66), were associated with impaired FMD.
The number of risk factors correlated with FMD, and an accumulation of coronary risk factors was related to impairment of endothelial function (37;53;67).

Relation between FMD and severity and extent of coronary atherosclerosis
Endothelial dysfunction of the brachial artery, expressed as FMD, correlated with the severity and extent of coronary atherosclerosis in 74 patients with angina pectoris (68) and 121 patients, who underwent coronary angiography (69).

In summary, FMD can identify individuals at risk before the development of clinically apparent cardiovascular disease. This suggests that the study of FMD may be used for the identification of novel risk factors for cardiovascular disease.

Disorders associated with impaired FMD

Table 1. Disorders associated with impaired FMD

Disorders Associated with impaired FMD
• CAD (68) (69;70) • Hypercholesterolemia (46) including familial hypercholesterolemia (55) • Hypertension (56) • Diabetes mellitus (58;59) • Homocyst(e)inemia, homocystinuria (62-64) • Neurophysiologic conditions, such as depression (65) • Chronic heart failure (71) • Alkoholism (72) • Acromegaly (60) • Growth Hormone deficiency/hypopituitarism (61) • Raynaud's phenomenon secondary to systemic sclerosis (73) • Hypothyroidism (74) • Hyperparathyroidism (75) • Polycytemia vera (76) • Low birth weight (77) • Systemic lupus erythematosus (78) • Behcet's Syndrome (79)

Reversibility of impaired FMD

Clinical studies of the effect of pharmacologic or other types of treatments are of importance in context with FMD, as this is the setting, where the advantages of the method are obvious.

- FMD in the brachial artery improved in response to reduction of atherosclerotic risk factors, i.e. smoking cessation (53)
- Physical exercise in healthy individuals, in patients with CAD, in patients with diabetes mellitus and in post-AMI patients improved FMD (80-84). In obese children and in obese adolescents, FMD improved after a program of 8 weeks of exercise (85;86).
- Short-term (6 weeks) dietary modification only, or diet plus a structured exercise program in overweight children enhanced FMD. Diet and exercise together were associated with a greater improvement in FMD than diet alone. FMD at 1 year was better in those children continuing exercise, compared with children who withdrew from the exercise program (87). Other investigators could not confirm this observation in asymptomatic adults with cardiovascular risk factors. Life style intervention for 3 months, containing exercise training combined with National Cholesterol Education Program step 1 diet and smoking cessation, did not improve FMD (88).
- It is very well documented that lipid-lowering therapy with statins improves FMD (89). Improvement of FMD occurred rapidly and could already be observed after 2 weeks' treatment, in healthy individuals (90).

FMD and prognosis

Healthy well-functioning endothelium protects against atherosclerosis. Therefore, it is not surprising that impaired endothelial vasomotor function (both in the coronary and the peripheral bed) had prognostic implications (70;91;92). In a prospective study of 187 patients undergoing non-emergency vascular surgery, the patients with decreased FMD (examined preoperatively) experienced significantly more postoperative cardiac events (death, myocardial infarction (MI), unstable angina /ischemic ventricular fibrillation, stroke, or elevated troponin I, reflecting myocardial necrosis) during the 30-day postsurgery period (91). After a 1.2-year follow-up of 199 patients with peripheral vascular disease, the same investigators reported a significantly higher rate of cardiovascular events in patients with impaired FMD. The risk was 9-fold grater in those with FMD < 8.1% (93). In another study, in which FMD was measured in 73 patients undergoing cardiac catheterization for chest pain, cardiovascular events (percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass graft surgery (CABG)) occurred more often in patients with impaired FMD (<10%), compared with patients with preserved FMD (>10%) over a follow-up period of 5 years (70). A low brachial artery FMD was an independent predictor of cardiovascular risk in 131 patients with peripheral arterial disease (PAD) during follow-up for about 2 years. Patients with FMD below the median (6.7%) had an independent 4.85-fold increase in risk (94). In another study of 152 patients with CAD, carotid atherosclerosis was measured as intima-media thickness of the common carotid artery, together with FMD. There was no correlation between intima-media thickness and FMD. Both FMD and "plaque burden" were independent predictors of adverse cardiac events during a 34-month follow-up. The worst prognosis was seen in those patients with severe grades of endothelial dysfunction concomitant with a high degree of carotid plaque (95). FMD can predict future fatal events. In a preliminary study of 518 consecutive ambulatory patients with suspected CAD, higher cardiovascular and non-cardiovascular mortality rates, during follow-up for no less than 2 years, were observed in those within the lowest tertile of FMD (96). An impaired FMD in 150 patients with chronic heart failure was associated with a moderately increased risk of death (OR 1.34, 95% CI 1.02 to 1.47) during a mean follow-up of 13 months (97). Only a few studies examine whether changes in FMD over time have a prognostic value. Modena et al. (98), in a study involving 400 postmenopausal women with hypertension, measured FMD before and 6 months after normalization of blood pressure. During follow-up for 67 months, event rates for cardiovascular events (death, acute pulmonary oedema, transient ischemic attack, and ischemic stroke) were 7-fold higher in those in whom FMD improved by <10%, compared with those who had improvements >10%. In contrast to most of the previous literature, only Fathi et al. (99) suggested that FMD is not an independent predictor of cardiovascular outcomes. They investigated 444 patients with presence of risk factors or known or suspected cardiovascular disease, and 75% of the patients had renal dysfunction, of which a substantial number was in dialysis and/or had been transplanted. The follow-up period was 24 months. Only in the subgroup of higher risk patients undergoing stress testing (277 patients), FMD, along with measurements of intima-media thickness and left ventricular mass, proved to be an independent predictor of cardiovascular events.

In conclusion, endothelial dysfunction, measured as impaired FMD, appears in the early stages of atherosclerosis, before any atherosclerotic changes are seen in the arteries. FMD is very closely associated with classical cardiovascular risk factors and correlates with the severity and extent of coronary atherosclerosis. Treatment of cardiovascular risk factors improves FMD in the brachial artery. Importantly, all interventions that have been shown to improve the outcome of vascular disease, including statins, angiotensin-converting enzyme inhibitors, smoking cessation and exercise, also improve FMD. In the light of this, endothelial

dysfunction could potentially be used as a surrogate marker in cardiovascular studies of risk reduction therapies. Recent studies have shown that FMD may have a prognostic value.

Chapter 2: Aims of the study

The aims of the present study were:

1. To examine, in a reproducibility study, whether we were capable of mastering the FMD method on a level comparable to that of other investigators.
2. To investigate whether or not FMD was associated with known and novel cardiovascular risk factors, especially presence of *C. pneumoniae* antibodies in blood.
3. To investigate the effect on FMD of 2 weeks intervention with macrolide antibiotic treatment in patients with acute coronary syndrome (ACS).

Chapter 3: Hypotheses

1. The occurrence of *C. pneumoniae* antibodies might, in parallel with other coronary risk factors, result in endothelial damage and impaired FMD.
2. Macrolide antibiotics could modulate FMD in patients with ACS.

Chapter 4: Design and methods

The first study was a reproducibility study. The second study was designed as an observational, cross-sectional study (EVIR). The third study was designed as a prospective, randomized, double-blind, placebo-controlled study.

Protocol - the three substudies: patient populations and ethics

Reproducibility study

To determine intra-observer variation of FMD, we recruited 19 healthy volunteers (men and women) among staff and students at The University Hospital of Hvidovre, Copenhagen, and 7 patients with chest pain and suspicion of AMI admitted to our department of cardiology. They were all examined with respect to FMD by the same observer (Beata Malmqvist) twice with days' intervals. Only healthy persons, who were on no medication (except anti-conception for women, see below), were invited, irrespective of smoking habits and a family history of cardiovascular disease.

All women were examined regardless of their menstrual cycle (49), and whether they were receiving oral hormonal therapy (anti-conception), or not. All participants were investigated under the same conditions. We expressed intra-observer variation (day-to-day variation) for FMD in two ways:

1. As mean and range of differences between the FMD measured on the two different days in the same subject by the same observer (100).
2. By analysis of agreement as described by Bland and Altman (101). In this analysis, the mean of two repeated measurements was plotted against the difference between these measurements, and the SD (standard deviation) of the mean difference was calculated, and mean of differences ± 2 SD was plotted to determine the limits of agreement (LA).

EVIR study

The patients who were recruited to EVIR were included consecutively from the Department of Cardiology at The University Hospital of Hvidovre, Copenhagen, in the period from November 1998 to March 2000. Most patients were admitted to the ward, but some patients were studied as outpatients.

The inclusion criterium was: Acute chest pain suggesting IHD and its evaluation by an exercise test. Exclusion criteria were: Inability to perform a conclusive exercise test, diabetes mellitus, chronic inflammatory diseases, acute infections, peripheral arterial occlusive disease, cancer, pregnancy, contraceptive hormone intake, postmenopausal hormone replacement therapy, ongoing drug or alcohol abuse, atrial fibrillation or left bundle branch block in the electrocardiogram, or participation in other studies, and inability to communicate in Danish or English.

Some 480 patients with acute chest pain, referred to a diagnostic exercise test, were screened for eligibility for participation. Altogether, approximately 250 patients were excluded: 2 in 5 due to an inconclusive exercise test, 1 in 5 due to diabetes and chronic inflammatory diseases (mainly COLD), 1 in 5 did not consent to participate and 1 in 5 was excluded for other reasons (postmenopausal hormone replacement therapy, atrial fibrillation or left bundle branch block in the electrocardiogram, pulmonary embolus, pericarditis, and inability to communicate in Danish or English).

In patients admitted to the emergency department, diagnostic workup included serial recording of 12-lead electrocardiograms, serial measurement of creatine kinase (CK) with myocardial band (MB) fractioning, and exercise testing after the pain had subsided. Coronary risk factors, such as plasma total cholesterol, low-density lipoprotein, high-density lipoprotein, triglycerides, blood pressure (BP), and body mass index (BMI), were measured at admittance, using conventional methods as employed in the department. The patients were interviewed about history on smoking, IHD, and hypertension. After the chest pains subsided the patients had an exercise test. Patients with AMI performed an exercise test during hospitalization for their AMI. Patients, in whom the chest pains subsided and who were not diagnosed with AMI, were discharged, and later did an exercise test in the out-patients' clinic. Exercise tests were carried out on a bicycle ergometer, according to the American College of Cardiology/American Heart Association guidelines for exercise testing (102). All patients, who successfully completed exercise testing, were examined with ultrasonography of the brachial artery, when the patients were in a stable condition, and had no chest pain. Patients with AMI were examined no earlier than the 5th day after the occurrence of the AMI. There was an interval between the debut of the acute episode of chest pain and the measurement of FMD of 27 ± 26 days (23 ± 24 days for patients with IHD, and 34 ± 26 days for patients without IHD, mean ± SD). Blood samples for detection of *C. pneumoniae* IgA and IgG

antibodies were drawn on the same day that the FMD assessment was carried out. The *C. pneumoniae* antibody results of 2 patients are missing.

AMI was diagnosed when two of the following three criteria were met: (1) typical chest pain persisting for > 30 minutes, (2) dynamic changes of the Q wave and the ST-T segment, compatible with AMI, and (3) elevation of the CK-MB to more than twice the upper limit of normal. For the diagnosis of IHD, one or several of the following criteria were required: prior MI or AMI, prior PTCA or CABG, or a positive exercise test. A positive exercise test was defined as the presence of exercise-induced anginal chest pain, accompanied by typical ST-segment deviations at 0.08 seconds after the J point, measuring 1 millimeter (mm) or more in at least 2 leads during or after exercise, or by only ST-segment deviations at 0.08 seconds after the J point, measuring 1 mm or more in at least 2 leads during or after exercise without symptoms (silent ischemia).
Demographic and clinical characteristics of the patients appear in Table 3.
Out of the 225 patients, 137 patients had IHD, and 88 were not diagnosed with IHD (i.e. no MI, negative exercise test). Of the 137 patients with IHD, 77 patients (56%) had acute myocardial infarction, 29 patients (21%) had prior myocardial infarction, 12 patients (9%) had prior PTCA, and 10 patients (7%) had prior CABG. In 98 of 137 patients, IHD was diagnosed for the first time during their hospitalization related to the episode with chest pain (77 had AMI and 21 did not have AMI, but a positive exercise test).
All patients received standard treatment during the acute phase (fibrinolysis in case of ST elevation myocardial infarction (STEMI), heparine, anti-platelet therapy, β-blockers, etc.). Some of the patients without the IHD-diagnosis were treated with anti-anginous medications. All in all, 12 patients were in treatment with β-blockers. Of these, eight were already in treatment before the acute episode of chest pain due to hypertension (4 patients), extrasystolia (1 patient), or as prophylaxis against either paroxysms of atrial fibrillation (1 patient) or hemicrania (1 patient), and as treatment of glaucoma (1 patient).
There were eight patients in treatment with calcium-blockers. Six of these patients were already in treatment before the acute episode of chest pain due to hypertension. The remaining four patients in treatment with β-blockers and two in treatment with calcium-blockers had their medication instituted during the acute hospitalization, in relation to the acute episode of chest pain, as prophylaxis against angina pectoris, in spite of a series of normal ECGs and normal levels of coronary enzymes. These patients continued the anti-ischemic treatment, until the diagnosis of IHD had been disproved by other diagnostic means (myocardial scintigraphy or coronary angiography (CAG)). Alltogether, twenty-six patients were put on prophylactic medication with anti-platelet agents during the acute episode of chest pain, and four of these were also administered nitrates. The treatment was not discontinued, untill either the exercise test, the myocardial scintigraphy or the CAG had disproved the diagnosis of IHD.

Prospective, randomized, double-blind, placebo-controlled study
The study was designed according to the CONSORT statement (103). The patients in this substudy were included consecutively from our department of cardiology between May 2000 and March 2001. Only men with ACS, stabilized after an ACS, were eligible for the study. ACS was diagnosed when two of the following three criteria were met: (1) typical chest pain persisting for > 30 minutes, (2) dynamic changes of the Q wave and the ST-T segment, compatible with AMI, and (3) elevation of the CK-MB to more than twice the upper normal limit, or when patients had chest pain associated with new or transient T-wave inversion or ST-segment depression or/and increase of troponin-T. Patients were excluded if they had diabetes mellitus, severe hepatic or renal failure,

cancer, chronic inflammatory disorders, were previously treated with macrolides, displayed hypersensitivity to macrolides, were currently treated with drugs that might interact with macrolides, had participated in other drug trials within the previous 4 weeks, or expected CABG or percutaneous coronary intervention (PCI) within 2-3 months, and if they were unable to communicate in Danish or English.

Of the 150 consecutive patients screened, 110 were excluded (26 had diabetes mellitus, 25 did not consent, 7 had inflammatory disease, 7 had been previously treated with macrolides, 17 participated in other drug trials, 6 had renal or hepatic failure, and the remaining 22 were excluded for various other reasons). Accordingly, 40 patients were randomized. Baseline characteristics are given in Table 16. Patients received stable doses of their usual medication for at last four weeks before the randomization. In the acute phase, all patients received medical intervention, and some of these were subsequently referred to invasive treatment, i.e. either PCI or CABG, for all patients according to department guidelines on treatment of ACS. Clinical history in the period from the actual episode of ACS to randomization appears in Table 16.

Randomization and treatment

The patients were randomised in three blocks of 12, 10, and 18 patients to receive 500 mg clarithromycin daily or placebo in a 1:1 ratio. The randomization of the patients and the dispensation of trial medication were organized by Glostrup Pharmacy. Twenty patients were given clarithromycin and 20 patients given placebo for 2 weeks. All other medications were kept constant throughout the study, if possible.

Medical history, physical examination, and ultrasonography of the brachial artery were performed at randomization, and 2 weeks (end of trial medication), and 6 weeks after randomization (4 weeks after completion of intervention with clarithromycin). Blood samples were drawn on the day of the FMD assessment at randomization, and 2 and 6 weeks after randomization. Each patient was carefully instructed to take the medication at fixed regular intervals. Treatment compliance was assessed by pill count.

Ethics

Written informed consent was obtained from all of the patients before enrollment in the two studies. The protocols were approved by the Regional Ethics Committee for Copenhagen (number: 01-337/98 and 02-087/99) and The Danish Data Protection Agency. The prospective, randomized, double-blind, placebo-controlled study was approved by The Danish Medicine Agency (number: 2612-1341).

ABBOT Laboratories provided the trial medication. No conflict of interests exists. The study was inspected by The Danish Medicine Agency (number: 2621-47).

Methods

General description of the flow-mediated dilation

The method was developed and first described in 1992 by Celermejer's group (32;104). High–resolution ultrasonography is used to measure changes in the diameter of the brachial artery in response to increased blood flow, and to nitroglycerin. The vasodilator response of the brachial artery during increased blood flow constitutes the basis of the method. Increased wall shear stress, due to the increase in blood flow, results in the production of the endothelium-derived vasodilators, including NO. The vasodilator response is predominantly caused by endothelial NO release (36). The blood pressure cuff, placed around the upper arm for 5 minutes, is used in order to occlude the brachial artery. When the cuff is released, the blood flow of the vessel is increased, resulting in endothelium-dependent flow-mediated dilation (FMD).

The non-invasive method allows for an evaluation of the endothelial function of the medium size systemic arteries, such as the brachial artery, although the coronary, the carotide and the iliac arteries remain the most important from a clinical perspective. Therefore, it is essential that the endothelial function of, i.e. the brachial artery, reflects the general state of these vessels. Atherosclerosis, however, is a universal process, and pathoanatomic studies confirm, that the brachial artery, too, is affected hereby. Histologic studies have found a high prevalence of atherosclerotic manifestations in the brachial artery, and a significant correlation between vascular lesions in this artery and in both the coronary and the carotide arteries (105). Importantly, endothelial dysfunction of the brachial artery, expressed as FMD correlates with measures of coronary endothelial dysfunction, indicating that endothelial dysfunction is a generalized process (106;107). More importantly, endothelial dysfunction of the brachial artery, expressed as FMD correlates with the severity and extent of coronary atherosclerosis (68;69) and is associated with known risk factors for IHD.

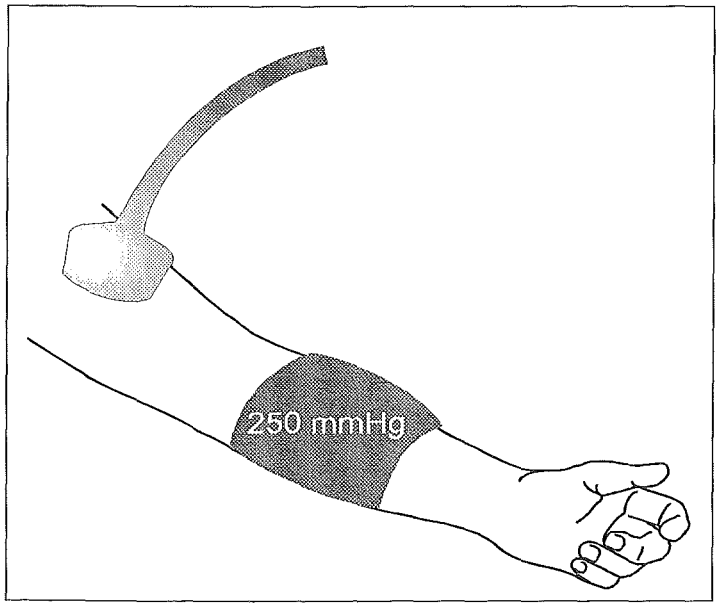
Ultrasonography is defined as soundwaves within the frequency area from 2 to 10 MHz, or even higher. The fundamental physical principle in ultrasonography is the pizo-electric effect of quarts and synthetic ceramics. This is exploited in the ultrasonographic transducer, where an electric impulse generates a mechanical impulse. The frequency depends on the size of the crystal. To obtain an ultrasonogram, it is required that many scanning lines are integrated, either through mechanical rotation of the ultrasonography crystal, thus getting a radiating sector scan via shielding, or through positioning of a large number of ultrasonography crystals side by side in the manner of a linear transducer. The frequency of the transducer determines its resolution and penetration, i.e. higher frequency results in higher resolution, but, conversely, less penetration. Generally, the frequency used for scanning of superficial structures is 5 MHz or higher, whereas scanning of more profound structures necessitates the use of lower frequencies.

FMD in the present study

FMD of the brachial artery was performed in all patients in the morning. The ultrasonographic examinations of the individual patients were always done at the same time of the day. All patients studied had abstained from alcohol, caffeine, and food for 8 hours before the study. All medications were withheld for 12 hours before the ultrasonographic examination. Patients were instructed to refrain from smoking. Each patient rested in the supine position for at least 30 minutes before the first rest scan was obtained. The brachial artery in the non-dominant arm was scanned in a longitudinal view 3 to 5 cm above the elbow. Blood flow was measured and images of the baseline brachial artery diameter were stored for subsequent analysis. The scanned area of the arm was marked so that it could be found again. The patient's arm was kept in the same position throughout

the scanning. The blood pressure cuff, placed around the forearm, was inflated to a pressure of 250 mmHg (Figure 2). After 5 minutes, the cuff was deflated. Blood flow was measured 15 seconds after cuff deflation, and images of the brachial artery were obtained from 45 seconds to 120 seconds after cuff release. Fifteen minutes later, baseline measurements were repeated. A tablet of glyceryl trinitrate (0.5 mg) was then administered sublingually, and 3 minutes later a scanning sequence was initiated where the artery was scanned for flow. Then diameter images were obtained once every minute for the next 10 minutes in order to determine the nitroglycerin-mediated dilation (NMD).
The duration of each examination session was approximately 2½ hours.
FMD of the brachial artery in the prospective, randomized, double-blind, placebo-controlled study was performed 3 times in each patient at the same time of the day, at randomization, and 2 and 6 weeks after randomization. All measurements of the ultrasonografic images were completed before the trial code was opened.

Figure 2. Illustration of the placement of the blood pressure cuff and the ultrasound probe on the arm where the images were taken



Collecting and processing of ultrasonographic data

The ultrasonographic examination was performed, using the 3000 ATL (Advanced Technology Laboratories, Inc., Bothell, Washington) scanner with a broadband (multiple-frequency) L12-5 MHz linear array transducer. Depth and gain settings were optimized to identify the lumen-to-vessel interface and were kept constant during each study. Images of the brachial artery diameter were recorded corresponding to the R-wave (end-diastole) on the electrocardiogram. All images of the brachial artery were numbered and stored on optical discs and then transferred to the computer hard disc for subsequent analysis. The analysis of images concerning the observational study (EVIR) was done by an investigator, who was blinded to the patients' clinical details, *C. pneumoniae* antibody status, and the sequence of ultrasound measurements (Birgitte Diness (BD)).
The analysis of images concerning the prospective, randomized, double-blind, placebo-controlled study was done randomly by another investigator, who was was blinded to treatment and the sequence of ultrasound measurements (Beata Malmqvist (BM)). Flow images were recorded on super-VHS videotapes. The brachial artery diameter was measured manually, using the program

Image-Pro-Plus™. The diameter of the brachial artery was measured from the anterior to the posterior "m line" at a fixed distance (52). A baseline diameter of the brachial artery was calculated on the basis of images recorded during 5 heartbeats. Each diameter measurement at 1 beat was performed at 5 points along the vessel (consisting of 5 measurements along approximately 1 centimeter of the longitudinal axis of the artery), so that a baseline diameter typically was calculated as the average diastolic diameter of 25 measurements. Calculation of FMD and NMD in a single patient/each examination required measurement of at least 30 images/pictures. In each image/picture, the diameter of the brachial artery was measured at 5 points. This corresponded to 150 measurements per examination per patient, all in all taking approximately 2½ hours. The total amount of measurements, executed in the EVIR (N = 225) and in the prospective, randomized, double-blind, placebo-controlled study (N = 40), corresponded to the recording of at least 10 350 ultrasonographic images/pictures, used for 1 552 500 measurements of the diameter of the brachial artery, all in all.

FMD was expressed as the percent change in diameter after induction of reactive hyperemia approximately at 60 seconds after cuff release, relative to the baseline diameter value (FMD%).

FMD% = ((vessel diameter after cuff release - baseline vessel diameter)/ baseline vessel diameter) x 100%

NMD was expressed as the percent change in diameter (maximal dilation, after sublingual administration of a 0.5 mg glyceryl trinitrate tablet) relative to the repeated baseline (re-baseline) diameter value (NMD%).

NMD% = ((vessel diameter after glyceryl trinitrate administration - repeated baseline vessel diameter)/ repeated baseline vessel diameter) x 100%

Figure 3. The brachial artery scanned along the longitudinal axis at baseline (D1), approximately 60 seconds after cuff release (D2), at re-baseline (D3), and 4 min after sublingual administration of a 0.5 mg glyceryl trinitrate tablet (D4) in a healthy subject (FMD = 6.6%, NMD = 25.5%).

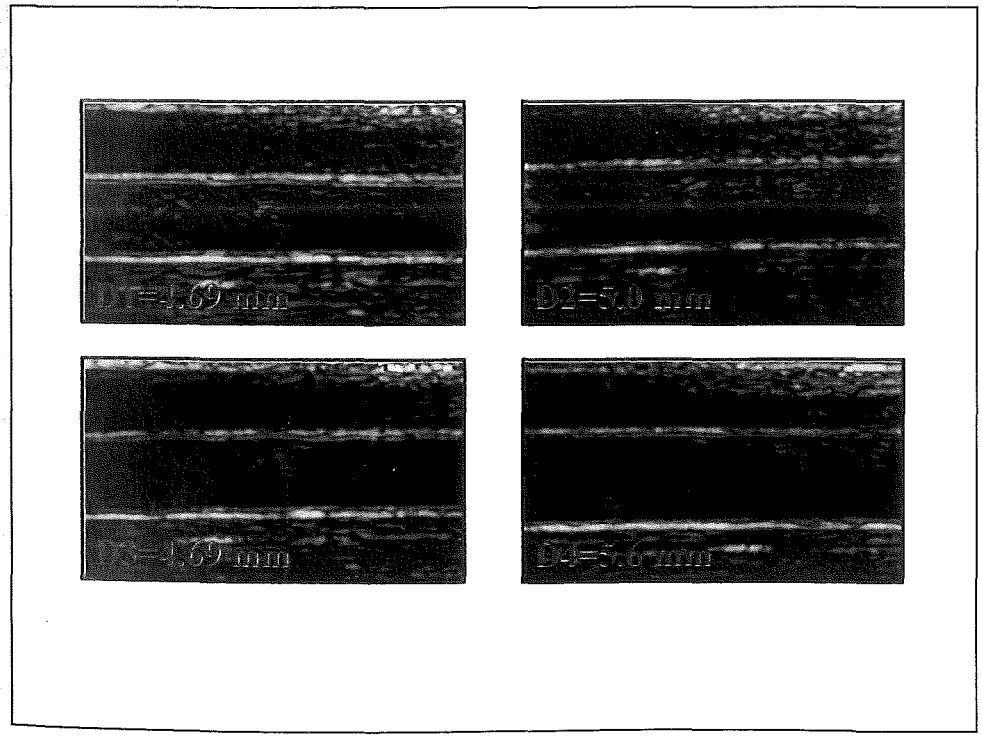
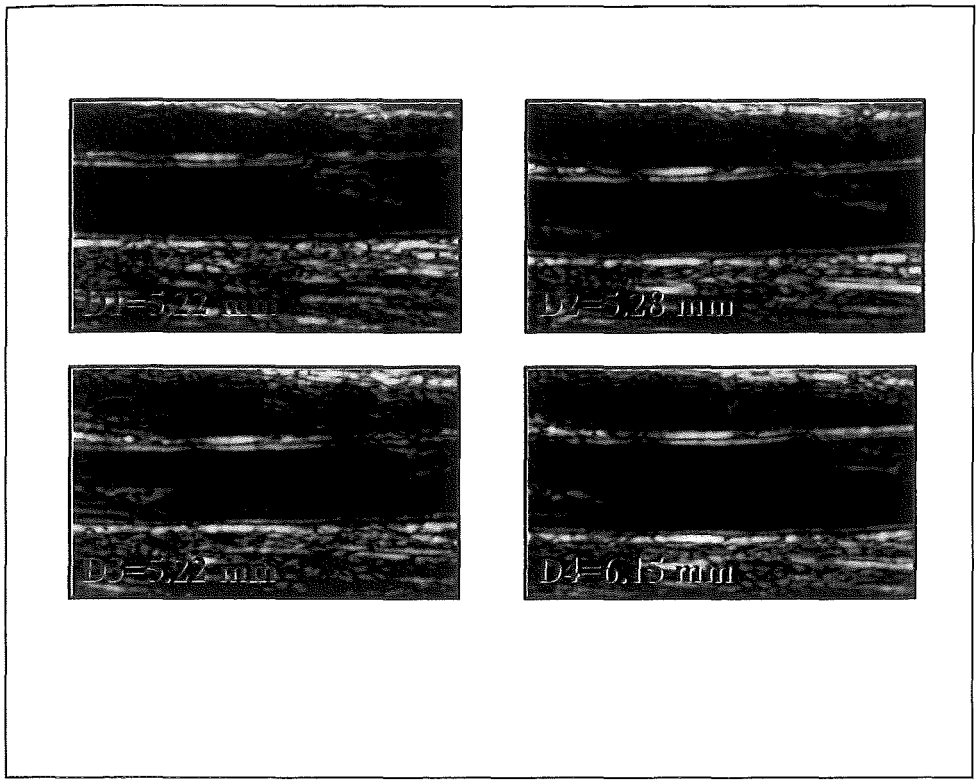


Figure 4. The brachial artery scanned along the longitudinal axis at baseline (D1), approximately 60 seconds after cuff release (D2), at re-baseline (D3), and 4 min after sublingual administration of a 0.5 mg glyceryl trinitrate tablet (D4) in a patient with IHD (FMD = 1.1%, NMD = 17.8%).



Baseline diameter confounds interpretation of FMD. First, if the baseline diameter changes, the resulting percent change in diameter might be affected. For example, a large increase in baseline diameter (from 3.0 to 3.3, = 10% increase) might result in a decrease in FMD that is a result of the change in resting tone, not the effect of the intervention on endothelial function. Baseline diameter size should also be reported together with FMD. Secondly, in the calculation of FMD the baseline diameter is included in the formula, making FMD very dependent of baseline diameter, unlike the absolute change. Consequently, the same change in absolute change will result in different FMDs in 2 patients with different diameters. For any given absolute change in the post-flow stimulus diameter, a larger baseline diameter yields a smaller measure of percent change. Third, smaller arteries appear to dilate relatively more than do larger arteries. The two last factors may result in bias, when comparing vasodilator responses between individuals and groups with different baseline diameters.

Other variables

Exercise tests were carried out on a bicycle ergometer in the clinical routine, according to the American College of Cardiology/American Heart Association guidelines for exercise testing (102). A positive exercise test was defined as the presence of exercise-induced anginal chest pain, accompanied by typical ST-segment deviations at 0.08 seconds after the J point, measuring 1 mm or more in at least 2 leads during or after exercise, or by only ST-segment deviations at 0.08 seconds

after the J point, measuring 1 mm or more in at least 2 leads during or after exercise without symptoms (silent ischemia).

Laboratory analyses

Blod samples for measuring plasma total cholesterol, low-density lipoprotein, high-density lipoprotein, and triglycerides were drawn at admittance, and were performed at the analytical unit of the Department of Biochemistry, Copenhagen University Hospital Hvidovre, applying routine methods. Blood samples for detection of *C. pneumoniae* IgA and IgG antibodies were drawn on the same day that the FMD assessment was carried out.

IgA and IgG antibodies against *C. pneumoniae* were measured using microimmunofluorescence assay based on *C. pneumoniae* antigen from Washington Research Foundation at an outside facility (Neisseria Unit, State Serum Institute, Copenhagen, Denmark) as previously described (108-112). As there is no consensus in the literature concerning which value defines seropositivity, we chose a priori a single cut-point to define seropositivity; seropositivity for *C. pneumoniae* IgA antibodies was defined as IgA titer ≥ 32 and seropositivity for *C. pneumoniae* IgG antibodies was defined as IgG titer ≥ 64, as suggested by several other investigators (23). High sensitive C-reactive protein (hsCRP) was measured using a highly sensitive latex-based immunoassay (Dade Behring, Newark, USA). The assay uses polystyrene particles coated with monoclonal antibodies to C-reactive protein (CRP). These particles agglutinate when mixed with samples containing CRP allowing the CRP concentration to be determined as a function of the intensity of scattered light in a nephelometer. This method has previously been shown to correlate highly with validated enzyme-linked immunosorbent assay (ELISA) and to be equally efficacious in classifying patients into different risk categories (113). In our laboratory setting, the within series coefficient of variation (CV%) was determined to be 2.3% (at a level of 2.1 mg/l) and 2.1% (at a level of 3.8 mg/l) and the between series CV% was 3.0% (at a level of 2.4 mg/l) and 5.2% (at a level of 11.9 mg/l).

Clinical parameters

Clinical characteristics were recorded both from interviews of patients and from their medical records. Blood pressure, and BMI were measured at admittance, using conventional methods as employed in the department. Hypertension was defined as self-reported history of hypertension or use of antihypertensive drugs.

Smokers were defined as current smokers or previous users of tobacco (quit less than 3 months earlier). Non-smokers were defined as subjects who had never used tobacco or who had abstained from smoking for ≥ 3 months before the study.

For the diagnosis of IHD, one or several of the following criteria were required: prior MI or AMI, prior PCI or CABG, or a positive exercise test. AMI was diagnosed when two of the following three criteria were met: (1) typical chest pain persisting for > 30 minutes, (2) dynamic changes of the Q wave and the ST-T segment, compatible with AMI, and (3) elevation of the CK-MB to more than twice the upper limit of normal.

Intervention

We chose a macrolide antibiotic – clarithromycin - in a long-acting formulation in order to improve compliance. It had been demonstrated that 14 days of treatment with clarithromycin is efficient in treating *C. pneumoniae* infection (114). Therefore, we chose a duration of antibiotic treatment of 2 weeks to avoid development of resistance.

Statistical analyses

Reproducibility study

Data were expressed as mean ± SD and median and range (in case of skewed data). Intra-observer variability analyses for FMD were reported in two ways:

- As mean of differences (and range of mean differences) between the FMD measured on the two different days in the same subject by the same observer,
- As a Bland-Altman plot of mean values of two repeated measurements. The accordance between the 2 measurements was examined using LA. LA was noted as the mean of differences ± 2 SD.

All analyses were carried out in Statistical Package for the Social Sciences (SPSS) version 11.5.

EVIR study

Continuous normally distributed variables were analyzed using the independent sample *t*-test for unpaired data. Analysis of normality was performed with the Kolmogorov-Smirnov test. When data were not compatible with a normal distribution, and the number of observations was low, the non-parametric test (Mann-Whitney test) was applied. The Chi-square test (or Fisher's exact test) was used to determine differences in categorical variables between groups. All tests were 2-sided. Statistical significance was established at $p < 0.05$. *C. pneumoniae* antibody titers were analyzed as categorical dichotomous variables with different cut-point values. We performed linear regression analyses to assess the relation between risk factors suspected of influencing endothelial function coded as independent variables, and FMD as the dependent variable. The requirements for the use of the linear regression analysis were controlled as described below. A linear relation between variables was controlled graphically by applying a scatter plot between the explaining variable and the response variable. A normal distribution of the response variable was verified by the Shapiro-Wilks test and graphically by a histogram. A normal distribution of the residuals of the dependent variable was controlled via two diagrams:

- A histogram of the normal curve of the standardized residuals,
- A P-P plot of the cumulated observed distribution of residuals versus the cumulated expected distribution.

Finally, the homogenicity of variance was controlled using a plot of the standardized residuals versus the expected values of the dependent variable. All analyses were carried out in SPSS 11.5.

Prospective, randomized, double-blind, placebo-controlled study

Data were analysed by a statistician blinded to the treatment code. The primary outcome measure – the effects of intervention on FMD and NMD - was assessed by applying the unpaired t-test on an intention-to-treat principle.

The effect of medication on IgA and IgG was investigated by applying a cumulative logit model to the ordinal titer at each visit in turn. Likewise, the effect of medication on hsCRP was investigated by applying a cumulative logit model to the ordinal response variable obtained by categorizing hsCRP into quartiles at each visit in turn. Two-sided p values < 0.05 were considered to indicate statistical significance. All analyses were carried out in Statistical Analysis System (SAS) 8.2, except the unpaired t-test and the analysis of normality for which the SPSS 11.5 was used.

The intervention code was not broken before all analyses had been finished.

The prospective, randomized, double-blind, placebo-controlled study was a small exploratory study and, therefore, a sample-size calculation was considered irrelevant.

Chapter 5: Results

Reproducibility study

We included 19 healthy volunteers and 7 males with chest pain, suspected of AMI. Of the 19 healthy volunteers, 11 were men and 8 women, at age 32 ± 7 years old (mean ± SD). There was no significant difference in age between healthy men and healthy women (32 ± 9 years for men, and 31 ± 4 for women (mean ± SD), $p = 0.76$). Healthy men had a considerably larger baseline diameter of the brachial artery than healthy women (compared data from occasion 1: 4.19 ± 0.44 mm vs. 3.29 ± 0.48 mm, $p = 0.001$), and a considerably smaller FMD than healthy women (compared data from occasion 1: 6.84 ± 1.44% vs. 9.62 ± 3.70%, $p = 0.04$ (mean ± SD)).

Men with chest pain, suspected of AMI were older than healthy men (61 ± 9 years vs. 32 ± 9 (mean ± SD), $p < 0.001$). There was no significant difference in baseline diameter of the brachial artery (4.59 ± 0.51 mm vs. 4.19 ± 0.44 mm, $p = 0.11$) and FMD (6.25 ± 2.07% vs. 6.84 ± 1.44%, $p = 0.49$) between men with chest pain, suspected of AMI, and healthy men, respectively (compared data from occasion 1).

The intra-observer variation of FMD was estimated among 7 patients with chest pain and 19 healthy volunteers. The observer (BM) performed FMD twice with days' intervals (2 - 24 days for the patients with chest pain and 1 - 239 days for the healthy, mean = 8 and 28 days, respectively). The results of intra-observer variation (day-to-day variation) for FMD are demonstrated in Table 2 and Figure 5 and 6. The FMD data and baseline diameter of the brachial artery data, used for quantification of the sources of intra-observer variation, are shown in the Appendix B.

Table 2. Intra-observer variation of FMD

	D1O1 (mm)	D1O2 (mm)	Differences D1O1-D1O2 (mm)	FMD1 %	FMD2 %	Differences FMD1-FMD2 %
7 patients with chest pain	4.59 (3.60-5.20)	4.58 (3.60-5.20)	0.01 (-0.19-0.30)	6.25 (3.85-10.18)	6.13 (3.85- 10.33)	0.12 (-1.67 - 1.41)
19 healthy subject	3.81 (2.73-5.0)	3.84 (2.65-5.10)	-0.03 (-0.5-0.18)	8.01 (4.88-17.86)	8.18 (4.76-17.86)	-0.18 (- 2.81 - 2.31)

Data are presented as mean and range.
D1 = baseline diameter; D1O1 = baseline diameter at 1. occasion; D1O2 = baseline diameter at 2. occasion; FMD = flow-mediated dilation; FMD1= FMD at 1. occasion; FMD2 = FMD at 2. occasion

Figure 5. Intra-observer variation of FMD in patients with chest pain, suspected of AMI. Bland-Altman plot of mean values of 2 repeated measurements of FMD in 7 patients with chest pain, suspected of AMI. The figur shows the difference between FMD, measured at two occasions by the same observer, in relation to the mean value of FMD at occasion 1. and 2. The accordance between the 2 measurements is examined using limits of agreement (LA). LA is noted as the mean of differences ± 2 SD. Limits of agreement lie between 2.22% and -1.98%. The mean difference is 0.12% and is not significantly different from zero. The solid line represents mean values and the pointed lines represent ± 2 SD (101).

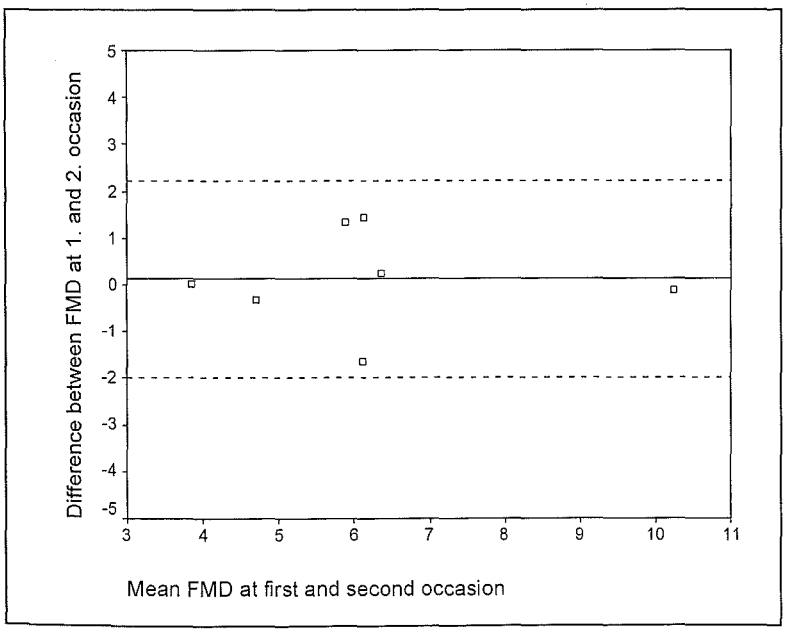
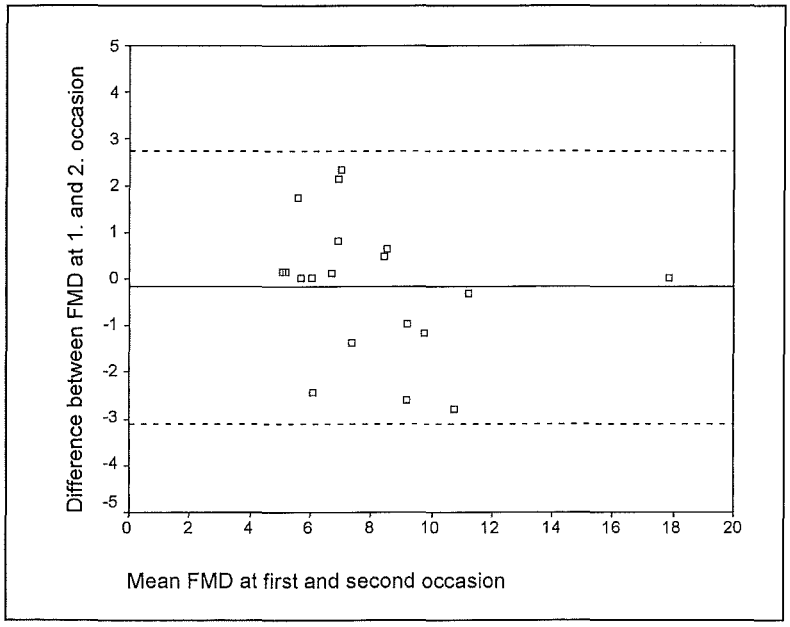


Figure 6. Intra-observer variation of FMD in healthy subjects. Bland-Altman plot of mean values of 2 repeated measurements of FMD in 19 healthy volunteers. The figur shows the difference between FMD, measured at two occasions by the same observer, in relation to the mean value of FMD at occasion 1. and 2. The accordance between the 2 measurements was examined using limits of agreement (LA). LA is noted as the mean of differences ± 2 SD. Limits of agreement lie between –3.11% and +2.75%. The mean difference is –0.18% and is not significantly different from zero. The solid line represents mean values and the pointed lines represent ± 2 SD.



EVIR study

Clinical characteristics

Of the 230 patients fulfilling the inclusion criteria, 5 patients were later excluded because of unsuccessful performance/measurement of FMD. Furthermore, the *C. pneumoniae* antibody results of 2 patients were missing, and these two patients are not included in the analysis, containing *C. pneumoniae* antibody results.

Demographic and clinical characteristics appear in Table 3.

Table 3. Demographic and Clinical Characteristics of Patients

	+ IHD n=137 (61%)	- IHD n=88 (39%)	P value
Age (yrs)	58 ± 12	48 ± 10	< 0.001
Men	107 (78%)	64 (73%)	
Women	30 (22%)	24 (27%)	0.357
Body mass index, kg/m²	27.3 ± 4.1	26.2 ± 3.9	0.051
Systolic BP, mmHg	136 ± 21	132 ± 22	0.112
Diastolic BP, mmHg	83 ± 11	86 ± 10	0.043
Laboratory test			
Cholesterol-total, mmol/l	5.5 ± 1.0	5.6 ± 1.0	0.689
Cholesterol-LDL, mmol/l	3.7 ± 0.9	3.7 ± 0.9	0.938
Triglycerides, mmol/l	1.7± 0.9	1.6 ± 0.8	0.405
Smokers, n (%)	76 (56%)	39 (44%)	0.102
C. pneumoniae antibody status, n (%)			
Patients with IgA titers ≥ 32*	66 (49%)	38 (43%)	0.404
Patients with IgG titers ≥ 64*	83 (61.5%)	50 (57%)	0.488
Clinical history, n (%)			
Hypertension	23 (17%)	18 (21%)	0.487
History of AMI	77 (56%)	0	
History of prior MI	29 (21%)	0	
History of prior PTCA	12 (9%)	0	
History of prior CABG	10 (7%)	0	
Medication at inclusion			
β-blockers	65 (47%)	12 (14%)	< 0.001
Calcium-blockers	45 (33%)	8 (9%)	< 0.001
ACE-inhibitors or Angiotensin II-receptor blockers	23 (17%)	5 (6%)	0.013**
Anti-platelet therapy	124 (90.5%)	26 (29.5%)	< 0.001
Nitrates	20 (15%)	4 (4.5%)	0.025**
Lipid-lowering drugs	36 (26%)	1 (1%)	< 0.001**
Time from debut of chest pain to measurement of FMD, days	23 ± 24	34± 26	0.001

Data are presented as mean values ± SD or number and percentage of patients. Continuous variables were analyzed, using a 2-tailed independent sample t-test. Categorical variables were compared, using the chi-square test (or Fisher's Exact test, indicated as **). Statistical significance was established at $p < 0.05$.
ACE = angiotensin-converting enzyme; AMI = acute myocardial infarction; BP = blood pressure; CABG = coronary artery bypass grafting; *C. pneumoniae* = *Chlamydia pneumoniae;* IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent; FMD = flow-mediated dilation; LDL = low-density lipoprotein; MI = myocardial infarction; PTCA = percutaneous transluminal coronary angioplasty
*= 2 blood tests for *C. pneumoniae* antibodies are missing.

Of the 225 patients finally included, 171 (76%) were men. One hundred and thirty seven (61%) were diagnosed with IHD. Patients with IHD, on average, were 10 years older than patients without IHD ($p < 0.001$), regardless of gender. The sex distribution was similar in patients with and without IHD. Patients without IHD had significantly ($p = 0.043$) higher diastolic blood pressure than patients with IHD. In other respects, there were no significant differences in risk factor levels.

***Chlamydia pneumoniae* antibody and IHD**

Distribution of the serological findings and clinical diagnosis appears from Table 4. One hundred and four patients (47%) and 133 (60%) were seropositive for *C. pneumoniae* IgA and IgG, respectively. Ninety-six patients (43%) were positive for both *C. pneumoniae* IgA and IgG. Among the 104 *C. pneumoniae* IgA seropositive patients, 96 were simultaneously *C. pneumoniae* IgG seropositive. In contrast, only 8 *C. pneumoniae* IgA seropositive patients (approx. 7.7%) were *C. pneumoniae* IgG seronegative in this combination of titers:

IgA = 32 and IgG = 16 (1 patient)
IgA = 32 and IgG = 32 (4 patients)
IgA = 64 and IgG = 0 (1 patient)
IgA = 64 and IgG = 32 (2 patients).

No significant differences were observed between patients with and without IHD in the prevalences of seropositivity for *C. pneumoniae* IgA, IgG and both IgA and IgG antibodies.

There is no consensus in the literature concerning which value defines seropositivity. In acknowledgment of this fact, consecutive titers of *C. pneumoniae* IgA and IgG were used in our study to dichotomize the entire population according to any given cut-off of *C. pneumoniae* IgA and IgG titer for evaluation of associations between titer and IHD (Table 5). We found a weak statistically significant association between IHD and high (≥ 256) *C. pneumoniae* IgA and IgG antibody titers ($p = 0.048$, $p = 0.011$, respectively).

Table 4. Distribution of *C. pneumoniae* IgA, IgG, and both IgA and IgG seropositivity according to presence or absence of IHD

	+IgA (n=104)	P value	+IgG (n=133)	P value	+IgA and +IgG (n=96)	P value
Men						
+IHD (n=107)	53 (50.5%)		66 (63%)		50 (48%)	
- IHD (n=64)	32 (50%)	0.952	40 (62.5%)	0.963	29 (45%)	0.771
Women						
+ IHD (n=30)	13 (43%)		17 (57%)		11 (37%)	
- IHD (n=24)	6 (25%)	0.161	10 (42%)	0.273	6 (25%)	0.359

Data are presented as number and percentage of patients. Variables were compared, using the chi-square test. Statistical significance was established at $p < 0.05$.
+IgA = *C. pneumoniae* IgA antibody titers ≥ 32; +IgG = *C. pneumoniae* IgG antibody titers ≥ 64; IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent.

Table 5. Distribution of *C. pneumoniae* antibody titers, the study population dichotomized into two subgroups (for any given cut-off), and IHD

Titer	+IHD, n (%)	-IHD, n (%)	P value
C. pneumoniae IgA	135	88	
<16	61 (45)	43 (49)	
≥16	74 (55)	45 (51)	0.590
≥32	66 (49)	38 (43)	0.404
≥64	41 (30)	20 (23)	0.211
≥128	26 (19)	10 (11)	0.117
≥256	19 (14)	5 (6)	0.048
C. pneumoniae IgG			
<16	33 (24)	27 (31)	
≥16	102 (76)	61 (69)	0.305
≥32	97 (72)	60 (68)	0.557
≥64	83 (62)	50 (57)	0.488
≥128	64 (47)	36 (41)	0.340
≥256	42 (31)	14 (16)	0.011
≥512	13 (10)	3 (3)	0.110*

Data are presented as number and percentage of patients. Variables were compared, using the chi-square test or Fisher's Exact test (indicated with *). Statistical significance was established at $p < 0.05$.
IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent.

Chlamydia pneumoniae antibody and gender

As earlier, consecutive titers of *C. pneumoniae* IgA and IgG were used to dichotomize the entire population according to any given cut-off of *C. pneumoniae* IgA and IgG titers for evaluation of associations between titer and gender, in patients with and without IHD (Table 6 and 7).
In patients with IHD, there was no difference in prevalence of *C. pneumoniae* IgA or IgG titers between men and women. In contrast, in the group of patients without IHD, the prevalence of *C. pneumoniae* IgA titers ≥ 16 and IgA titers ≥ 32 was significantly higher in men than in women (58% men vs. 33% women, $p = 0.041$ and 50% men vs. 25% women, $p = 0.035$, respectively for IgA ≥ 16 and IgA ≥ 32). This was observed, too, for *C. pneumoniae* IgG titers, including titer ≥ 64 and 128, but the difference was not significant.

No difference was observed in prevalence of seropositivity for *C. pneumoniae* IgA, IgG, and both IgA and IgG between genders in the group of patients with IHD (Table 6). Data for both IgA and IgG seropositivity are not shown. In contrast, there was a significant difference in prevalence of seropositivity for *C. pneumoniae* IgA antibodies in men (50%) and women (25%) without IHD (p =

0.035, as described above). In this same group of patients without IHD, this was also found for *C. pneumoniae* IgG (62.5% for men and 42% for women), but the difference was not significant (p = 0.079, Table 7). The prevalence of seropositivity for both *C. pneumoniae* IgA and IgG was 45% for men and 25% for women without IHD (p = 0.083, not shown in the table).

Tabel 6. Distribution of *C. pneumoniae* antibody titers in patients with IHD, the study population dichotomized into two subgroups (for any given cut-off), and gender

Titer	Men +IHD, n (%)	Women +IHD, n (%)	P value
C. pneumoniae IgA	105	30	
<16	47 (45)	14 (47)	
≥16	58 (55)	16 (53)	0.853
≥32	53 (50.5)	13 (43)	0.490
≥64	33 (31)	8 (27)	0.661
≥128	23 (22)	3 (10)	0.192*
≥256	17 (16)	2 (7)	0.243*
≥512	3 (3)	1 (3)	1.000*
C. pneumoniae IgG			
<16	26 (25)	7 (23)	
≥16	79 (75)	23 (77)	0.872
≥32	74 (70.5)	23 (77)	0.506
≥64	66 (63)	17 (57)	0.539
≥128	51 (49)	13 (43)	0.612
≥256	35 (33)	7 (23)	0.297
≥512	11 (10.5)	2 (7)	0.732*

Data are presented as number and percentage of patients. Variables were compared, using the chi-square test or Fisher's Exact test (indicated with *). Statistical significance was established at $p < 0.05$.
IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent.

Table 7. Distribution of *C. pneumoniae* antibody titers in patients without IHD, the study population dichotomized into two subgroups (for any given cut-off), and gender

Titer	Men -IHD, n (%)	Women -IHD, n (%)	P value
C. pneumoniae IgA	64	24	
<16	27 (42)	16 (53)	
≥16	37 (58)	8 (33)	0.041
≥32	32 (50)	6 (25)	0.035
≥64	18 (28)	2 (8)	0.084*
≥128	9 (14)	1 (4)	0.274*
≥256	5 (8)	0	0.317*
≥512	1 (2)	0	1.000*
C. pneumoniae IgG			
<16	17 (27)	10 (42)	
≥16	47 (73)	14 (58)	0.171
≥32	47 (73)	13 (54)	0.084
≥64	40 (62.5)	10 (42)	0.079
≥128	30 (47)	6 (25)	0.063
≥256	13 (20)	1 (4)	0.100*
≥512	3 (5)	0	0.559*

Data are presented as number and percentage of patients. Variables were compared, using the chi-square test or Fisher's Exact test (indicated with *). Statistical significance was established at $p < 0.05$.
IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent.

Chlamydia pneumoniae antibody and FMD

FMD and serology findings are summarized in Table 8 and Table 9. There were no differences in FMD, in either of the sexes, in patients with and without positive *C. pneumoniae* antibody titers for either IgA or IgG. This also applied to patients with both positive IgA and IgG, and to patients who were either IgA or IgG positive, or both IgA and IgG negative. These findings were observed in patients with and without IHD. There were no differences in FMD in patients with *C. pneumoniae* IgA and IgG antibody titers ≥ 256, and in patients with *C. pneumoniae* IgA and IgG antibody titers < 256 (Table 10). Again, this was also the case, when comparing the group of patients with combined IgA and IgG ≥ 256 versus the group of patients with IgA or IgG ≥ 256, or both IgA and IgG < 256 (Table 11). The findings were observed in patients with and without IHD.

Table 8. Relation between seropositivity for *C. pneumoniae* IgA and IgG antibody titers and FMD, NMD, and baseline diameter, according to gender in patients with and without IHD

	+IgA	-IgA	P value	+IgG	-IgG	P value
+ IHD						
Men						
FMD (%)	4.0 ± 2.7	4.4 ± 2.4	0.535	4.0 ± 2.6	4.5 ± 2.4	0.311
NMD (%)	19.1 ± 5.5	19.7 ± 5.2	0.544	19.2 ± 5.3	19.7 ± 5.6	0.681
D1 (mm)	4.5 ± 0.5	4.5 ± 0.6	0.856	4.5 ± 0.5	4.5 ± 0.6	0.870
Women						
FMD (%)	4.2 ± 2.4	3.9 ± 2.7	0.731	4.0 ± 2.5	4.1 ± 2.8	0.940
NMD (%)	21.7 ± 7.3	16.9 ± 5.6	0.051	19.4 ± 6.8	18.6 ± 6.9	0.748
D1 (mm)	3.6 ± 0.3	3.7 ± 0.5	0.520	3.6 ± 0.3	3.7 ± 0.6	0.580
- IHD						
Men						
FMD (%)	5.4 ± 3.0	5.3 ± 2.1	0.923	5.6 ± 2.8	4.9 ± 2.1	0.244
NMD (%)	19.1 ± 5.3	20.1 ± 5.2	0.461	19.3 ± 5.0	20.2 ± 5.7	0.533
D1 (mm)	4.4 ± 0.6	4.5 ± 0.6	0.690	4.5 ± 0.7	4.4 ± 0.4	0.277
Women						
FMD (%)	7.0 ± 2.3	6.1 ± 3.1	0.518	6.6 ± 2.2	6.1 ± 3.3	0.708
NMD (%)	20.1 ± 9.1	20.2 ± 5.5	0.972	18.6 ± 7.7	21.2 ± 5.3	0.329
D1 (mm)	3.3 ± 0.4	3.5 ± 0.3	0.374	3.5 ± 0.4	3.4 ± 0.2	0.773

Data are presented as mean values ± SD. Continuous variables were analyzed using a 2-tailed independent sample t-test. Statistical significance was established at $p < 0.05$.
D1 indicates baseline diameter of the brachial artery; FMD = flow-mediated dilation; IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent; NMD = nitroglycerin-mediated dilation. +IgA = *C. pneumoniae* IgA antibody titers ≥ 32; +IgG = *C. pneumoniae* IgG antibody titers ≥ 64.

Table 9. Relation between seropositivity for both *C. pneumoniae* IgA and IgG antibody titers and FMD, NMD, and baseline diameter, according to gender in patients with and without IHD

	Both +IgA and +IgG	+IgA or +IgG or both neg	P value
+ IHD			
Men			
FMD (%)	4.0 ± 2.7	4.4 ± 2.4	0.499
NMD (%)	19.1 ± 5.7	19.6 ± 5.1	0.612
D1 (mm)	4.5 ± 0.5	4.5 ± 0.6	0.780
Women			
FMD (%)	4.5 ± 2.5	3.8 ± 2.6	0.433
NMD (%)	20.8 ± 7.3	18.0 ± 6.3	0.291
D1 (mm)	3.6 ± 0.3	3.7 ± 0.5	0.659
- IHD			
Men			
FMD (%)	5.6 ± 2.9	5.1 ± 2.2	0.481
NMD (%)	19.1 ± 5.4	20.1 ± 5.2	0.476
D1 (mm)	4.4 ± 0.6	4.5 ± 0.6	0.814
Women			
FMD (%)	7.0 ± 2.3	6.1 ± 3.1	0.518
NMD (%)	20.1 ± 9.1	20.2 ± 5.5	0.972
D1 (mm)	3.3 ± 0.4	3.5 ± 0.3	0.374

Data are presented as mean values ± SD. Continuous variables were analyzed using a 2-tailed independent sample t-test. Statistical significance was established at $p < 0.05$.
D1 indicates baseline diameter of the brachial artery; FMD = flow-mediated dilation; IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent; NMD = nitroglycerin-mediated dilation. +IgA = *C. pneumoniae* IgA antibody titers ≥ 32; +IgG = *C. pneumoniae* IgG antibody titers ≥ 64.

Table 10. Relation between high (≥ 256) versus low (< 256) *C. pneumoniae* IgA and IgG antibody titers and FMD, NMD, and baseline diameter in patients with and without IHD

	IgA ≥ 256	IgA < 256	P value	IgG ≥ 256	IgG < 256	P value
+ IHD						
FMD (%)	4.1 ± 2.5	4.2 ± 2.5	0.832	3.8 ± 2.8	4.3 ± 2.4	0.210
NMD (%)	18.8 ± 5.2	19.4 ± 5.8	0.815	18.4 ± 5.3	19.7 ± 5.8	0.358
D1 (mm)	4.5 ± 0.5	4.3 ± 0.7	0.235	4.4 ± 0.5	4.3 ± 0.7	0.179
- IHD						
FMD (%)	5.8 ± 2.6	5.6 ± 2.7	0.857	6.2 ± 2.9	5.5 ± 2.6	0.249
NMD (%)	19.8 ± 5.0	19.8 ± 5.6	0.964	20.1 ± 6.0	19.7 ± 5.5	0.600
D1 (mm)	4.5 ± 0.1	4.2 ± 0.7	0.227	4.3 ± 0.6	4.2 ± 0.7	0.281

Data are presented as mean values ± SD. Continuous variables were analyzed using Mann-Whitney test. Statistical significance was established at $p < 0.05$.
D1 indicates baseline diameter of the brachial artery; FMD = flow-mediated dilation; IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent; NMD = nitroglycerin-mediated dilation.

Table 11. Relation between <u>both</u> high *C. pneumoniae* IgA and high IgG antibody titers (≥ 256) versus either low IgA or IgG, or both (< 256) and FMD, NMD, and baseline diameter in patients with and without IHD

	<u>Both</u> IgA≥256 and IgG≥256	IgA<256 or IgG<256 or both<256	P value
+ IHD			
FMD (%)	4.2 ± 2.6	4.2 ± 2.5	0.997
NMD (%)	18.3 ± 5.3	19.4 ± 5.7	0.564
D1 (mm)	4.3 ± 0.7	4.3 ± 0.7	0.116
- IHD			
FMD (%)	6.8 ± 2.7	5.6 ± 2.7	0.400
NMD (%)	21.6 ± 6.1	19.7 ± 5.6	0.525
D1 (mm)	4.6 ± 0.1	4.2 ± 0.7	0.284

Data are presented as mean values ± SD. Continuous variables were analyzed using Mann-Whitney test. Statistical significance was established at $p < 0.05$.
D1 indicates baseline diameter of the brachial artery; FMD = flow-mediated dilation; IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent; NMD = nitroglycerin-mediated dilation.

Because there is no consensus in the literature concerning which value defines seropositivity, we dichotomized the entire population, according to any given cut-off of *C. pneumoniae* IgA and IgG titer, for evaluation of associations between titer and FMD. FMD was similar in two subgroups of patient populations, dichotomized for any given cut-off of *C. pneumoniae* IgA and IgG antibody titers. These findings were observed in patients with and without IHD. The FMD data are presented in the Appendix C (Table 1C, Table 2C and Table 3C).

To evaluate a possible linear association between *C. pneumoniae* IgA and IgG antibody titer level and FMD, we performed 2 models of linear regression analysis. First, *C. pneumoniae* titers were entered into the model as ordinal variables, coded as 0 for absence of titer, and 1, 2, 3, 4, 5, 6, 7, and 8, for titers of 16, 32, 64, 128, 256, 512, 1024, and 2048, respectively. Secondly, *C. pneumoniae* titers were entered into the model as continuous variables, coded as log(2) for the titers: 16, 32, 64, 128, 256, 512, 1024, and 2048, respectively (a titer of 0 were excluded). There was no association between *C. pneumoniae* IgA and IgG antibody titer level and FMD (Figure 7 and Figur 8).

Figure 7.

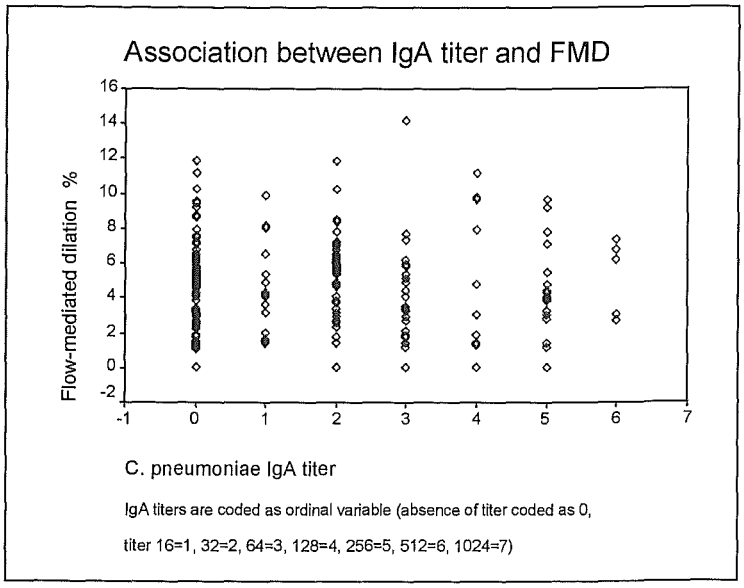
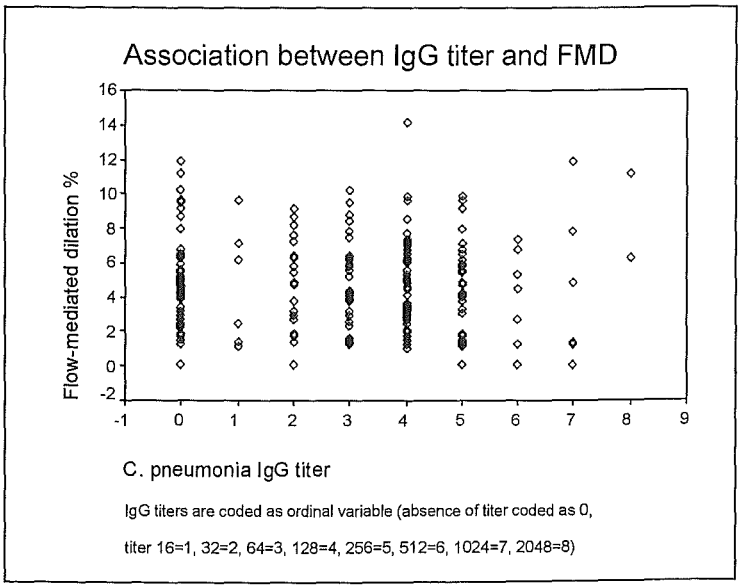


Figure 8.



FMD and IHD

FMD was significantly lower in men with IHD, compared to men without IHD (4.2 ± 2.5% vs. 5.3 ± 2.6%, p = 0.005). In women, essentially similar findings were made (4.0 ± 2.6% vs. 6.3 ± 2.9%, p = 0.003). The relation between IHD-diagnosis and baseline brachial artery diameter, FMD, and nitroglycerin-mediated dilation is summarized in Table 12. Women had a significantly smaller baseline brachial artery diameter than men (3.6 ± 0.4 mm vs. 4.5 ± 0.6 mm, p < 0.001). FMD was lower in patients with IHD than in patients without IHD (4.2 ± 2.5% vs. 5.6 ± 2.7%, p < 0.001). NMD was similar in the two groups.

There was no significant difference in FMD and NMD between men and women with IHD. In men and women without IHD similar findings were made. Women with IHD had significantly larger brachial artery diameter than women without IHD (p = 0.044).

Table 12. FMD, NMD, and baseline diameter, according to gender of patients with and without IHD

	+ IHD	- IHD	P value
All			
FMD (%)	4.2 ± 2.5	5.6 ± 2.7	< 0.001
NMD (%)	19.3 ± 5.7	19.8 ± 5.6	0.538
D1 (mm)	4.3 ± 0.6	4.2 ± 0.7	0.096
Men			
FMD (%)	4.2 ± 2.5	5.3 ± 2.6	0.005
NMD (%)	19.4 ± 5.4	19.6 ± 5.3	0.759
D1 (mm)	4.5 ± 0.6	4.5 ± 0.6	0.492
Women			
FMD (%)	4.0 ± 2.6	6.3 ± 2.9	0.003
NMD (%)	19.0 ± 6.7	20.1 ± 6.4	0.537
D1 (mm)	3.7 ± 0.4	3.4 ± 0.3	0.044

Data are presented as mean values ± SD. Continuous variables were analyzed, using a 2-tailed independent sample t-test. Statistical significance was established at p < 0.05.

D1 indicates baseline diameter of the brachial artery; FMD = flow-mediated dilation; IHD = ischemic heart disease; +IHD = IHD present; -IHD = IHD absent; NMD = nitroglycerin-mediated dilation.

Association between FMD and risk factors of atherosclerosis - results from univariate and multiple linear regression analyses

Variables associated with FMD

In the univariate linear regression analysis, including all patients (Table 13), variables significantly associated with FMD were age ($p < 0.001$), and presence of IHD ($p < 0.001$). To account for the possible explanatory effect on FMD of variables as age, IHD, smoking, cholesterol, *C. pneumoniae* IgA antibodies, and *C. pneumoniae* IgG antibodies, a multiple linear regression analysis was performed (results shown in Table 14 for model included *C. pneumoniae* IgA, and Table 15 for model included IgG respectively). In the multiple linear regression analyses, including all patients (and with gender as an explanatory variable), we found that only age and IHD were statistically significantly associated with FMD ($p < 0.001$ for age, and $p = 0.043$ for IHD, where *C. pneumoniae* IgA was included (Table 14) and $p < 0.001$ for age, and $p = 0.046$ for IHD, where *C. pneumoniae* IgG was included (Table 15)). After gender stratification, the only statistically significant variables associated with FMD in *men* were age ($p = 0.001$). In *women* none of these variables were statistically significantly associated with FMD. Thus, there was no association between FMD and *C. pneumoniae* antibody titers. We did not find an association between FMD and cholesterol.

Table 13. Results of univariate linear regression analysis for FMD as response variable. Explanatory variables were: age, and cholesterol coded as continuous variables, and presence of IHD, smoking, seropositivity for *C. pneumoniae* IgA, seropositivity for *C. pneumoniae* IgG, and gender as dichotomous categorical variables. Results reported for all patients (with gender as categorical variable) and after gender stratification

	All		Men		Women	
Parameter	β	P value	β	P value	β	P value
Age	-0,063	< 0.001	-0.060	< 0.001	-0.077	0.020
Presence of IHD	-1.442	< 0.001	-1.140	0.005	-2.276	0.003
Smoking	-0.418	0.241	-0.377	0.345	-0.364	0.659
Total cholesterol	-0.139	0.420	-0.289	0.152	0.123	0.723
Seropositivity for IgA *C. pneumoniae*	-0.162	0.652	-0.173	0.666	0.077	0.927
Seropositivity for IgG *C. pneumoniae*	-0.110	0.764	-0.032	0.938	-0.178	0.825
Gender	0.436	0.296				

IHD = ischemic heart disease, seropositivity for *C. pneumoniae* IgA = cut-point ≥ 32; seropositivity for *C. pneumoniae* IgG = cut-point ≥ 64.
Baseline: men, non-smokers, absence of IHD, seronegative for *C. pneumoniae*.

Table 14. Results of multiple linear regression analysis for FMD as response variable. Explanatory variables were: age, and cholesterol coded as continuous variables, and presence of IHD, smoking, seropositivity for *C. pneumoniae* IgA, and gender as dichotomous categorical variables. Results reported for all patients (with gender as categorical variable) and after gender stratification. Shown for seropositivity for *C. pneumoniae* IgA

	All		Men		Women	
Parameter	β	P value	β	P value	β	P value
Age	-0.057	<0.001	-0,057	0.001	-0.071	0.075
Presence of IHD	-0.792	0.043	-0.493	0.263	-1.640	0.061
Smoking	-0.664	0.073	-0.677	0.100	-1.176	0.188
Total cholesterol	-0.062	0.718	-0.219	0.266	0.342	0.349
Seropositivity for IgA *C. pneumoniae*	0.229	0.518	0.126	0.747	0.875	0.301
Gender	0.400	0.326				

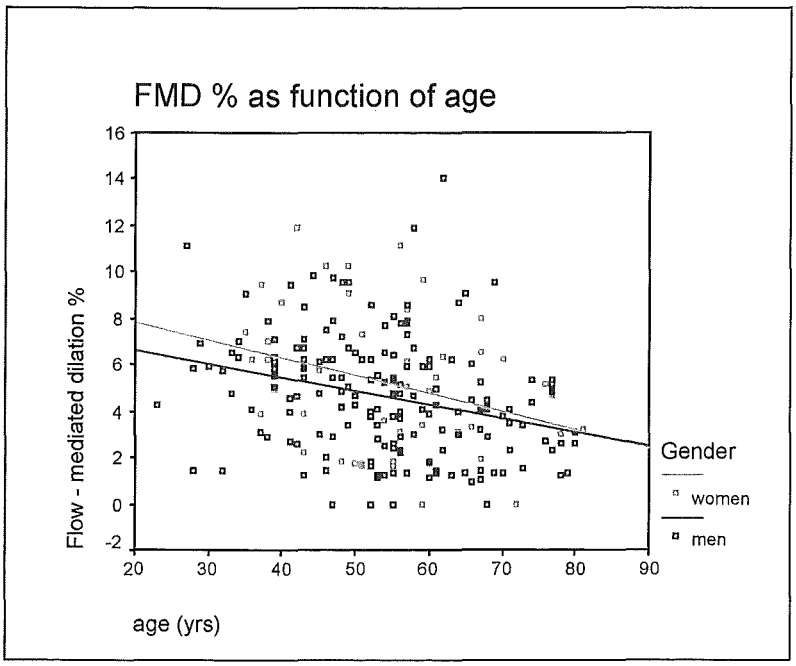
IHD = ischemic heart disease; seropositivity for *C. pneumoniae* IgA = cut-point ≥ 32.
Baseline: men, non-smokers, absence of IHD, seronegative for IgA *C. pneumoniae*.

Table 15. Results of multiple linear regression analysis for FMD as response variable. Explanatory variables were: age, and cholesterol coded as continuous variables, and presence of IHD, smoking, seropositivity for *C. pneumoniae* IgG, and gender as dichotomous categorical variables. Results reported for all patients (with gender as categorical variable) and after gender stratification. Shown for seropositivity for *C. pneumoniae* IgG

	All		Men		Women	
Parameter	β	P value	β	P value	β	P value
Age	-0.059	< 0.001	-0.059	0.001	-0.073	0.076
Presence of IHD	-0.782	0.046	-0.481	0.274	-1.547	0.077
Smoking	-0.650	0.076	-0.663	0.103	-1.114	0.216
Total cholesterol	-0.070	0.680	-0.229	0.246	0.348	0.344
Seropositivity for IgG *C. pneumoniae*	0.323	0.371	0.288	0.477	0.607	0.467
Gender	0.414	0.309				

IHD = ischemic heart disease; seropositivity for *C. pneumoniae* IgG = cut-point ≥ 64.
Baseline: men, non-smokers, absence of IHD, seronegative for IgG *C. pneumoniae*.

Figure 9. Flow-mediated dilation % as function of age with respect to gender



Prospective, randomized, double-blind, placebo-controlled study

Clinical characteristics

Forty patients were randomized. Twenty patients received clarithromycin 500 mg daily, and 20 patients were given placebo. Baseline characteristics are given in Table 16. The groups were well balanced, except for a difference in systolic BP ($p = 0.049$) and BMI ($p = 0.044$). We did not adjust for systolic BP and BMI, because our previous study with 225 patients did not show an association between FMD and systolic BP, and FMD and BMI (115).

CAVE!

Table 16. Baseline Characteristics of Randomized Patients

	Clarithromycin (n=20)	Placebo (n=20)
Age (yrs)	63 ± 11	65 ± 12
Body mass index, kg/m²	28.1 ± 5.0	25.2 ± 3.6
Systolic BP, mmHg	128 ± 16	139 ± 19
Diastolic BP, mmHg	84 ± 13	88 ± 10
Laboratory test		
Cholesterol-total, mmol/l	4.8 ± 0.8	4.9 ± 1.0
Cholesterol-HDL, mmol/l	1.2 ± 0.3	1.3 ± 0.5
Cholesterol-LDL, mmol/l	2.9 ± 0.8	2.8 ± 0.9
Triglycerides, mmol/l	1.4 ± 0.4	1.7 ± 0.7
Smokers, n (%)	10 (50%)	8 (40%)
C. pneumoniae antibody status, n (%)		
Patients with IgA titer ≥ 32	4 (20)	3 (15%)
Patients with IgG titer ≥ 64	8 (40%)	10 (50%)
Clinical history before actual episode of ACS, n (%)		
Prior MI	5 (25%)	5 (25%)
Prior PCI	1 (5%)	3 (15%)
Prior CABG	4 (20%)	4 (20%)
Clinical history in the period from the actual episode of ACS to randomization		
STEMI, n (%)	6 (30%)	4 (20%)
PCI, n (%)	6 (30%)	5 (25%)
CABG, n (%)	5 (25%)	4 (20%)
Time from PCI to randomization (months)	5.9 ± 2.6	4.8 ± 2.7
Time from CABG to randomization (months)	4.1 ± 2.7	3.7 ± 2.0
Time from actual episode of ACS to randomization for all patients (months)	4.4 ± 3.3	5.5 ± 3.7
Medication at inclusion, n (%)		
β-blockers	16 (80%)	13 (100%)
Calcium-blockers	4 (20%)	6 (30%)
ACE inhibitors or Angiotensin II-receptor blockers	6 (30%)	7 (35%)
Anti-platelet therapy	19 (95%)	20 (100%)
Nitrates	4 (20%)	2 (10%)
Lipid-lowering drugs	16 (80%)	14 (70%)

Data are presented as mean values ± SD or number and percentage of patients. ACE indicates angiotensin-converting enzyme; ACS = acute coronary syndrome; BP = blood pressure; CABG = coronary artery bypass graft surgery; HDL = high-density lipoprotein; LDL = low-density lipoprotein; PCI = percutaneous coronary intervention; MI = myocardial infarction; STEMI = ST elevation myocardial infarction.

Follow-up, adverse events, and compliance

Adverse events were reported in four patients in the placebo and four in the clarithromycin group. None were serious, and none caused hospitalization. Opening the trial code before end of trial was not necessary. All patients were compliant with medication and visits. In 39 patients compliance was 100%. One patient returned a single tablet. None of the smokers gave up during the study. No patients were lost during follow-up, and none discontinued the intervention.

Ultrasonografic results (FMD and NMD)

Brachial artery diameter at rest did not differ between the two groups before trial drug administration and did not significantly change after 2 weeks and 6 weeks. Before trial drug administration, FMD did not differ significantly between groups. After two weeks of treatment, the unadjusted FMD was not significantly different in the 2 groups. Six weeks after randomization (4 weeks after completion of intervention with clarithromycin), the FMD was higher in the clarithromycin group compared to the placebo group (6.2% vs. 4.9%, p = 0.070) (Table 17). NMD was not affected significantly by clarithromycin intervention.

Table 17. Ultrasonografic findings at baseline, and 2 weeks and 6 weeks after randomization to 2 weeks clarithromycin 500 mg daily or placebo

	Clarithromycin (n=20)	Placebo (n=20)	P value
FMD, %			
Baseline	5.0 ± 1.8	4.9 ± 2.3	
2 Weeks	5.5 ± 2.0	5.1 ± 2.3	0.557
6 Weeks	6.2 ± 2.4	4.9 ± 2.2	0.070
D1, mm			
Baseline	4.7 ± 0.6	4.5 ± 0.5	
2 Weeks	4.7 ± 0.6	4.5 ± 0.5	0.218
6 Weeks	4.7 ± 0.5	4.5 ± 0.5	0.225
NMD, %			
Baseline	18.1 ± 5.8	17.1 ± 5.1	
2 Weeks	18.7 ± 5.7	17.7 ± 5.2	0.556
6 Weeks	18.6 ± 6.0	17.4 ± 5.2	0.507

Data are presented as mean values ± SD. Variables were analyzed using the unpaired t-test. Statistical significance was established at $p < 0.05$.
D1 indicates baseline diameter of the brachial artery; FMD = flow-mediated dilation; NMD = nitroglycerin-mediated dilation

FMD data of individual patients, from the clarithromycin and the placebo group, divided according to *C. Pneumoniae* IgA and IgG, at entry and after 6 weeks, are shown in Figure 10, 11, 12 and 13.

Figure 10.

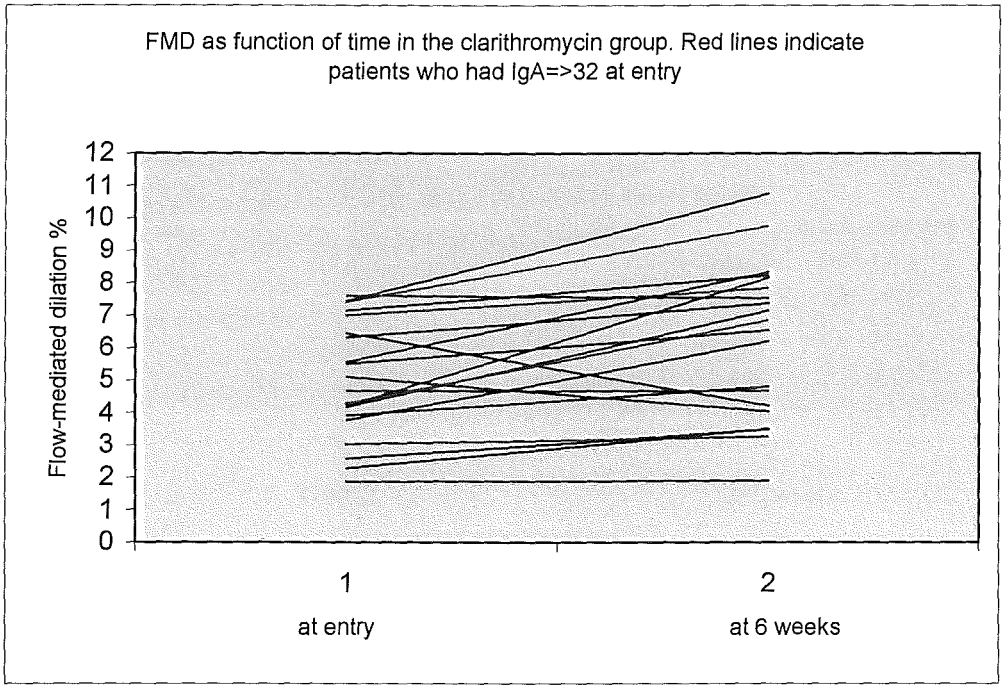


Figure 11.

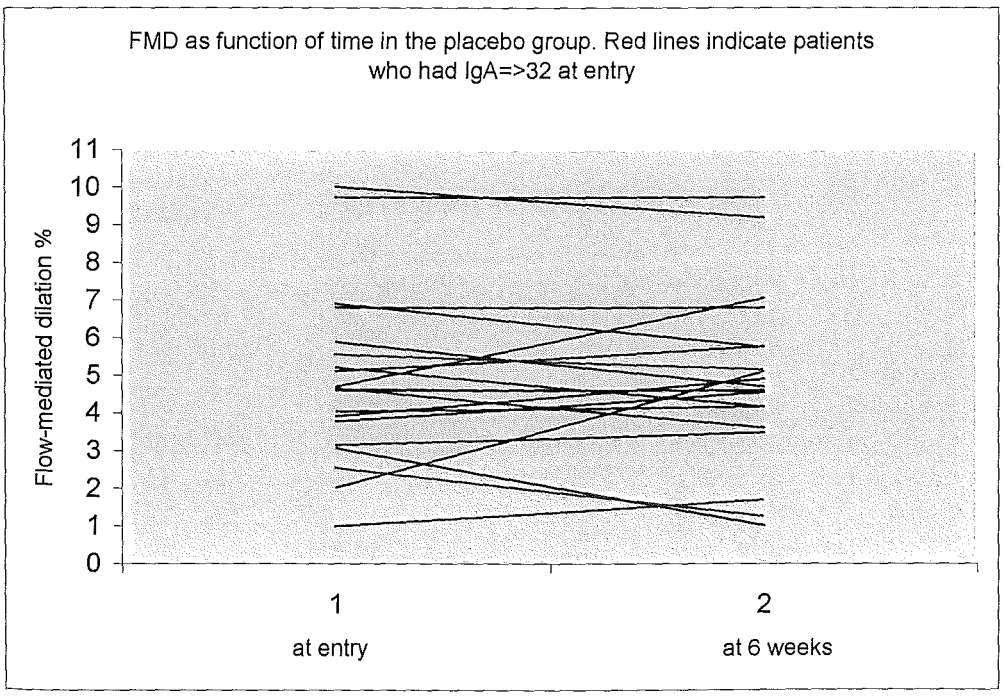


Figure 12.

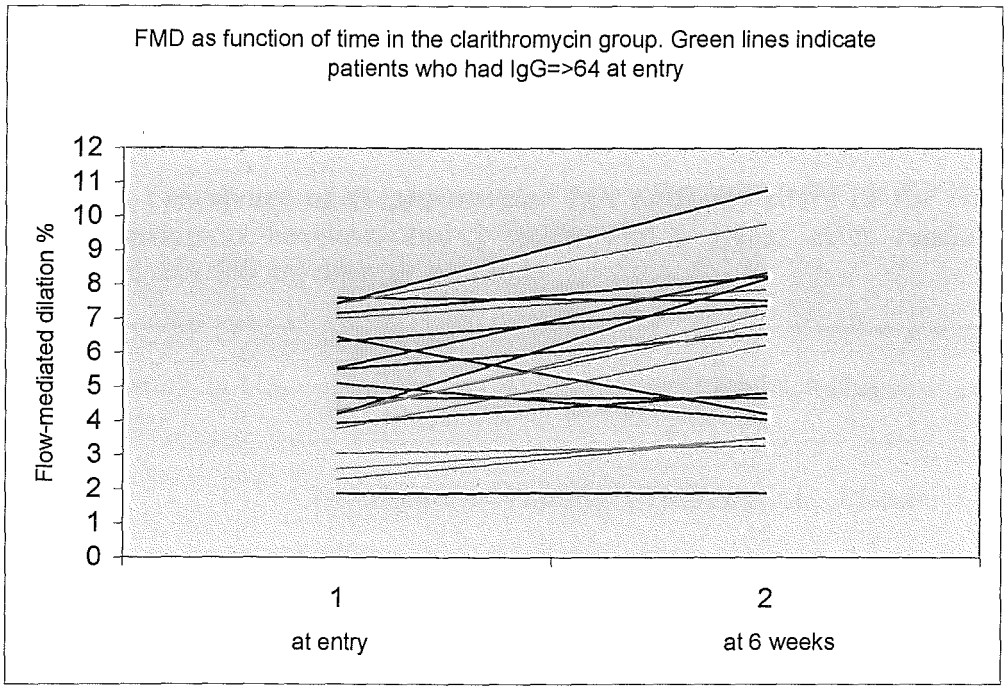
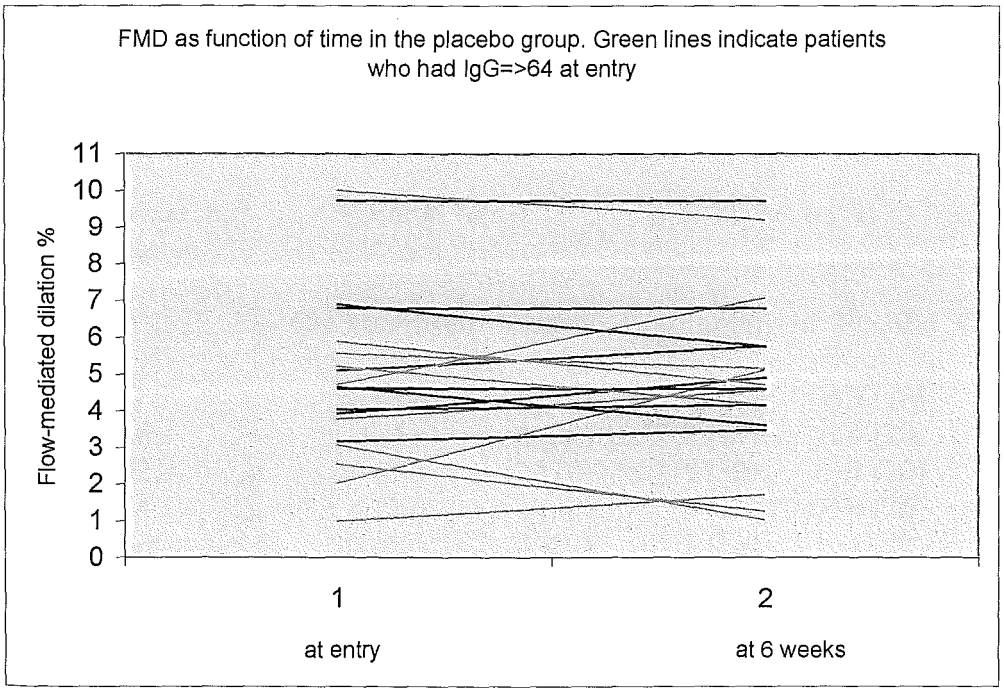


Figure 13.



Serological results

C. pneumoniae antibodies

The prevalence of *C. pneumoniae* antibody titer did not differ between the two groups at randomization. The *C. pneumoniae* antibody titers neither changed in the clarithromycin nor in the placebo group during the study (Table 18 and Table 19).

Table 18. Prevalence of *C. pneumoniae* IgA antibody titers in the clarithromycin and the placebo group at baseline, and 2 weeks and 6 weeks after randomization to 2 weeks clarithromycin 500 mg daily or placebo

C. pneumoniae IgA antibody titer	Entry, n (%)		2 weeks, n (%)		6 weeks, n (%)	
	Clarithromycin	Placebo	Clarithromycin	Placebo	Clarithromycin	Placebo
0	13 (65)	12 (60)	14 (70)	12 (60)	15 (75)	12 (60)
16	3 (15)	5 (25)	1 (5)	4 (20)	0	4 (20)
32	2 (10)	2 (10)	2 (10)	3 (15)	2 (10)	3 (15)
64	1 (5)	1 (5)	0	1 (5)	0	0
128	1 (5)	0	2 (10)	0	2 (10)	1 (5)
Missing	0	0	1(5)	0	1 (5)	0

Table 19. Prevalence of *C. pneumoniae* IgG antibody titers in the clarithromycin and the placebo group at baseline, and 2 weeks and 6 weeks after randomization to 2 weeks clarithromycin 500 mg daily or placebo

C. pneumoniae IgG antibody titer	Entry, n (%)		2 weeks, n (%)		6 weeks, n (%)	
	Clarithromycin	Placebo	Clarithromycin	Placebo	Clarithromycin	Placebo
0	5 (25)	4 (20)	4 (20)	3 (15)	5 (25)	3 (15)
16	1 (5)	4 (20)	2 (10)	4 (20)	2 (10)	6 (30)
32	6 (30)	2 (10)	6 (30)	4 (20)	7 (35)	2 (10)
64	4 (20)	6 (30)	3 (15)	4 (20)	3 (15)	2 (10)
128	3 (15)	3 (15)	3 (15)	4 (20)	1 (5)	6 (30)
256	0	0	0	0	0	1 (5)
512	1 (5)	1 (5)	1 (5)	1 (5)	0	0
1024	0	0	0	0	1 (5)	0
Missing	0	0	1(5)	0	1 (5)	0

The effect of clarithromycin intervention was independent of *C. pneumoniae* IgA and IgG seropositivity, defined as IgA titer ≥ 32, or IgG ≥ 64 and did not affect *C. pneumoniae* antibody titers. The effect of clarithromycin on *C. pneumoniae* IgA and IgG seropositivity was investigated by applying a cumulative logit model to the ordinal titer at each visit in turn yielding an odds-ratio which (if different from one) indicates that it is more likely to be in the higher (lower) category of seropositivity (Table 20 and 21). The cumulative logit model acts as a series of logistic regressions (or plain odds-ratios for each possible categorization of IgA (IgG) into two groups e.g.
0 and 16, 32, 64, 128, 256, 512, 1024
0, 16 and 32, 64, 128, 256, 512, 1024
0, 16, 32 and 64, 128, 256, 512, 1024
0, 16, 32, 64 and 128, 256, 512, 1024
0, 16, 32, 64, 128 and 256, 512, 1024
0, 16, 32, 64, 128, 256 and 512, 1024
0, 16, 32, 64, 128, 256, 512 and 1024

The resulting Odds-ratio is an average (weighted) of these odds ratios.

Table 20. For IgA modeling the odds ratio that clarithomycin enhances seropositivity

Visit	Odds-ratio based on cumulative logit	P value	Ordinary odds-ratio based on IgA < 32 vs IgA ≥ 32	P value (Fisher)
1	0.94	0.89	1.42	0.68 (1.00)
2	0.67	0.65	1.1	0.94 (1.00)
3	0.42	0.34	1.1	0.94 (1.00)

The ordinary odds-ratio was achieved by dichotomizing *C. pneumoniae* IgG antibodies into two groups:

- Positive: IgA ≥ 32
- Negative: IgA < 32

While it appears that there is a discrepancy between the two methods (nothing significant) it should be noted that more than 75% of the patients have IgA < 32 making inferences questionable.

Table 21. For IgG modeling the odds ratio that clarithomycin enhances seropositivity

Visit	Odds-ratio based on cumulative logit	P value	Ordinary odds-ratio based on IgG < 64 vs IgG ≥ 64	P value (Fisher)
1	0.91	0.87	0.67	0.53 (0.75)
2	0.88	0.63	0.66	0.60 (0.75)
3	0.58	0.46	0.41	0.22 (0.32)

The ordinary odds ratio was achieved by dichotomizing *C. pneumoniae* IgG antibodies into two groups:

- Positive: IgG ≥ 64
- Negative: IgG < 64

Neither the cumulative logit model, nor the ordinary odds-ratio proved any effect of clarithromycin on *C. pneumoniae* IgG antibodies, although it would appear that seropositivity decreases with time (odds-ratio tended to be lower), when using clarithomycin. However, there is no evidence to this.

High sensitive C-reactive protein

Clarithromycin intervention had no effect on hsCRP level (Table 22).

Table 22. The distribution of hsCRP expressed as mean, median and range (min and max) in the clarithromycin and the placebo group at baseline, and 2 weeks and 6 weeks after randomization to 2 weeks administration of clarithromycin 500 mg daily or placebo

HsCRP (mg/l)	Entry		2 weeks		6 weeks	
	Clarithromycin	Placebo	Clarithromycin	Placebo	Clarithromycin	Placebo
Mean±SD	3.82 ± 5.84	3.40 ± 3.27	4.01 ± 4.92	3.82 ± 4.01	4.87 ± 6.30	2.42 ± 1.83
Median	1.62	2.29	1.78	2.96	2.61	1.90
Range	0.84-23.80	0.84-13.50	0.84-15.50	0.84-15.80	0.84-24.40	0.84-6.85

Chapter 6: Discussion

Reproducibility study

Like other investigators (42), we found that healthy women had a smaller baseline diameter of the brachial artery than healthy men. Furthermore, these women had a significantly higher FMD than the men. The latter finding was probably explained by the inverse relationship between FMD and vessel size at baseline (at rest), and by the fact that smaller arteries appeared to dilate relatively more than larger arteries. A favorable effect of estrogenes on FMD was plausible (49). We found that the values of FMD in healthy individuals were comparable to those obtained in other studies (47;67;116).

A significant difference in FMD between men with chest pain and healthy men was not observed, in spite of the fact that the healthy men were significantly younger than the men with chest pain, and had a smaller, although not significantly so, vessel diameter. Celermajer et al (41) found a decline in FMD with advancing age. FMD was preserved in men until 40 years of age. In our pilot study, FMD was similar in younger men (32 years of age) and in older men (61 years), suggesting an earlier decline. Other investigators found that FMD was similar in men 35 and 55 years old (42). However, it should be noted that the sample size, especially of the group of patients with chest pain, was very small. Furthermore, our pilot study was not designed to investigate neither gender differences in FMD nor age–related changes in FMD. The aim of the study was to evaluate reproducibility in determining FMD. The intra-observer variability was within accepted limits. In our hands, the method seemed to be of similar validity as reported by other groups (104;116;117).

EVIR study

The present study demonstrated a weak association between high IgA and IgG *C. pneumoniae* antibody titers and IHD, but failed to demonstrate any association between FMD and *C. pneumoniae* antibodies. It is commonly accepted that FMD reflects the presence of risk factors for IHD. However, in the present cohort, we were unable to demonstrate this possible association between elevated *C. pneumoniae* antibodies and endothelial function.

The exercise test was applied in order to discriminate between presence and absence of IHD. The exercise test can detect significant obstructive coronary disease. It cannot be ruled out that many patients, who were classified as not having IHD, did in fact have IHD. All presented to the hospital with chest pain, and many, presumably, had some degree of CAD, although it was non-obstructive, because the exercise tests were negative. Hence, this segment of patients might have acted as confounders. The exercise test was applied for the study, because the method is ubiquitous, inexpensive, non-invasive and safe. It is also easy to do and, generally, does not cause much discomfort to the patients. Finally, CAG was not done at our hospital.

Furthermore, patients defined as not-having IHD are not without risk factors for IHD. Some had been diagnosed with hypertension (21%) and hypercholesterolemia (1%). In a small part of the patients, it was more complex to disprove the diagnosis of IHD. The diagnostic program was supplemented with myocardial scintigraphy or CAG. This explains, why the patients continued the anti-ischemic treatment in spite of a normal exercise test, until the additional tests disproved the diagnosis of IHD. However, it cannot be excluded that these patient, to a lesser extent, had IHD, although it was not diagnosed.

Many patients without IHD were given medications related to the treatment of IHD or hypertension. It was normal procedure in our department that all patients with ACS were started on medication with anti-platelet therapy and β-blockers or calcium-blockers. Some of the patients without IHD already had the treatment discontinued during the hospitalization, others continued up to the moment, when the IHD-diagnosis was disproved by a normal exercise test. Finally, a few patients continued this medication, until the diagnosis of IHD was disproved by myocardial scintigraphy or CAG. The patients who continued the treatment, until the diagnosis of IHD was disproved, and in whom the FMD-measurement was done either in close relation to the exercise test, or before the diagnosis of IHD was disproved by other diagnostic tests, contributed to the increased consumption of anti-ischemic medication in the non-IHD group.

Since patients with ACS, requiring acute transportation to a centre with invasive facilities for the purpose of PCI or CABG, could not do an exercise test, they were, consequently, not invited to participate in the study. Such patients could have had the most severe endothelial dysfunction and the highest antibody titers and, thus, might have made it easier to detect a correlation.

All patients received standard treatment during the acute phase, and anti-ischemic treatment was administered to the patients with IHD. If FMD was measured shortly after the acute event and the initiation of medication, the patients could not be expected to have reached a steady-state condition with respect to medication, but, as demonstrated earlier (118), this should not have influenced the results of the measurement of FMD.

Like Celermejer et al., we found that FMD was inversely correlated to age (41), with a greater vasodilator response in young persons. As patients without IHD were younger than patients with IHD (48 years vs. 58 years), the difference in FMD between these groups might partially be caused by the difference in age and might, therefore, in reality be less pronounced.

We reported the FMD response at approximately 60 seconds after cuff deflation in all patients, as did the majority of investigators (89-91). It has been postulated that the peak diameter change occured approximately at that point of time after cuff deflation in healthy subjects (47), but in CAD patients, the change occurred approximately 88 seconds after cuff deflation (119). Only 35% of the CAD patients had a maximum response at 60 seconds (119). When we calculated FMD, based on the maximal response in our study population, it did not change our conclusions.

The microimmunofluorescence test is considered the golden standard for laboratory diagnosis of *C. pneumoniae* infection (112). As described in chapter 4, the interpretation of serological data is very difficult, because there is no agreement, which titer cut-point best distinguishes between a positive and negative result. For IgA titers, a level ≥ 32 has been widely accepted. This level of IgA titers appears to reflect chronic infection the best (6). For IgG, the situation is more complex. Many studies used an IgG level ≥ 64 (8;23;120;121), although several others used a level ≥ 128 (6;122;123). We defined seropositivity, a priori, as IgA ≥ 32 and IgG ≥ 64.

It is important to note, that measurements of *C. pneumoniae* antibodies in sera from our patient population, were not performed with the aim of demonstrating acute or chronic infection with *C. pneumoniae*. Since Saikku et al., in 1988, demonstrated an association between an increased level of antibodies and IHD, a high level of IgG and /or IgA *C. pneumoniae* antibodies has been regarded as an indicator of an increased risk of cardiovascular events (6). As a matter of fact, it should be added that proper evaluation of a *C. pneumoniae* antibody titer, for demonstration of acute or chronic infection, necessitates measurements of IgM antibodies and consecutive measurements of IgG antibodies, in order to detect a possible increase in titers.

In this study, we observed that the rate of *C. pneumoniae* IgA seropositivity, defined as titer ≥ 32, was 49% in patients with IHD, and 43% in patients without IHD. The prevalence of *C. pneumoniae* IgG seropositivity, defined as titer ≥ 64, was 61.5% and 57% in patients with IHD and without IHD, respectively. As described in chapter one, a meta-analysis of prospective studies (9) found no

association between *C. pneumoniae* seroprevalence and cardiovascular events. The prevalence of *C. pneumoniae* IgG seropositivity in our study corresponds well with that reported by other investigators (124). We observed a gender difference in prevalence of seropositivity of *C. pneumoniae* IgA, IgG and both IgA and IgG, simultaneously. We observed this difference only in the group of patients without IHD. The prevalence of positive *C. pneumoniae* IgA, IgG and both IgA and IgG antibodies was higher in men than in women without IHD, suggesting that men are more susceptible to *C. pneumoniae* infections than women. Our results are in accordance with those of other investigators, who, in larger study populations than our, found that the *C. pneumoniae* titer was associated with male gender (124-126). The difference in prevalence of seropositivity of *C. pneumoniae* antibodies in men and women without IHD must be taken with some reservation, as the number of men included differed markedly from the number of women. The aim of our studies was neither to examine the prevalence of the *C. pneumoniae* titers, nor potential gender differences in the prevalence of titer. Furthermore, the size of our patient population would have been far too limited for such a study aim. It is also imperative to notice, that in the analysis of both IgA and IgG, it was the IgA effect that dominated, because of the fact that patients, who were IgA positive, were almost always also IgG positive.

Some investigators have suggested that no single infectious agent *per se* plays a role in atherogenesis. Rather, a combination of several viral and bacterial agents plays a decisive role in atherogenesis (2). Zhu et al. (3) discovered a linear relation between CAD and IgG antibodies to an increasing number of pathogens, including cytomegalovirus, *C. pneumoniae*, hepatitis A virus, and herpes simplex virus type 1 and 2.

Sharma et al. suggested that elevated serum markers of infection and/or inflammation were associated with functional abnormalities of the vasculature in healthy subjects (127). However, we did not find this to be the case in our population. A new study, using invasively assessed endothelial function of the coronary arteries, demonstrated that pathogen burden, rather than the type of organism involved, was more likely to be associated with endothelial dysfunction and presence and severity of coronary artery disease (128).

The relationship between serum antibody titers and the presence of *C. pneumoniae* in atheromatous vessel walls is still uncertain. There are conflicting reports on the correlation between the organisms detected in atheroma and serum antibody titers (14). It is conceivable that an assay of circulating *C. pneumoniae* DNA may be a more precise marker of the infection. We did not measure *C. pneumoniae* DNA in our study.

There was no correlation between *C. pneumoniae* IgA and IgG on one side and FMD on the other. On the other hand, we cannot rule out that *C. pneumoniae* infection may play a role in plaque instability and rupture, although this is not supported by other studies, such as ACADEMIC, either (129).

One of the limitations of our study was that we did not keep continuous records of all screened patients with regard to the number of patients with ACS, who were transferred to invasive centres and were not referred to exercise tests, patients who fulfilled the inclusion criteria, but did not want to participate, the number of patients willing to participate, but who later turned out to fulfil the exclusion criteria, and, finally, the number of patients performing an insufficient exercise test. The reported number of approximately 250 excluded patients was calculated on the basis of the documentation of the number of exercise tests performed in the inclusion period from november 1998 to march 2000, with the exception of patients included in the EVIR study.

The strength of the EVIR study is based on the fact, that a relatively substantial number of patients of both sexes were included. Further, this study population with chest pain reflected the daily clinical reality. We prospectively defined *C. pneumoniae* seropositivity without the potential for later manipulation.

Prospective, randomized, double-blind, placebo-controlled study

We showed that 2 weeks of oral clarithromycin intervention, in patients stabilized after an episode of ACS, resulted in greater increase in FMD in patients from the clarithromycin group, compared to placebo, and that the potentially beneficial effect could be observed 4 weeks after conclusion of the intervention. However, the difference in FMD between the two groups, at the end of the study, was close to, but did not reach statistical significance ($p = 0.07$) at the 5% level.

When we decided to include patients with ACS into the study, it was based on the expectation that infection with *C. pneumoniae* might result in endothelial damage and impaired FMD. Assuming that *C. pneumoniae* plays a role in the development of ACS and has a negative influence on endothelial function, an intervention with antibiotics directed against *C. pneumoniae* in patients during the course of ACS might stabilize the endothelium and, potentially, result in a larger degree of improvement in FMD, than would presumably be observed in patients without acute dynamics in their IHD. When the protocol was written, only 2 studies (23;130) had examined and demonstrated the effect of antibiotics in patients with IHD. Gupta et al. included 60 patients with *C. pneumoniae* IgG $\geq$ 64 in their study more than 6 months after MI (23), and Gurfinkel et al. 202 included patients with ACS (UAP and non-Q AMI), independent of baseline *C. pneumoniae* titer (130). They demonstrated the beneficial effect of antibiotics in their respective patient populations.

On average, there was an interval of 4.9 ± 3.5 months between the episode of ACS and the randomization. At least one month separated the invasive procedures (PCI, CABG or CAG) from the ensuing randomization. We believe that neither the pharmacological treatment, which was unchanged for no less than 4 weeks, nor the invasive procedures could have had any impact on the ultrasonographies with respect to the measurement of endothelial function. The acute event *per se* could not have had any influence on the endothelial function, either, as a minimum of 4 weeks passed from the acute episode of ACS, until the ultrasonographic measurement.

The patients, who participated in the prospective interventional study, were older than the patients in the observational study (64 vs. 58 years). Both patient populations inhabited the same Greater Copenhagen suburb area, and the inclusion and exclusion criteria did not diverge in any important aspects, apart from the fact that women were barred from entering the interventional study. One explanation of the difference in age might be, that 25 patients declined to participate in the interventional study, which was more comprehensive (3 visits), i.e. patients of younger age, and possibly still in the work force, were more inclined to choose not to enter the study, while the older patients, who were likely retired, were more willing to accept. Unfortunately, information on the occupational status of the patients was not gathered. Another explanation of the difference in age was the small number of patients in the interventional study (40 patients), compared to the Evir-study (137 patients with IHD).

In the interventional study, there were fewer patients, who were *C. pneumoniae* IgA seropositive (17.5%), than in the EVIR study (46%). No difference in the distribution of *C. pneumoniae* IgG was observed (59% vs. 65%, Evir patients vs. patients in the interventional study). Additionally, 43% of the entire Evir population was both IgA and IgG seropositive, compared to only 17.5% of the population in the interventional study. Patients, who were *C. pneumoniae* IgA seropositive, were nearly always IgG seropositive, too. Hence, there seemed to be an IgA-effect manifesting itself. The small number of patients in the interventional study may offer a partial explanation of the difference in distribution of *C. pneumoniae* IgA between the 2 populations.

We included the patients irrespective of their *C. pneumoniae* titer, which was not measured until after conclusion of the study. In our trial, the *C. pneumoniae* titer did not change during treatment, and the effect on FMD was independent of IgA and IgG *C. pneumoniae* seropositivity at

randomization. In one study, the effects of antibiotics with respect to reduction of cardiovascular events appeared to occur regardless of *C. pneumoniae* serology (130). In another study, a 36% reduction in readmission with UAP was observed in patients receiving antibiotics for 12 weeks, and this effect was independent of *C. pneumoniae* seropositivity at study entry (131). This suggested that IgA and IgG titers might be inadequate markers for guiding treatment. We, too, demonstrated, as a number of other investigators, that antibiotics did not affect *C. pneumoniae* titer (129;130). The effect might therefore be unrelated to the antimicrobial effect. Secondly, this effect of clarithromycin on the endothelium did not seem to be mediated through a reduction of the inflammatory response, expressed as hsCRP. It is important to notice, that determination of hsCRP in patients with cardiovascular disease, is not done with the intention of disclosing infection. Since Ridker et al. in 1998 (132) demonstrated an association between an increased hsCRP and IHD, a high level of hsCRP has been regarded as a marker of an increased risk of cardiovascular episodes. However, it should be stressed that the operational interval of hsCRP is lower than in tests used for detecting an infection in a normal clinical setting. The value of a normal hsCRP for patients with IHD has not been determined. It is unknown whether a hsCRP level of 2 mg/L *per se* confers more or less of a risk than a level of 3 mg/L.

We have not studied other inflammatory markers. It is well established that macrolides, in addition to their antichlamydial action, also have anti-inflammatory properties (27;28), and that the immunologic response is enhanced during atherogenesis. Phagocytes and their products (oxidants, enzymes and cytokines) are key effectors of inflammation. Macrolides have a direct effect on neutrophile function and the production of cytokines involved in the inflammation cascade. Macrolides directly inhibit in-vitro oxidant production by phagocytes, and modify chemotaxis (both inhibition and an increase). Macrolides induced alteration of cytokine production involved in the inflammation cascade (decreased production of IL-1, IL-6, IL-8, TNF and increased production of IL-10) (28). We believe that a treatment period of two weeks is generally too short to modulate the immune response adequately. However, it is well known that flow-mediated brachial artery vasoactivity responds rapidly to a given intervention. Thus, the effect of statines on FMD was detected after only 2 weeks of treatment (90). Therefore, we expected that an intervention over 2 weeks would have sufficed in influencing FMD. A longer duration of antibiotic therapy is probably important to evaluate bactericidal and anti-inflammatory effects. The aim of the study was to evaluate the effect of macrolides on endothelial function, rather than deciding whether it was an anti-inflammatory, bactericidal or some other effect exerting its influence. Nevertheless, keeping in mind the differences in FMD four weeks after termination of antibiotic treatment in our study, gives rise to the speculation that, albeit of only two weeks duration, the treatment with clarithromycin might have sufficed in exerting an immunomodulating effect, via its antichlamydial action, ultimately resulting in the observed improvement in FMD. This intriguing topic warrants a whole new series of studies for proper elucidation.

Nevertheless, it cannot be excluded that clarithromycin, in fact, apart from *C. pneumoniae,* treated other microorganisms, that might have been of importance in the atherogenic process. We did not measure other titers. Like many investigators in cardiovascular research, we did not apply *C. pneumoniae* IgA and IgG to demonstrate acute or chronic infection, but, as mentioned before, rather as a marker that had been found to have a slight association with IHD (7). Prospective studies, however, failed to confirm this association with certainty. Only once did we measure *C. pneumoniae* titers, but not IgM, which is the antibody subclass to measure in acute infections by definition. Some investigators have used the presence of IgG antibodies of ≥ 512 as an indication of acute infection, but the positive correlation between a single high serum antibody titer and the detection of the *C. pneumoniae* bacteria in atheromatous tissues has been poor (11;13;14;16).

We decided to choose an intervention period of 2 weeks for the following reasons: First, to avoid development of resistance. Secondly, it was demonstrated, that 14 days of treatment with clarithromycin was efficient in treating *C. pneumoniae* infection (114). Thirdly, a short 3-days' course of therapy with the anti-chlamydial macrolide antibiotic azithromycin improved the outcome of patients with post-MI (23). Finally, FMD responded rapidly to a given intervention (90).
The patients were in a stable phase of ACS, when we interventioned. It is possible that an earlier intervention in the acute phase might have had a greater effect. It cannot be excluded that the stage of atherosclerotic vascular disease is likely to play a role in modulating the response of the individual to a given intervention. In a rabbit model of atherosclerosis (nasopharyngeal inoculation with *C. pneumoniae*), early treatment with azithromycin prevented atherosclerotic plaque progression, while delayed treatment proved ineffective (133).
FMD is a surrogate marker for cardiovascular risk. FMD is associated with coronary risk factors, is useful in assessing response to treatment, and may be of prognostic value (70;91). Therefore, an effect on FMD may be considered beneficial. Only few studies examined the effect of antibiotics on FMD. One randomized trial included 40 patients with stable documented coronary heart disease and *C. pneumoniae* IgG antibody titers of 16 or greater (134). After 5 weeks of azithromycin treatment, the FMD improved from 2.66% to 4.78%, independent of the presence of low (< 32) or high (≥ 32) *C. pneumoniae* antibody titers. There was no change in CRP. Another study, that investigated FMD of the radial artery in 40 males with peripheral arterial occlusive disease and *C. pneumoniae* IgG antibody titers of 128 or greater (135), demonstrated a beneficial effect of roxithromycin on FMD after 1 month. However the effect was not significant ($p = 0.07$) and disappeared after 6 months. A third study of the effect of antibiotics on FMD showed no changes in FMD, neither after 2 weeks, nor 3 months of azithromycin treatment, in 58 patients with stable and documented CAD (136). We demonstrated only a weak effect of clarithromycin on FMD in stable patients after ACS, as did Wiesli et al. (123;135) in patients with peripheral arterial occlusive disease. In contrast to that particular study, we observed an increase in FMD four weeks after the termination of intervention. However, we decided a´ priori to compare FMD between the groups in a very conservative way by applying a robust simple unpaired t-test.
One has to keep in mind that the various measures, that may improve FMD, cannot automatically be transferred to the clinical setting. FMD can be used as a surrogate marker for cardiovascular risk. Our study, however, cannot be used for changing the present recommendations for a possible use of antibiotics for patients stabilized after ACS. Large intervention trials with macrolides detected either small effects or none at all (129;131;137-140). Only a few small clinical intervention trials in humans with atherosclerosis showed that treatment with macrolides had a protective effect on coronary events in *C. pneumoniae* seropositive patients (23;24). Our results should be interpreted with caution and, ideally, confirmed in a larger trial. The study has some limitations:
The number of patients in the prospective, randomized, double-blind, placebo-controlled study was small. Although our sample size was relatively small, it was comparable to a number of published studies, addressing the effects of their interventions on FMD (123;134;141-144).
The study was a small exploratory study. To minimize variation, we included only men, because FMD in women varies with hormonal status (49). Consequently, the results are applicable only to men.
The duration of intervention in our study was 2 weeks. This was shorter than in other trials (129;138;140). However, the regimen, used in our study and in other antibiotic trials, was not chosen in order to evaluate the effectiveness of eradication of *C. pneumoniae* in patients with IHD.

Conclusions and future perspectives

In this thesis a weak association has been demonstrated between high IgA and IgG *C. pneumoniae* antibody titers and IHD. We failed to demonstrate an association between FMD and *C. pneumoniae* antibody titers. We did not find a relationship between IgA and IgG *C. pneumoniae* antibody titers and FMD, irrespective of presence or absence of IHD. Infection with *C. pneumoniae* (as evidenced by the occurrence of *C. pneumoniae* antibodies) did not result in endothelial damage and impaired FMD. This, however; does not exclude that *C. pneumoniae* may play a role in plaque instability and rupture. In the prospective, randomized, double-blind, placebo-controlled study, 2 weeks of oral clarithromycin intervention, in patients stabilized after an episode of ACS, resulted in greater increase in FMD in patients from the clarithromycin group, compared to placebo. Futhermore, the possible beneficial effect could be observed 4 weeks after conclusion of the intervention. However, the difference in FMD between the two groups, at the end of the study, was close to, but did not reach statistical significance ($p = 0.07$) at the 5% level. Whether this effect of clarithromycin, on the long-term prognosis in IHD patients, can be observed, will be clarified, when the results of the CLARICOR study are at hand.

Future perspectives could involve measurements of FMD for the evaluation of new farmacologic agents and, possibly, other types of intervention to reduce cardiovascular disease. In favour of this perspective speaks the fact, that FMD has prognostic value and that a strong correlation exists between improved FMD and reduced cardiovascular risk. It may be possible, that only in genetically susceptible people does *C. pneumoniae* accelerate atherogenesis. Thus, the effect of antibiotics may be diluted, if a proper selection or targeting of susceptible individuals is not performed. A future scenario may imply that patients, during investigation for IHD, are subjected to a genetic/immunologic test, determining which patients would benefit the most from a given antibiotic and/or immunomodulating therapy, directed against *C. pneumoniae* and derived effects thereof. Presently, we are already screening patients who have symptoms and risk factors for IHD. There is increasing focus on prophylactic measures in healthy people and on primary intervention. FMD can be used as a relatively inexpensive tool to screen for cardiovascular risk. But we still lack data on the prognostic value of FMD in the large general populations, especially in the segments with very low-risk of cardiovascular disease. No data are available evaluating sensitivity and predictive values for the general healthy population. Furthermore, a normal range for FMD has not been established. Only data concerning FMD in selected high-risk populations exist.

Identifying healthy individuals without any other known risk factors for IHD, who might potentially benefit from intervention, appears to be obvious. However, the use of FMD for screening the general health of the population has been limited by the lack of large prospective trials evaluating FMD as a screening tool in the general population. And by the lack of trials demonstrating, that improving FMD, in a given population, via a specific type of intervention decreases the cardiovascular risk of the same population. This latter thesis has not been tested directly. We only have evidence that many of the interventions reduce cardiovascular risk, and that these interventions improve FMD. A further limitation is that FMD is easily affected by environmental factors, i.e. mental stress, postprandial lipaemia, circadian rytm, short-term exercise and a number of other factors. There is a need for longitudinal studies elucidating how FMD changes or does not change with longer intervals of time. Studies of healthy individuals, focusing on long-term follow-up of prevalence of cardiovascular events and the prognostic value of FMD, should be carried out. A longitudinal prospective analysis of our data will, presumably, contribute to the determination of the potential prognostic value of FMD. We are currently doing this analysis.

At the present time application of FMD is limited to research. Clinical application of FMD requires the solving of several technical and non-technical issues.

Danish summary

Der er en stigende evidens for, at infektion og inflammation er involveret i patogenesen for aterosklerose og myokardieinfarkt. Infektion med bakterien *Chlamydia pneumoniae* (*C. pneumoniae*) synes at være impliceret i patogenesen for iskæmisk hjertesygdom (IHS). *C. pneumoniae* antistoffer er vist at have en svag association til IHS og til kendte risikofaktorer for aterosklerose. *C. pneumoniae* er fundet i aterosklerotiske plaques fra koronar- og karotis-karrene hos mennesker. *C. pneumoniae* fremskynder udvikling af aterosklerose i dyreeksperimentelle modeller. Intervention med makrolidantibiotika hos *C. pneumoniae* inficerede forsøgsdyr forsinker og hæmmer udviklingen af aterosklerotiske læsioner. Hypotesen, at en infektion med *C. pneumoniae* kunne være en mulig ætiologisk og/eller patogenetisk faktor for aterosklerose og IHS, er blevet underbygget af små interventionsstudier, som har vist en reduktion af nye hjertetilfælde blandt patienter med IHS, som blev behandlet med makrolidantibiotika.

Endotelfunktionen spiller en meget vigtig rolle i aterogenesen. Bevaret, velfungerende endotel har en række vigtige funktioner, herunder regulering af vaskulær tonus via produktion af vasoaktive substanser, blandt andet nitrogenoxid. En gentagen og kontinuerlig påvirkning af endotelet med skadelige faktorer resulterer i tab af disse vigtige funktioner og dermed i et dysfungerende endotel (endotelial dysfunktion). Endotelfunktionen kan måles non-invasivt ved hjælp af ultralyd af arteria brachialis. Det er vist, at endotelfunktionen i arteria brachialis korrelerer godt med endotelfunktionen i koronarkarrene og er associeret med kendte risikofaktorer for IHS. Ultralydsmetoden til bestemmelse af endotelfunktionen i arteria brachialis udnytter endotels vasodilatoriske respons på en øget blodgennemstrømning, flow-medieret dilatation (FMD). Endoteldysfunktion forekommer meget tidligt i aterogenesen, og før man kan se makroskopiske anatomiske forandringer. Kendte risikofaktorer for IHS som alder, hyperkolesterolæmi, diabetes mellitus, og rygning, er associeret til endoteldysfunktion og nedsat FMD. Det er overordentligt veldokumentet, at FMD forbedres, når de klassiske kardiovaskulære risikofaktorer reduceres. FMD er korreleret til prognosen og kan forudsige kardiovaskulære hændelser. Som følge heraf kan FMD måske fungere som en proxy for koronar aterosklerose og IHS.

Denne afhandlings formål var at undersøge, om tilstedeværelsen af C. *pneumoniae* IgA and IgG antistoffer kunne være associeret til nedsat FMD, og om intervention med makrolidantibiotika (clarithromycin) hos patienter med IHS kunne bedre FMD.

Vi har udført 2 studier. Første studie er en observationel tværsnitsundersøgelse af 225 patienter, henvist med brystsmerter og mistanke om akut koronar syndrom (AKS). Vi har opdelt populationen i 2 grupper: patienter med IHS og patienter, som har gennemført en normal og sufficient arbejdstest og aldrig tidligere har fået påvist IHS (uden IHS). FMD, samt forekomsten af positive *C. pneumoniae* IgA and IgG antistoffer i de to grupper, blev sammenlignet. Middelværdien af FMD hos patienter med IHS versus uden IHS var 4.2% vs. 5.6%. Students t-test for uparrede data viste, at FMD i de to grupper var signifikant forskellige ($p < 0.001$). Seropositivitet for *C. pneumoniae* IgA antistoffer blev defineret som en IgA titer ≥ 32. Seropositivitet for *C. pneumoniae* IgG antistoffer blev defineret som en IgG titer ≥ 64. Forekomsten af positive *C. pneumoniae* IgA og IgG (hver for sig) antistoffer i de to grupper af patienter (med IHS og uden IHS) blev sammenlignet. Chi-square test viste, at der ingen forskel var i forekomsten af seropositivitet for *C. pneumoniae* IgA og IgG mellem patienter med IHS versus uden IHS ($p = 0.404$ for IgA and $p = 0.488$ for IgG). FMD hos patienter med positive *C. pneumoniae* IgA and IgG antistoffer blev sammenlignet med FMD hos patienter med negative *C. pneumoniae* IgA and IgG antistoffer. Der var ingen forskel i FMD blandt

C. pneumoniae seropositive versus seronegative. Det var gældende både for *C. pneumoniae* IgA og for IgG antistoffer. Disse fund blev observeret hos mænd og kvinder med og uden IHS.
Vi har vist, at patienter med IHS havde dårligere FMD end patienter uden IHS. Alder og forekomsten af IHS var signifikant associeret med FMD ved en multipel lineær regressionsanalyse, hvor andre risikofaktorer for aterosklerose, som rygning, kolesterol, køn og *C. pneumoniae* IgA and IgG antistoffer, indgik. Nye risikofaktorer for IHS, som C. *pneumoniae* IgA og IgG seropositivitet, er ikke associeret med FMD eller IHS. Kun patienter med meget høje titre af *C. pneumoniae* IgA og IgG antistoffer ($\geq$ 256) havde hyppigere IHS diagnosen end patienter med titre < 256. Antallet af patienter med så høje titre er dog meget lille, hvorfor resultatet bør tages med forbehold. Specielt var der ikke signifikant forskel på FMD mellem patienter med *C. pneumoniae* IgA og IgG antistoftitre $\geq$ 256 og patienter med *C. pneumoniae* IgA og IgG antistoftitre < 256. Fra den første undersøgelse konkluderes, at tilstedeværelsen af positive *C. pneumoniae* antistoffer ikke er associeret med nedsat FMD. Vi fandt heller ikke association mellem *C. pneumoniae* titer-niveau og FMD. Der er ikke korrelation mellem *C. pneumoniae* IgA og IgG på den ene side og FMD på den anden. Der var ingen forskel i forekomsten af seropositivitet for *C. pneumoniae* IgA og IgG mellem patienter med IHS versus uden IHS. En mulig svag association mellem høje *C. pneumoniae* antistoffer (titer $\geq$ 256) og IHS, som vi fandt, kan ikke bevises at være medieret via en eventuel skadelig effekt af infektion med *C. pneumoniae* på endotelet. På den anden side kan vi ikke udelukke, at infektion med *C. pneumoniae* kunne spille en rolle for plaqueinstabilitet og ruptur.
Det andet studie var en randomiseret dobbeltblindet intervention med makrolidantibiotika (clarithromycin) versus placebo hos 40 patienter med AKS. Efter stabilisering blev patienterne behandlet med 500 mg clarithromycin eller placebo 1 gang daglig i 2 uger. FMD, samt *C. pneumoniae* IgA og IgG titre, blev målt 3 gange: før interventionen og henholdsvis 2 og 6 uger efter interventionsstart. Før administrationen af projektmedicin var FMD ikke signifikant forskellig mellem grupperne. Efter to ugers behandling var der ikke signifikant forskel på FMD i de 2 grupper. Seks uger efter randomiseringen (4 uger efter afslutningen på interventionen med clarithromycin) var FMD højere i clarithromycin-gruppen end i placebo-gruppen (6.2% vs. 4.9%, p = 0.07). Den potentielt gavnlige effekt kunne observeres 4 uger efter afslutningen af interventionen. Effekten af clarithromycin-intervention var uafhængig af *C. pneumoniae* IgA og IgG seropositivitet ved start af interventionen. *C. pneumoniae* IgA- og IgG- antistof-titrene ændredes hverken i clarithromycin- eller i placebo-gruppen i løbet af studiet.
Resultaterne fra det andet studie antyder, at FMD er reversibel efter intervention med makrolidantibiotika. Effekten er ikke afhængig af tilstedeværelsen af positive *C. pneumoniae* IgA- og IgG- titre. Den langsigtede effekt af makrolidantibiotika på FMD er dog ikke klarlagt. Hvorvidt disse resultater eventuelt kunne få kliniske konsekvenser, må afgøres ved at intervenere med makrolidantibiotika hos et større antal af patienter og gennem en langvarig opfølgning af FMD og med fokus på udviklingen af kliniske aterosklerotiske manifestationer. Enviderere er der behov for yderligere at dokumentere, hvorvidt FMD kunne have en prognostisk betydning. Disse studier er undervejs.

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Appendix A

Table 1 A. Overview of randomized (placebo-controlled) antibiotic trials for secondary prevention of coronary and peripheral artery disease

Study (reference)	Publication Year	Study population, no of patients randomized	Antibiotic (duration of treatment)	Endpoint (follow up)
Gupta et al. (23)	1997	60 males, more than 6 months post-MI, *C. pneumoniae* IgG titer ≥64	A (3 days)	Reduction in MI, UAP and death at 18 months, decrease in *C. pneumoniae* titer in the azithromycin group
ACADEMIC (129)	2000	302 patients with stable CAD, *C. pneumoniae* IgG titer ≥ 16	A (3 months)	No change in cardiac event rate (death, cardiac arrest, MI, stroke, UAP and unplanned coronary revascularization) at 24 months, but reduction of CRP and IL-6 at 6 months, no effect on *C. pneumoniae titer*
WIZARD (139)	2003	7747 patients with stable CAD, with previous MI, *C. pneumoniae* IgG titer ≥ 16	A (3 months)	No change in death, reinfarction, coronary revascularization procedures and hospitalization for angina at 14 months follow-up
AZACS (145)	2003	1439 patients with ACS, regardless *of C. pneumoniae* titer	A (5 days)	No change in MI, recurrent myocardial ischaemia requiring revascularisation, and death at 6 months
ACES (138)	2000/2004	4016 patients with angiographic evidence of at least one coronary artery stenosis > 50 %, or a documented MI, regardless of *C. pneumoniae* titer	A (1 year)	No change in cardiac event rate (death from CAD, MI, revascularization procedures, and hospitalization for UAP) at 3.9 years follow-up
CLARIFY (140)	2002	148 patients with non Q-AMI or UAP. No information about serological status with regard to *C. pneumoniae*	C (3 months)	41 % reduction in the RR of cardiovascular event (combination of death, myocardial infarction, unstable angina, ischemic stroke and critical limb ischemia) by clarithromycin during a follow-up averaging 555 days (absolute risk reduction=14.9%)

ROXIS (24)	1999	202 UAP and non Q-AMI, enrolling of patients independent of *baseline C. pneumoniae* titer	R (1month)	Reduction in combined end-points of MI, recurrent ischemia and death at 1month and 3 months but not at 6 months; reduction in CRP levels, no effect on *C. pneumoniae* titer
ISAR-3 (146)	2001	1010 patients with elective or urgent coronary stenting, regardless of *C. pneumoniae* titer	R (4 weeks)	No change in restenosis rate, revascularization procedures and death at 1 years; Only in patients with high *C. pneumoniae* titers (IgG ≥ 512), roxithromycin reduced the rate of restenosis
Leowattana et al. (147)	2001	84 patients with ACS, regardless of *C. pneumoniae* titer	R (1month)	No significant difference of cardiac events (cardiovascular death, unplanned coronary revascularization and recurrent angina/MI) at 90 days follow-up
Wiesli et al. (123)	2002	40 males with PAD and *C. pneumoniae* IgG titer ≥ 128	R (1month)	Reduction in revascularization, improvement in walking distance at 2.7 years follow-up. Regression of soft carotid plaque size (no change in CRP, IL-6 and TNF-α) after 6 months
ANTIBIO (148)	2003	872 patients with AMI, regardless of *C. pneumoniae* titer	R (6 weeks)	No change in death, MI, resuscitation, stroke, revascularization or angina leading to hospitalization at 12-month
Jan Kaehler et al. (149)	2003	327 consecutive patients undergoing coronary angioplasty, regardless of *C. pneumoniae* titer	R (6 weeks)	No change in cardiovascular mortality, non-fatal MI, and symptomatic restenosis at 1 year follow-up
PROVE-IT (137)	2002/2004	4162 patients with ACS, no information about serological status with regard to *C. pneumoniae* titer	G in combination with statins (2 year), 2x2 factorial design	No change in cardiac event rate (death, MI, UAP, and revascularization) at 2 years follow-up

STAMINA (131)	2002	325 patients with ACS, regardless of *C. pneumoniae* titer	1 of 3 treatment regimens for 1 week: 1. Combination of A and M and O 2. Combination of X and M and O 3. Placebo	36% reduction in readmission with UAP in patients receiving antibiotics at 12 weeks, this effect persisted during 1 year of follow-up. The effect was independent of *C. pneumoniae* seropositivity at study entry. No difference in mortality. Reduction in CRP levels in patients receiving amoxicillin (only in patients with UAP, but not in MI)
CLARICOR (150)	2001	4600 patients with stable CAD, regardless of *C. pneumoniae* titer	C (14 days)	Ongoing, follow-up 2 years Endpoint: cardiovascular events Expected in 2004
CROAATS (151)		340 patients with documented MI and 2 seropositive serum samples (*C. pneumoniae* IgG titer ≥ 20), obtained with an interval of 2 months	A (3 treatment cycli for 3 days, starting on days 1, 10, and 20)	Ongoing, follow-up 18 months Endpoint: death from CAD, MI, revascularization procedures, and hospitalization for UAP Expected in 2004
MARBLE (152)		1240 patients with known CAD on waiting list for elective CABG, randomized while waiting for surgery, no information about serological status with regard to *C. pneumoniae*	A (3 months) in addition to concomitant antianginal medication	Ongoing Endpoint: cardiovascular events Expected in 2003 ?

A = azithromycin; C = clarithromycin; G = gatifloxacin; M = metronidazole; O = omeprazole; R = roxithromycin; X = amoxicillin;
ACS = acute coronary syndrome; AMI = acute myocardial infarction; CABG = coronary artery bypass graft surgery; CAD = coronary artery disease; *C. pneumoniae* = *Chlamydia pneumoniae*; CRP = C- reactive protein; IgG = immunoglobulin G; IL-6 = interleukin-6; MI = myocardial infarction; PAD = peripheral arterial disease; RR = relative risk; TNF-α = tumor necrotizing factor-α; UAP = unstable angina pectoris.

Table 2 A. Overview of population based case-control trials and interventional trials without intervention in the control group (retrospective)

Study (reference)	Publication Year	Study population, no of patients randomized	Antibiotic (duration of treatment; time from exposure to index/event date)	Clinical effect
Meier et al. (153)	1999	3 315 AMI without clinical risk factors prior to the MI, and 13 139 control subjects	Exposure to tetracyclines, quinolones, macrolides, sulfonamides, penicillins, and cephalosporines within 3 years prior to the date of MI	Cases (first-time AMI-patients) were less likely to have used tetracyclines (OR 0.70, 95%CI 0.55-0.90) and quinolones (OR 0.45, 95%CI 0.21-0.95). No difference in use of the other types of antibiotics
Torgano et al. (154)	1999	84 patients with chronic IHD, regardless of *C. pneumoniae* titer and *Helicobacter pylori* titer	Clarithromycin (14 days) in patients with *C. pneumoniae* positive titers, indicating past or chronic infection (IgG ≥ 64 or IgA ≥ 32); combination of omeprazole (30 days), tinidazole (7 days) and clarithromycin (14 days) in patients with *Helicobacter pylori* positive titers	After 6 months treated patients experienced a significant decrease in fibrinogen, CRP and *C. pneumoniae* and *Helicobacter pylori* - antibody titers
Jackson et al. (155)	2000	1 796 patients with AMI with a history of cardiovascular risk factors, and 4 882 control subjects	Use of erythromycin, tetracycline and doxycycline during the previous 5 years before the first MI	No difference in use of the three antibiotics among AMI patients and controls
Herings et al. (156)	2000	628 patients without a history of cardiovascular risk factors and 1615 control subjects	Effect of high-dosage fluoroquinolones on the incidence of a first time MI during follow-up of 4.5 years	Protective effect of the use of high-dosage fluoroquinolones on the incidence of a first time MI (OR 0.34, 95%CI 0.12-0.93)

Østergaard et al. (157)	2001	Healthy 634 users of macrolides and 3 827 users of penicillins	Using of macrolides vs. penicillins	Reduction in hospitalization for cardiovascular disease in the first 3 months among users of macrolides (RR 0.48, 95%CI 0.27-0.88) compared to users of penicillins. No difference after 3 months
Pilote et al. (158)	2002	26 195 AMI patients with age ≥ 65 years	Group using antichlamydial antibiotics, group using sulfa-derivative antibiotics, to which *C. pneumoniae* is not sensitive, and group using neither of the above classes of antibiotics (2 exposure models (1) during the first 3 months after AMI and (2) during the 6 months before AMI).	Exposure to antichlamydial antibiotics during the 3 months after AMI was associated with a small survival benefit (2-years mortality rate), whereas exposure during the 6 months before AMI did not affect survival
Brassard P et al. (159)	2003	1 047 elderly patients (age ≥ 65 years) with hypertension and MI and 5 235 control subjects	Antibiotics with antichlamydial activity (makrolides, quinolones, tetracyclines) vs. antibiotics withhout antichlamydial activity (cephalosporins, penicillins) 14 years before MI	No association between antibiotic use and MI, but a trend toward greater protection (risk of acute MI) of antibiotics with antichlamydial activity (OR 0.68, 95%CI 0.46-1.00)

AMI = acute myocardial infarction; CI = confidence intervals; *C. pneumoniae* = *Chlamydia pneumoniae*; CRP = C- reactive protein; IgA = immunoglobulin A; IgG = immunoglobulin G; MI = myocardial infarction; OR = odds ratio; RR= relative risk.

Table 3 A. Overview of randomized placebo-controlled antibiotic trials for improvement of FMD, specific markers of endothelial activation, and general markers of inflammation

Study (reference)	Publication Year	No of patients randomized, study population	Antibiotic (duration of treatment)	Endpoint-endothelial function
Semaan et al. (160)	2000	40 patients with CAD and *C. pneumoniae* IgG titer ≥16	A (3 months)	Prolonged treatment with azithromycin did not significantly affect the plasma levels of soluble VCAM-1, ICAM-1, and E-selectin over a period of 6 months
Parchure et al. (134)	2002	40 men with stable and documented CAD (> 50% lumen diameter reduction of at least one coronary artery) and *C. pneumoniae* IgG titer ≥16	A (5 weeks)	Improvement of FMD, independent from the presence of low (<32) or high (≥32) *C. pneumoniae* antibody titers, reduction in E-selectin and von Willebrand factor, but no change in CRP at 5 weeks
Kuvin et al. (136)	2003	58 men and women with stable and documented CAD (> 50% lumen diameter reduction of at least one coronary artery); enrolling of patients independent of baseline *C. pneumoniae* titer	A (2 treatment protocols: 14 days and 3 months)	No effect on FMD
Hillis et al. (161)	2004	141 patients with ACS, regardless of *C. pneumoniae* titer	A (5 days)	Significant reduction in ICAM-1 levels, but no effect on VCAM-1, IL-6, or CRP after follow-up of 3 months. No effect on *C. pneumoniae* IgA or IgG titers

Sinisalo et al. (162)	1998	34 non-smoking men, with mild hypertension or moderate hypercholesterolaemia and a previous CABG, regardless of *C. pneumoniae* titer	D (4 months)	No effect on basal nitric oxide production, *C. pneumoniae* antibodies or coronary heart disease risk factors after follow-up 6 months
Wiesli et al. (135)	2002	40 males with PAD and and *C. pneumoniae* IgG titer ≥ 128	R (1 month)	Some (not significant, p=0.07) improvement of FMD of radial artery in 11 roxithromycin-treated patients after 1 month, the effect disappeared after 6 months

A = azithromycin; D = Doxycycline; R = roxithromycin; ACS = acute coronary syndrome; CABG = coronary artery bypass graft surgery; CAD = coronary artery disease; *C. pneumoniae* = *Chlamydia pneumoniae*; CRP = C- reactive protein; FMD = flow-mediated dilation; IgA = immunoglobulin A; IgG = immunoglobulin G; IL-6 = interleukin-6; VCAM = vascular cell adhesion molecule; ICAM = intercellular cell adhesion molecule; PAD = peripheral arterial disease.

Appendix B

Table 1B. FMD in 7 patients with chest pain, suspected of AMI at 2 different occasions measured by the same observer

	Age (yrs)	D1O1 (mm)	D1O2 (mm)	Difference D1O1-D1O2 (mm)	FMD1%	FMD2%	Difference FMD1-FMD2%
1	55	4.78	4.97	-0.19	6.49	6.24	0.25
2	52	4.72	4.78	-0.06	6.57	5.23	1.34
3	68	4.53	4.60	-0.07	6.84	5.43	1.41
4	51	4.91	4.84	0.07	10.18	10.33	-0.15
5	71	3.60	3.60	0.00	5.28	6.94	-1.67
6	71	5.20	5.20	0.00	3.85	3.85	0.00
7	61	4.40	4.10	0.30	4.55	4.88	-0.33
Mean±SD	61±9	4.59±0.51	4.58±0.55	0.01±0.15	6.25±2.07	6.13±2.10	0.12±1.05
Range	51-71	3.60-5.20	3.60-5.20	-0.19-0.30	3.85-10.18	3.85-10.33	-1.67-1.41

Data are presented as mean ± SD and range.
D1 = baseline diameter; D1O1 = baseline diameter at occasion 1; D1O2 = baseline diameter at occasion 2; FMD = flow-mediated dilation; FMD1 = FMD at occasion 1; FMD2 = FMD at occasion 2

Table 2B. FMD in 19 healthy volunteers at 2 different occasions measured by the same observer

	Age (yrs)	D1O1 (mm)	D1O2 (mm)	Difference D1O1- D1O2 (mm)	FMD1 %	FMD2 %	Difference FMD1-FMD2%
1W	31	3.25	3.08	018	9.38	12.20	-2.81
2W	38	3.05	3.40	-0.35	8.20	5.88	2.31
3W	31	3.31	3.35	-0.04	8.68	8.21	0.47
4W	25	2.73	2.65	0.08	9.17	10.38	-1.20
5W	30	4.25	4.20	0.05	6.47	4.76	1.71
6W	26	3.30	3.30	0.00	6.06	6.06	0.00
7W	31	2.80	2.80	0.00	17.86	17.86	0.00
8W	36	3.60	3.50	0.10	11.11	11.43	-0.32
9M	27	4.23	4.10	0.13	8.88	8.23	0.64
10M	23	4.60	4.65	-0.05	8.70	9.68	-0.98
11M	35	4.50	4.35	0.15	6.67	8.05	-1.38
12M	37	3.90	4.00	-0.10	5.13	5.00	0.13
13M	33	3.80	3.80	0.00	7.89	10.53	-2.63
14M	23	3.80	3.90	-0.10	5.26	5.13	0.13
15M	23	4.10	4.10	0.00	4.88	7.32	-2.44
16M	30	3.50	3.50	0.00	5.71	5.71	0.00
17M	43	4.10	4.60	-0.50	7.32	6.52	0.80
18M	52	5.00	5.10	-0.10	8.00	5.88	2.12
19M	27	4.57	4.63	-0.06	6.76	6.67	0.09
Mean±SD	32±7	3.81±0.64	3.84±0.67	-0.03±0.16	8.01±2.91	8.18±3.26	-0.18±1.47
Range	23–52	2.73-5.00	2.65–5.10	-0.50–0.18	4.88-7.86	4.76-7.86	-2.81-2.31

Data are presented as mean ± SD and range.
D1 = baseline diameter; D1O1 = baseline diameter at occasion 1; D1O2 = baseline diameter at occasion 2; FMD = flow-mediated dilation; FMD1 = FMD at occasion 1; FMD2 = FMD at occasion 2; M = men; W = women

Appendix C

Table 1C. Association between *C. pneumoniae* antibody titers, the study population dichotomized into two subgroups (for any given cut-off), and FMD

C. pneumoniae titer

IgA	n	FMD %	IgA	n	FMD %	P value
< 16	104	4.8 ± 2.5	≥ 16	119	4.6 ± 2.8	0.494
< 32	119	4.8 ± 2.5	≥ 32	104	4.6 ± 2.8	0.594
< 64	162	4.9 ± 2.5	≥ 64	61	4.3 ± 3.0	0.034
< 128	187	4.8 ± 2.6	≥ 128	36	4.4 ± 3.1	0.277
< 256	199	4.8 ± 2.7	≥ 256	24	4.4 ± 2.5	0.541
< 512	218	4.7 ± 2.7	≥ 512	5	5.2 ± 2.1	0.528
IgG			IgG			
< 16	60	4.9 ± 2.6	≥ 16	163	4.7 ± 2.7	0.533
< 32	66	4.9 ± 2.6	≥ 32	157	4.7 ± 2.7	0.611
< 64	90	4.8 ± 2.6	≥ 64	133	4.7 ± 2.7	0.750
< 128	123	4.8 ± 2.5	≥ 128	100	4.6 ± 2.9	0.666
< 256	167	4.9 ± 2.6	≥ 256	56	4.4 ± 3.0	0.269
< 512	207	4.8 ± 2.6	≥ 512	16	4.5 ± 3.8	0.533

Data are presented as mean values ± SD. Continuous variables were analyzed using Mann-Whitney test. Statistical significance was established at $p < 0.05$.
FMD indicates flow-mediated dilation; n = number of patients

Table 2C. Association between *C. pneumoniae* antibody titers, the study population dichotomized into two subgroups (for any given cut-off), and FMD in patients without IHD

C. pneumoniae titer

IgA	n	FMD	IgA	n	FMD	P value
<16	43	5.5 ± 2.5	≥16	45	5.7 ± 2.9	0.841
<32	50	5.6 ± 2.5	≥32	38	5.6 ± 3.0	0.916
<64	68	5.5 ± 2.4	≥64	20	6.1 ± 3.5	0.601
<128	78	5.4 ± 2.6	≥128	10	6.8 ± 3.3	0.141
<256	83	5.6 ± 2.7	≥256	5	5.8 ± 2.6	0.857
<512	87	5.6 ± 2.7	≥512	1	6.7	0.591

IgG			IgG			
<16	27	5.4 ± 2.6	≥16	61	5.7 ± 2.7	0.559
<32	28	5.4 ± 2.6	≥32	60	5.7 ± 2.7	0.693
<64	38	5.3 ± 2.7	≥64	50	5.8 ± 2.7	0.482
<128	52	5.3 ± 2.5	≥128	36	6.0 ± 2.8	0.277
<256	74	5.5 ± 2.6	≥256	14	6.2 ± 3.0	0.249
<512	85	5.6 ± 2.6	≥512	3	6.3 ± 5.0	0.696

Data are presented as mean values ± SD. Continuous variables were analyzed using Mann-Whitney test. Statistical significance was established at $p < 0.05$.
FMD indicates flow-mediated dilation; n = number of patients

Table 3C. Association between *C. pneumoniae* antibody titers, the study population dichotomized into two subgroups (for any given cut-off), and FMD in patients with IHD

C. pneumoniae titer

IgA	n	FMD	IgA	n	FMD	P value
<16	61	4.4 ± 2.5	≥16	74	4.0 ± 2.5	0.375
<32	69	4.2 ± 2.5	≥32	66	4.1 ± 2.6	0.715
<64	94	4.5 ± 2.5	≥64	41	3.4 ± 2.4	0.012
<128	109	4.3 ± 2.5	≥128	26	3.5 ± 2.4	0.100
<256	116	4.2 ± 2.5	≥256	19	4.1 ± 2.5	0.832
<512	131	4.1 ± 2.5	≥512	4	4.8 ± 2.3	0.525
IgG			IgG			
<16	33	4.6 ± 2.5	≥16	102	4.0 ± 2.5	0.300
<32	38	4.5 ± 2.7	≥32	97	4.0 ± 2.5	0.427
<64	52	4.4 ± 2.5	≥64	83	4.0 ± 2.5	0.397
<128	71	4.4 ± 2.5	≥128	64	3.9 ± 2.6	0.283
<256	93	4.3 ± 2.4	≥256	42	3.8 ± 2.8	0.210
<512	122	4.2 ± 2.4	≥512	13	4.1 ± 3.6	0.630

Data are presented as mean values ± SD. Continuous variables were analyzed using Mann-Whitney test. Statistical significance was established at $p < 0.05$.
FMD indicates flow-mediated dilation; n = number of patients

Appendix D

Supplement to the paper: **"*Chlamydia Pneumoniae* Antibodies and Endothelial Function as Assessed by Flow-Mediated Dilation in Patients With Chest Pain With and Without Ischemic Heart Disease." Beata B Malmqvist, Birgitte R Diness, Kim Krogsgaard, Lars H. Thomassen, Gorm B Jensen, & the IAMA Group. The American Journal of Cardiology 2003; 91; 982-985.**

I have the following comments concerning the inconsistency between the paper above and the ph.d.-thesis: " **Flow-mediated dilation. The link between *Chlamydia pneumoniae* antibodies and ischemic heart disease. The effect of clarithromycin on endothelial function in men with acute coronary syndrome."**

First, in table 8 and 12 of the thesis, corresponding to table 4 and 5 of the paper, there is a difference in FMD% between the 2 pieces of work. In the first draft to "The American Journal of Cardiology", we reported the FMD% measured at the time of the maximum response after deflation of the BP-cuff. The reviewers, however, wanted us to report the response at 60 seconds, so that our study might be compared to most other studies using the same method. Consequently, as far as the patients are concerned, whose maximum response time deviated the most from 60 seconds, I adjusted the maximum FMD% values to the FMD% values at 60 seconds after deflation of the BP-cuff (which did not influence our conclusions at all). Unfortunately, I did not do a back up of the extended database file of FMD% values at approximately 60 seconds of response time. As the hard disc of my computer broke down in the summer of 2003, I had to regenerate the file subsequently.

Secondly, the results from the univariate and the multiple linear regression analyses also differ between the 2 pieces of work (this applies to both smoking, being significant in the univariate linear regression analysis in the paper, but now becoming non-significant, and to the p-value for IHD in the multiple linear regression analysis – which in the paper is statistically non-significant, but becomes significant in the thesis). One reason for this has been given above (as the FMD% is the response variable, only slight changes in the FMD% are required to change the estimates), another is due to the changed definition of smokers, as explained below.

Thirdly, in the paper we define smokers as subjects who are either currently consuming tobacco products or have quitted smoking less than 3 months ago (smoking 1), whereas non-smokers are subjects who have either never consumed tobacco or have given up smoking 3 months or more previously. This definition is "incorrect" in the text of the paper when compared to the information of the tables. It should read (in accordance with table 1 of the paper): Smokers are defined as subjects who are either current or former consumers of tobacco products. Non-smokers are subjects who have never smoked tobacco (smoking 2). For the calculations of the paper we employed this definition (smoking 2), upon which all results cited in the paper are based.
I checked both variables in the univariate and the multiple linear regression analysis. No significant difference in our conclusions is found ("smoking 1" ($p = 0.241$ in the univariate analysis) and "smoking 2" ($p = 0.057$ in the univariate analysis of the thesis, and $p = 0.043$ in the paper). Both are non-significant in the multiple linear regression analysis. The significance of other parametres is not altered – this particularly applies to age and IHD).
In order to be consistent, I have subsequently used the "smoking 1" definition in the thesis. Hence, the change in the number of smokers, and the change of p-value in table 8 (of the thesis) corresponding to table 1 of the paper.