ABSTRACT

Chlamydophila pneumoniae is a Gram-negative obligate intracellular eubacteria, with a biphasic developmental cycle and two distinct morphological forms: the extracellular infectious elementary body and the intracellular replicating reticulate body.

C. pneumoniae is an aetiological agent of respiratory infection also suspected to play an immuno-pathogenetic role in atherosclerosis by contributing to inflammation and plaque instability.

Phospholipases D (PLDs) are enzyme involved in lipid metabolism and others events which can direct or indirect impact on virulence and inflammatory response. To better understand the role of *C. pneumoniae* PLD (CpPLD) in cell biology and during the infection, the *Cppld* gene was cloned and expressed in *Escherichia coli* and the recombinant protein rCpPLD was purified.

This generated protein was highly immunogenic in mice and capable to elicit anti-CpPLD antibodies in the general population exposed to *C. pneumoniae*.

In vitro experiments of gene transcription and expression in Hep-2 infected cells, showed that *Cppld* gene was expressed highly to early and late chlamydial development, and the CpPLD protein was localized at the periphery of inclusions at 72 h post infection.

Enzymatic activity was also investigated. The rCpPLD was able to synthesize cardiolipin from 2 molecules of phosphatidyl-glycerol, demonstrating that the CpPLD was a cardiolipin sinthase enzyme.

Furthermore, the purpose of this study was to evaluate the serological response to the rCpPLD in patients with acute coronary syndromes (ACS) and in healthy blood donors. All serum samples were screened by microimmunefluorescence (MIF). the positive samples were categorized as subjects with presumptive *C. pneumoniae* infection or past exposure (only specific IgG) and chronic infection (presence of specific IgG and IgA).

MIF-negative sera showed antibodies against rCpPLD. In MIF-positive subjects antibodies against to rCpPLD were consistently found in sera of ACS patients with chronic infection.

Additionally, it was recognized an immunodominant epitope in position 233-252 aa (P5) of the CpPLD protein which strongly reacted with ACS sera. The CpPLD protein and its P5 peptide could be plausible antigens for the diagnosis of *C. pneumoniae* chronic infections in ACS patients. These data suggest that rCpPLD may be a useful tool for future studies concerning the role that this enzyme plays in the pathology and immune response to *C. pneumoniae* infection.

ABSTRACT

Chlamydophila pneumoniae è un batterio Gram-negative ed intracellulare obbligato, con un ciclo di sviluppo bifasico, in quanto assume due diverse forme: la forma extracellulare infettiva chiamata, corpo elementare e la forma intracellulare replicativa chiamata, corpo reticolare.

C. pneumoniae è l'agente eziologico delle infezioni respiratorie e sembra giocare un ruolo immuno-patogenico nell'aterosclerosi contribuendo all'infiammazione ed instabilità della placca.

Le Fosfolipasi D (PLDs) sono enzimi coinvolti nel metabolismo lipidico ed in altri eventi che direttamente o indirettamente agiscono sulla virulenza e la risposta infiammatoria.

Per capire meglio il ruolo della PLD di *C. pneumoniae* (CpPLD) nella biologia cellulare e durante l'infezione, il gene *Cppld* è stato clonato ed espresso in *Escherichia coli* e la proteina ricombinante rCpPLD è stata purificata. Questa proteina si è dimostrata molto immunogenica e capace di individuare anticorpi anti-CpPLD di una popolazione generale di soggetti esposti a *C. pneumoniae*.

Esperimenti *in vitro* di trascrizione genica ed espressione in cellule Hep-2 infettate, ha dimostrato che il gene *Cppld* è molto espresso all'inizio e alla fine del ciclo di sviluppo della Chlamydia e che la proteina CpPLD è localizzata alla periferia del corpo d'inclusione a 72 ore dall'infezione.

Della proteina CpPLD è stata studiata anche l'attività enzimatica. rCpPLD è stata in grado di sintetizzare cardiolipina da 2 molecole di fosfatidil glicerolo, dimostrando di essere un enzima cardiolipina sintetasi.

Inoltre, lo scopo di questo studio è stato anche quello di valutare la risposta sierologica alla rCpPLD in pazienti con sindrome coronarica acuta (ACS) ed in sangue di donatori sani.

Tutti i sieri esaminati sono stati analizzati per microimmunofluorescenza (MIF). I campioni positivi sono stati categorizzati come soggetti con presuntiva infezione da *C. pneumoniae* o passata esposizione (solo specifiche IgG) ed infezione cronica (presenza di specifiche IgG e IgA). Nessun siero MIF-negativo ha mostrato anticorpi contro rCpPLD. In soggetti MIF-positivi, anticorpi contro rCpPLD sono stati consistentemente trovati in sieri di pazienti affetti da ACS con un'infezione cronica.

In più è stato individuato un epitopo immunodominante P5 (aa 233-252) della proteina CpPLD, il quale reagisce fortemente con sieri di soggetti con ACS. La CpPLD ed il peptide P5 potrebbero essere utilizzati come antigeni per la diagnosi di *C. pneumoniae* in pazienti ACS con infezione cronica. Questi dati suggeriscono che rCpPLD potrebbe essere utile per futuri studi in merito al ruolo che quest'enzima gioca nella patologia e nella risposta immunitaria all'infezione da *C. pneumoniae*.

KEY WORDS

Chlamydia pneumoniae; Polymerase chain reaction (PCR); Atherosclerosis; **ACS** Acute coronary syndromes; phospholipase D; cardiolipin (CL); CL synthase enzyme; real-time PCR; Linear B cell epitope; 3D structural modelling.

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FACOLTA' DI MEDICINA

DOTTORATO DI RICERCA IN

MICROBIOLOGIA MEDICA E IMMUNOLOGIA

XXI CICLO

CHARACTERIZATION of PHOSPHOLIPASE D PROTEIN of *Chlamydophila pneumoniae* and IMMUNOLOGICAL RESPONSE in PATIENTS with ACUTE CORONARY SYNDROMES

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Anno Accademico 2008-2009

INDEX

AAA	Abdominal aortic aneurysm
ACS	Acute coronary syndromes
AD	Alzheimer's disease
AMI	Acute myocardial infarction
CDC	Centre for Disease Control
CHD	Coronary heart disease
CL	Cardiolipin synthase
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
PCR	Polymerase chain reaction
CRP	C-reactive protein
EB	Elementary body
HC	Healthy control
HSPs	Heat shock proteins
MIF	Microimmmunofluorescence
MOMP	Major outer membrane protein
MS	Multiple sclerosis
PLD	Phospholipase D
Pss	Phosphatidyl serine synthase
RB	Reticulate body
T3S	Type III secretion
TIA	Transient ischaemic attack

CHARACTERIZATION of PHOSPHOLIPASE D PROTEIN of *Chlamydophila pneumoniae* and IMMUNOLOGICAL RESPONSE in PATIENTS with ACUTE CORONARY SYNDROMES

1. INTRODUCTION

1.1 CHLAMYDIAE

Chlamydiae are gram-negative obligate intracellular eubacteria, and include three species pathogens for humans: *Chlamydia pneumoniae* (Grayston *et al.* 1989), *C. trachomatis* that comprises two human biovars (trachoma and lymphogranuloma venereum) with a total of 18 serovars, and *C. psittaci*.

Originally chlamydiae were first considered as protozoa or a virus. Subsequently, they were taxonomically included into their own order *Chlamydiales*, with one family, *Chlamydiaceae*, and a single genus, Chlamydia.

When the Approved Lists of Bacterial Names was published in 1980, the *Chlamydiaceae* had just two species. At that time, all bacteria with chlamydia-like chemical characteristics, morphology and developmental replication belonged to either *Chlamydia trachomatis* or *Chlamydia psittaci* in the order *Chlamydiales* (Moulder 1982).

During the 1980s, the development of new techniques DNA-based provided the differentiation of chlamydial groups and contributed to the creation of two additional species, C*hlamydia pneumoniae* (Grayston *et al.* 1989) and *Chlamydia pecorum* (Fukushi *et al.* 1992).

Genetic data, including phylogenetic analyses using the ribosomal operon, were also consistent with the presence of nine groups in the *Chlamydiaceae* (Everett *et al.* 1997) that have *16S rRNA* gene sequences that were > 90% identical (Pettersson *et al.* 1997; Takahashi *et al.* 1997).

In the mid-1980s, an atypical strain of *C. psittaci* was found to be responsible for the epidemic of mild pneumonia that had occurred in Finland in 1978 (Saikku *et al.* 1985). This new strain was called TWAR, which was an acronym of the first two Seattle isolates TW-183, isolated in 1965 from the eye of a child during a trachoma vaccine trial in Taiwan, and AR-

39, isolated in 1983 from a throat swab of a university student with pharyngitis in Seattle (Grayston *et al.* 1986).

Previously, strain TWAR was synonymous with the designation of *Chlamydia pneumoniae* (Kuo *et al.* 1995).

Successively, *Chlamydia pneumoniae* was transferred in other specie in the family *Chlamydiaceae,* and was identified as *Chlamydophila pneumoniae* (Grayston *et al.* 1989).

1.2 GENOME of *C. pneumoniae*

The genome of *C. pneumoniae* was completely sequenced. The genome of *C. pneumoniae* CWL029 (Refseq: NC_000922) consists of 1,230,230 nt that include 1122 genes, 43 structural RNAs and 1052 protein coding sequences (http://www.ncbi.nlm.nih.gov).

Comparison of the *C. pneumoniae* with the *C. trachomatis* genome will provide an understanding of the common biological processes required for infection and survival in mammalian cells. Genomic differences are implicated in the unique properties that differentiate the two species in disease spectrum. Analysis of the 1,230,230 nt *C. pneumoniae* genome revealed 214 protein-coding sequences not found in *C. trachomatis*. The apparent low level of DNA homology between *C. trachomatis* and *C. pneumoniae*, in contrast to analogous cell structures and developmental cycles, predicts that comparative analysis of the two genomes will significantly enhance the understanding of both pathogens.

Identification of genes present in one species but not the other is of particular importance for the biology, virulence and pathogenesis. Obviously, the identification of genes shared between the two species supports the requirement for these capabilities in biological systems that have, over long-term association with mammalian host cells, evolved to reduce metabolic capacities while optimizing survival, growth and transmission of these unique pathogens.

It is evident that there is a high level of functional conservation between *C. pneumoniae* and *C. trachomatis*.

Orthologue genes between *C. pneumoniae* and *C. trachomatis* were identified for 859 (80%) of the predicted coding sequences (Kalman *et al*. 1999).

The central metabolic pathways from the *C. pneumoniae* genome sequence are the same as those identified for *C. trachomatis*.

C. pneumoniae has a glycolytic pathway and a linked tricarboxylic acid cycle; although likely functional, these processes are incomplete as genes for citrate synthase, aconitase and isocitrate dehydrogenase were not identified. *C. pneumoniae* has a complete glycogen synthesis and degradation system, supporting a role for glycogen synthesis and use of glucose

derivatives in chlamydial metabolism. Furthermore, genes encoding essential functions in aerobic respiration are present, and electron flux may be supported by pyruvate, succinate, glycerol-3-phosphate and NADH dehydrogenases, NADH-ubiquinone oxidoreductase and cytochrome oxidase.

C. pneumoniae also contains the V (vacuolar) - type ATPase operon and the two ATP translocases found in *C. trachomatis*.

The type-III secretion virulence system required for invasion by several pathogenic bacteria and found in the *C. trachomatis* genome in three chromosomal locations is also present in the *C. pneumoniae* genome. Each component is well conserved and genes such as a predicted serine/threonine protein kinase and other genes physically linked to those encoding structural components of the type-III secretion apparatus, are also highly similar between the two species, suggesting the functional roles in modifying cellular biology are fundamentally conserved.

Recently different *C. pneumoniae* strains were sequenced for comparative analyses. The identity in restriction sites in physical maps of the two strains of *C. pneumoniae* (J138 and CWL029) has been interpreted to mean that strains are highly conserved in their genomic organization and gene order (Fig. 1) (Shirai *et al.* 2000).

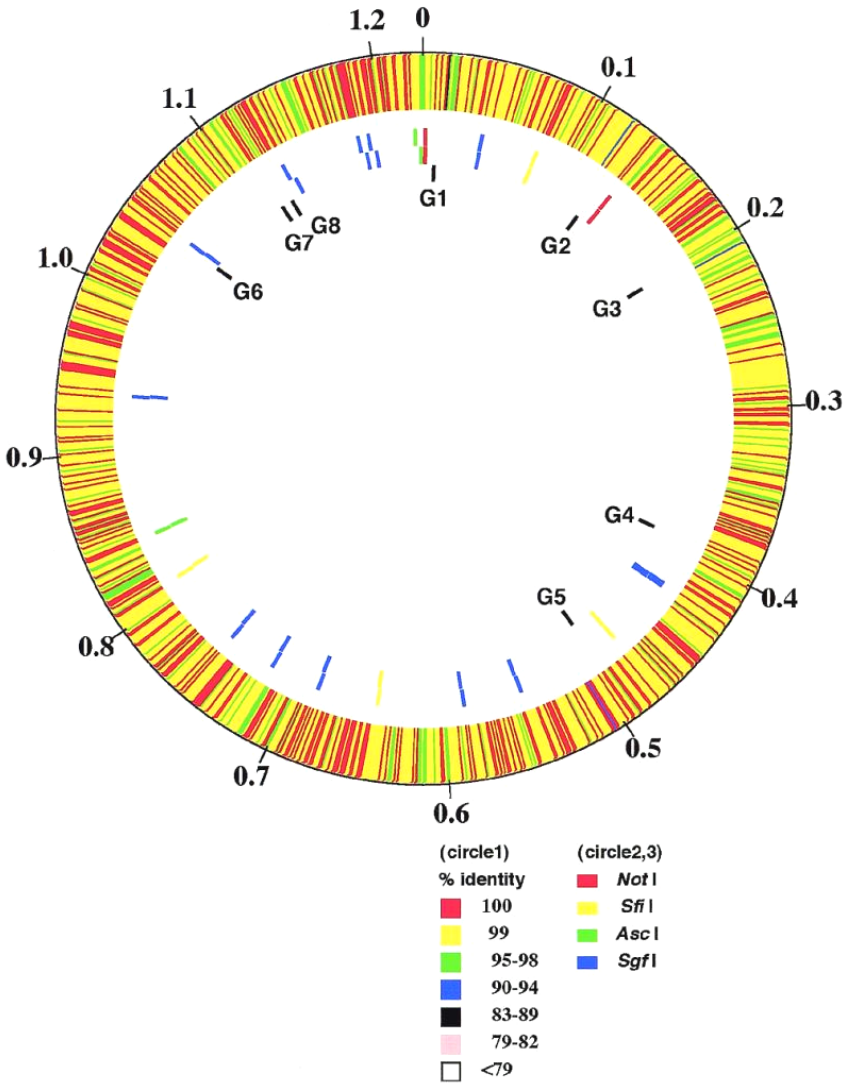


Fig.1. Genomic organization of *C. pneumoniae* (Shirai *et al.* 2000)

The sequence comparison showed that three DNA segments, ranging in size from 27 to 84 nt, are unique to the J138 genome (G2, G4 and G6), while five DNA segments, ranging in size from 89 to 1649 nt, are unique to the CWL029 genome (G1, G3, G5, G7 and G8).

With the exception of these eight regions, the nucleotide sequences throughout the genomes are almost identical (identity, 99.9%), and the orders of the homologous sets of genes are the same.

Recent analysis of the genomic polymorphism of chlamydial species revealed that the genome of *C. pneumoniae* is highly conserved, whereas that of *C. trachomatis* is diverse (Meijer *et al.* 1999). Identification of the minimal genetic diversity in the whole genome will facilitate understanding of the biology of *C. pneumoniae*. The genome sequence of *C. pneumoniae* AR39, which has found to be identical to the sequence of CWL029 except a

truncated ORF (~300 bp shorter in AR39) provides additional information on the genomic stability of this organism (Read *et al.* 2000). Previous genomic-sequence comparison of the two chlamydial species revealed that the *C. pneumoniae* CWL029 genome contains 187,711 additional nucleotides including 214 coding sequences compared with the *C. trachomatis* serovar D genome (Kalman *et al.* 1999; Stephens *et al.* 1998) and the low level of similarity for individual encoded proteins between the orthologs from the two species is consistent with the recent reclassification of genus *Chlamydia* based on the phylogenetic analysis.

1.3 DEVELOPMENTAL CYCLE

Chlamydiae are intracellular bacteria that have a biphasic developmental cycle with two distinct morphological forms. The extracellular infectious form (0.3 μm) is called elementary body (EB), and the intracellular replicating form (1.0 μm) is called reticulate body (RB).

Infectious EBs start the cycle by attaching to a susceptible host cell membrane. They infect host cell via parasite-specified phagocytosis or receptor-mediated endocytosis and when inside the cell, the chlamydiae remain within an enlarging intracellular vacuole, a characteristic inclusion keep away from lysosomal fusion (Fig. 2). During the first few hours, EBs differentiate into metabolically active RBs and using the host cells energy and nutrient resources, RBs begin to multiply by binary fission. After multiple rounds of division, RBs start to transform back to EBs. Finally, by exocytosis or host cell lysis, the infectious EBs are released into the cytoplasm, to initiate new cycles in new host cells (Hatch 1999). In cell culture conditions, the duration of the developmental cycle is between 48 and 72 hours. In natural infections, the situation is more complicated, and the normal development of *Chlamydia* is easily disturbed. Certain circumstances (nutrient deficiency, interferon-gamma, antibiotics) may result in morphological alterations of RBs and the emergence of enlarged, atypical chlamydial forms (Beatty *et al.* 1993). These aberrant forms may persist inside the host cell in a viable but culture-negative state for a long time. The cycle of both normal and altered development of *Chlamydia* is presented in Fig. 2.

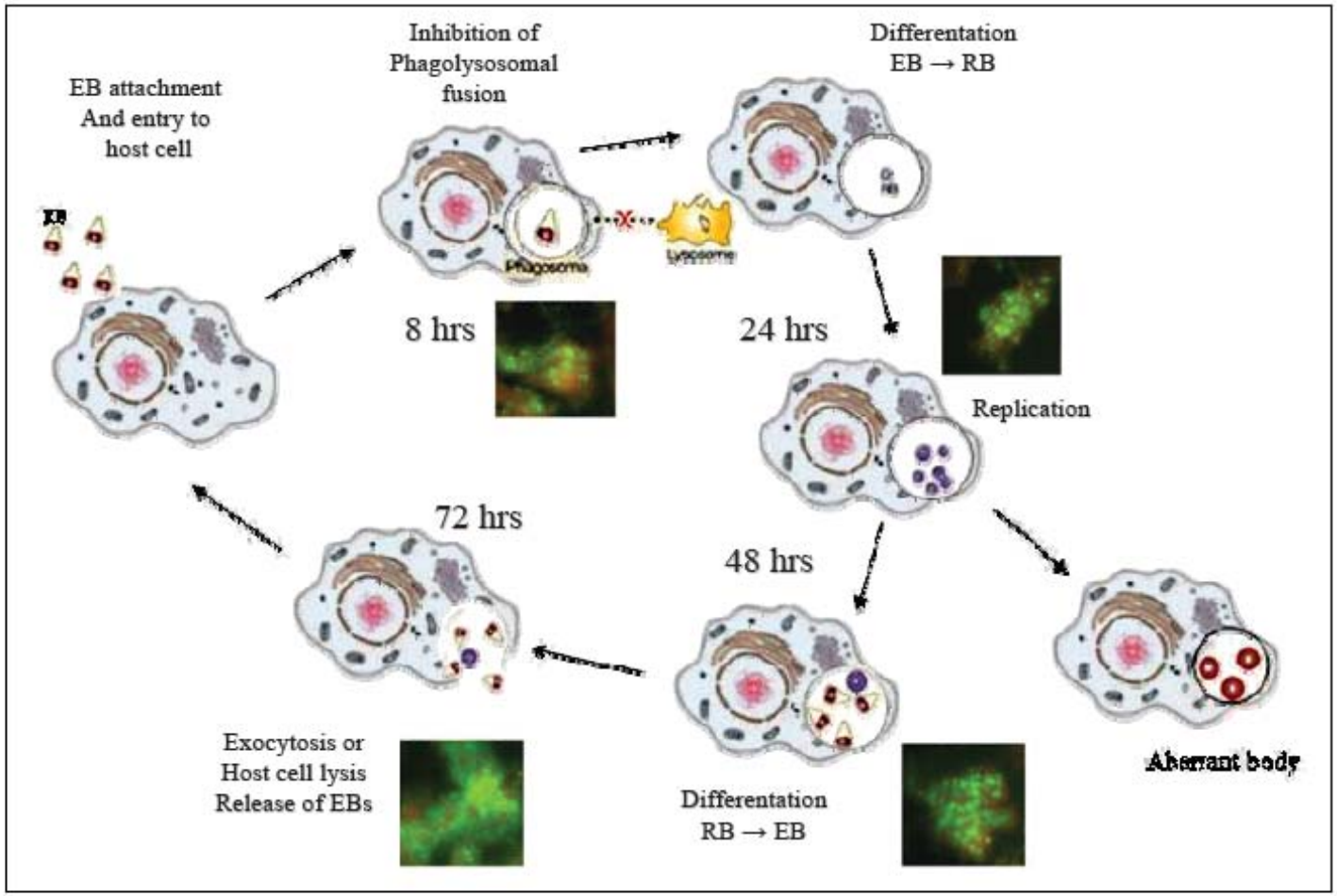


Fig. 2 Developmental cycle of Chlamydia

1.4 STRUCTURE

At all stages of development, chlamydial cells appear to be surrounded by a double membrane, a characteristic feature of gram-negative bacteria. However, unlike other gram-negative bacteria, chlamydiae don't have a peptidoglycan layer in the space between the two membranes (Barbour *et al.* 1982; Fox *et al.* 1990).

On the other hand, they contain penicillin-binding proteins, and the presence of peptide crosslink analogous to those between peptidoglycan backbones has been suggested (Barbour *et al.* 1982).

The genomic sequence revealed the presence of genes for peptidoglycan synthesis, membrane assembly and recycling (Stephens *et al.* 1998) and that peptidoglycan has been suggested to be needed in RB cell division (Brown *et al*. 2000).

Lipopolysaccharide (LPS), which is a general endotoxin in gram-negative bacteria, is localised on the surface of *Chlamydia*, both at EBs and RBs (Birkelund *et al.* 1989). Chlamydial LPS is structurally similar to the rough form of LPS found in enterobacteria, having both a cross-reactive epitope and a genus-specific epitope (Nurminen *et al.* 1983; Brade *et al.* 1987). The structure of LPS is not identical in all chlamydial species, and compared to the LPS of enterobacteria, chlamydial LPS has much lower endotoxin activity (Nurminen *et al.* 1983; Brade *et al.* 1987; Ingalls *et al.* 1995).

Chlamydia has a different kind of outer membrane proteins (Omp). The most abundant of them is the major outer membrane protein (MOMP) of 38 to 42 kDa, comprising about 60% of OMPs (Caldwell *et al.* 1981).

MOMP contains serovar-, subspecies- and species-specific epitopes that can be identified by monoclonal antibodies (Campbell *et al.* 1990; Perez Melgosa *et al.* 1991).

MOMP is surface-localised not only on *C. trachomatis* and *C. psittaci*, as first thought (Knudsen *et al.* 1999), but also on *C. pneumoniae* (Wolf *et al.* 2001). However, the MOMP appears to be less immunogenic and antigenically complex than that of the other chlamydiae (Campbell *et al.* 1990, Perez Melgosa *et al.* 1991).

Others important proteins are OMP2, a 60-kDa cysteine-rich protein, and OMP3a small cysteine-rich protein, is synthesised late in the developmental cycle and it is not exposed at the surface of *Chlamydia* (Collett *et al.* 1989) .

The OMP2 protein has been suggested to be surface-exposed (Ting *et al.* 1995, Stephens *et al.* 2001) and is the structural element for the hexagonally arrayed structures, and only seen at the inner surface of the outer membrane complex (Mygind *et al.* 1998).

Chlamydia also contains heat shock proteins (HSPs). The genes encoding HSP10, HSP60 and HSP70 have been cloned and sequenced (Morrison *et al.* 1989, Danilition *et al.* 1990, LaVerda and Byrne 1997). These genes are continuously expressed throughout the developmental cycle.

The HSPs are highly conserved within chlamydial species, including *C. pneumoniae* (Kikuta *et al.* 1991; Kornak *et al.* 1991). All three HSPs can be found in the outer membrane complexes of both EBs and RBs and chlamydial HSP60 and HSP70 are highly immunogenic during natural infection (Brunham *et al.* 1994).

In the inclusion membrane, there exists a group of proteins called inclusion membrane proteins (Inc). The first of them was demonstrated in *C. psittaci* by Rockey *et al.* (Rockey *et al.* 1995) and named IncA. Since then, six other Incs, from IncB to IncG, have been characterised (Bannantine *et al.* 1998; Scidmore-Carlson *et al.* 1999). The genome contains an even higher number of hypothetical Inc proteins (Rockey *et al.* 2000). The potential to export such a high number of Incs to the inclusion membrane suggests that the inclusion membrane may have several functions in vesicle trafficking, inclusion development, avoidance of lysosomal fusion, nutrient acquisition and signalling associated with EB-RB-EB reorganisation.

1.5 CLINICAL MANIFESTATIONS

C. pneumoniae is a respiratory pathogen that causes both upper and lower respiratory tract diseases.

The majority of infections are asymptomatic or mild infections (Saikku 1992b; Miyashita *et al.* 2001b). Involvement of infection has been described in common cold, persistent cough, pharyngitis, sinusitis and otitis media (Blasi 2000). Pneumonia and acute bronchitis are the most frequently recognised lower respiratory tract diseases associated with infection (Kuo *et al.* 1995). In addition to acute respiratory infections, several chronic respiratory tract inflammatory diseases have also been associated with infection. These include chronic bronchitis and chronic obstructive pulmonary disease (COPD) as well as sarcoidosis (Blasi 2000, Saikku 2002).

In patients with COPD, the persistence of microorganisms in the respiratory tract may facilitate access of different pathogens to the lower airways, and long-standing infection might trigger what is traditionally described as the vicious circle of chronic bronchitis.

Chronic *C. pneumoniae* infection has been found to be common in cases of chronic bronchitis, and it could contribute to disease progression through a toxic effect on bronchial

epithelial cells, impairing ciliary function, and increasing chronic inflammation via proinflammatory cytokine production (Blasi *et al.* 1993; von Hertzen *et al.* 1997).

The possibility of chronic colonization in patients with COPD is supported by serology, electron microscopy and immunohistochemistry (Theegarten *et al.* 2000; Wu *et al.* 2000).

In addition to respiratory tract infections, *C. pneumoniae* has been associated with cardiovascular diseases.

Sub-acute inflammatory conditions, such as endocarditis, myocarditis and vasculitis, have been reported to follow the infection (Saikku 2002). The association of infection with coronary heart disease (CHD), acute coronary syndromes (ACS) and acute myocardial infarction (AMI) was discovered in 1988 by Saikku *et al.* (Saikku *et al.* 1988a). A number of papers have been published to support the theory of association between infection and atherosclerosis and its complications, such as AMI, stroke, transient ischaemic attack (TIA) and abdominal aortic aneurysm (AAA) (Ngeh *et al.* 2002).

The first indication that *C. pneumoniae* has an association with atherosclerosis and CHD dates back to 1986 (Grayston *et al.* 1986), and it association has been shown by seroepidemiology, immunohistochemistry, polymerase chain reaction (PCR), electron microscopy and tissue culture. Animal models of atherosclerosis have been used to investigate the role of *C. pneumoniae* in the initiation and progression of atherosclerotic disease (Mussa *et al.* 2006). The results of some treatment trials using antibiotics for the prevention of cardiovascular events in animal models of atherosclerosis encouraged secondary prevention trials in humans (Muhlestein *et al.* 1998; Fong 2000).

Small-scale studies indicated that antibiotic treatment may prevent adverse cardiovascular events. However, large clinical trials failed to demonstrate any effect (O'Connor *et al.* 2003; Grayston *et al.* 2005).

A comprehensive review analysed the epidemiological and experimental evidence accumulated over the last 20 years linking *C. pneumoniae* to atherosclerosis (Watson *et al.* 2008). The authors conclude that *C. pneumoniae* is neither sufficient in itself nor necessary to cause atherosclerosis or its clinical consequences in humans. However, it is highly likely to be a modifiable risk factor that may be a target of future therapies.

C. pneumoniae is also detected with higher frequency in the cerebrospinal fluid of multiple sclerosis (MS) patients and more pronounced evidence of central nervous system (CNS) inflammation/demyelination (Grimaldi *et al.* 2003). These findings led to the hypothesis that *C. pneumoniae* might act as a cofactor that is able to increase already established inflammatory and demyelinating processes and promote more active disease. Published data

suggest the need for longitudinal observations, and clinical trials with *C. pneumoniae*-specific antibiotics to clarify its exact role in MS infected patients (Stratton *et al.*2006).

Studies that have used PCR to detect the bacterium in the brains of Alzheimer's disease (AD) patients have yielded conflicting results (Balin *et al.*1998; Gieffers *et al.* 2000).

A recent study demonstrated that brain tissue samples from a high proportion of patients with AD are PCR-positive for *C. pneumoniae*, but those from age-/sex-matched non-AD controls are not. Moreover, the organism is viable within the brains of patients with AD, indicating metabolic activity of the organism in those tissues (Gerard *et al.* 2006). A randomized, placebo controlled, multicentre clinical trial has been performed to determine whether a 3-month course of doxycycline and rifampicin can reduce the decline of cognitive function in patients with AD (Loeb *et al.* 2004).

There was no clear relationship between the results and treatment in terms of eradication of chronic *C. pneumoniae* infection, suggesting that the activity of the two drugs may be related to non-antibiotic effects. None of these observations demonstrates a causal relationship between CNS infection with *C. pneumoniae* and the neuropathogenesis characteristic of AD, but they do open the way to further investigations.

1.6 PATHOGENESIS

Different chlamydial species as well as different biovars infect *in vitro* different cell types.

C. pneumoniae has been shown to be able to infect and multiply in endothelial cells, smooth muscle cells, monocytes/macrophages and lymphocytes (Kaukoranta-Tolvanen *et al.* 1994; Gaydos *et al.* 1996; Fryer RH 1997; Haranaga *et al.* 2001). The dissemination of the bacterium has been studied in mouse models. After intranasal inoculation, the bacteriumspreads systemically in mice, and it can be isolated from lungs, spleen and peritoneal macrophages (Yang *et al.* 1995). Intravenous and subcutaneous inoculations also result in disseminated infections. It has further been shown, that *C. pneumoniae* has an ability to disseminate systematically via infected macrophages along hematogenous and lymphatic routes (Moazed *et al.* 1998).

The host defence mechanisms seem to be unable to eradicate *Chlamydia* or to provide protection from re-infections. Therefore, repeated infections with *Chlamydia* are common (Ward 1995).

In addition a classic replicative life cycle, which represents acute chlamydial infection, *Chlamydia* may enter in a persistent state within the host cell that is characterized by metabolic and structural changes. Persistent *C. pneumoniae* organisms have an atypical

ultrastructural morphology with inclusions that show only a few enlarged bodies or aberrant structure and an altered gene expression profile. For example, there is an altered gene expression of outer membrane proteins OMP1 and OMP2 or inclusion membrane proteins (Mathews *et al.* 2001; Kutlin *et al.* 2001; Molestina *et al*. 2002).

Byrne *et al.* (Byrne *et al.* 2001) demonstrated that persistence show enhanced expression of the genes required for DNA replication, but not of the genes essential for bacterial cell division. Chlamydial type III secretion (T3S) system appears to remain fully functional during chronic infection, so may continuously release effector proteins into the host cell cytoplasm, ensuring a *C. pneumoniae* adjusted environment.

This is of particular interest, as *C. pneumoniae* was reproducibly recovered from atherosclerotic lesions (Maass *et al.* 1998). Chronic persistent infection of vascular cells indeed leads to proproliferative and proinflammatory phenotypes within the infected vasculature *in vitro* and *in vivo*. However, attempts to eradicate *C. pneumoniae* in patients with cardiovascular diseases have all failed (O'Connor *et al.* 2003; Grayston *et al.* 2005; Anderson *et al.* 2004). This can easily be explained by the fact that the persistent state is completely refractory to antibiotic treatment (Gieffers *et al.* 2001). In fact, first-choice anti-chlamydial drugs may even induce chlamydial persistence under certain conditions (Gieffers *et al.* 2004).

The *C. pneumoniae* T3S appears to be expressed and functional in acute, as well as in chronic, infection (Peters *et al.* 2007; Slepenkin *et al.* 2003) and thus may represent a prominent virulence factor. The major role of a functional T3S may also be in securing growth and development of the pathogen by modifying apoptosis signals or some other transcriptional regulation important for chlamydial survival.

The chlamydial injectisome consists of approximately 25 proteins, which can be predicted from sequence homologies. Their examination will result in a better understanding of chlamydial pathogenesis and may result in new therapeutic strategies.

An important defence mechanism against invading pathogens is the cell death by apoptosis. Whereas in acute chlamydial infection host cell lysis is obviously crucial for infective EBs to be released from the host cell, in chronic infection it has been shown to successfully escape from this programmed cell death by inhibiting apoptotic signalling cascades within various host cell populations, monocytes, epithelial cells or microglial cells (Airenne *et al.* 2002; Boelen *et al.* 2007). It is important to know that the pro-apoptotic BH3- proteins, which stimulate apoptotic events, can be efficiently degraded by *C. pneumoniae*. Furthermore, experiments showed that the release of the apoptotic trigger protein cytochrome c can be

inhibited by chlamydiae, and caspase-3 activity is reduced in *C. pneumoniae*-infected HeLa cells.

In fact, in early pulmonary infection, *C. pneumoniae* is ingested by neutrophil granulocytes where the bacterium is able to survive and multiply by delaying the programmed host cell apoptosis via inhibition of procaspase-3 and enhanced antiapoptotic interleukin (IL)-8 expression (van Zandbergen *et al.* 2004). Transmission of the bacterium to mononuclear cells and systemic dissemination can result and many studies assume a central role of the transcription factor nuclear factor kappa-B (NF-jB) in anti-apoptotic effects.

However, its impact still remains controversial (Paland *et al.* 2006).

When *C. pneumoniae* infects cells is possible to see an enhanced expression of numerous inflammatory chemokines and cytokines (Ikejima *et al.* 2006). Under *in vitro* conditions, vascular endothelial cells and smooth muscle cells are highly susceptible to infection, and develop a pro-atherosclerotic and pro-proliferative phenotype (Gaydos *et al.* 1996; Godzik *et al.* 1995).

This *in vitro* constellation might lead to complex vascular alterations *in vivo*, endothelial dysfunction, destruction of endothelial integrity and the basal membrane by proliferating smooth muscle cells, and maintenance of atherosclerotic plaques and subsequent plaque rupture. This underlines the need to associate a role of *C. pneumoniae* in nearly all stages of atherogenesis.

In addition, *C. pneumoniae* seems to have relevant pro-mitogenic properties.

A connection between vascular cell proliferation, a hallmark of atherogenesis, and chlamydial infection would certainly highlight the possible relevance of bacterial infection in atherosclerotic development.

The proliferation-promoting potency of *C. pneumoniae*, in particular chlamydial HSP60, is able to induce vascular smooth muscle cell proliferation in a p44/42–mitogen-activated protein kinase-dependent manner (Sasu *et al.* 2001). Even human HSP60, which co-localizes with chlamydial HSP60 in atherosclerotic lesions, might contribute to *C. pneumoniae*-dependent vascular cell proliferation (Hirono *et al.* 2003).

Coombes and Mahony (Coombes *et al.* 1999), examined the complex cell–cell communication within the vascular wall and reported that the supernatant of *C. pneumoniae*-infected endothelial cells contained one or more soluble factors causing vascular smooth muscle cell proliferation in a time- and dose-dependent manner.

In diseases associated with infection, antibody responses to chlamydial HSP60 in asthma (Hahn *et al.* 2000) and arteriosclerosis (Ciervo *et al.* 2002, Mahdi *et al.* 2002) have been reported.

Biological basis for *C. pneumoniae* involvement in asthma and chronic airway inflammation indicates that gene products (mainly HSP60) and the activation of transcription factors (notably nuclear factor kappa-B), are responsible for the activation of most cellular elements in bronchial tissue (epithelium, endothelium, monocytes–macrophages, smooth muscle cells), resulting in a cascade of cytokine release and adhesion molecule upregulation, which favours cellular influx into the airways, persistent infection and airway remodelling.

The HSP60 is highly conserved and an autoimmune response to human HSP60 may have a role in chlamydial pathogenesis. In fact, antibodies again to chlamydial HSP60 have been shown to cross-react with human HSP60, and both chlamydial and human proteins are localised in atherosclerotic plaque macrophages (Kol *et al.* 1998). It has also been found that chlamydial HSP60 induces foam cell formation by inducing oxidation of LDL in monocytes (Kalayoglu *et al.* 1999).

1.7 EPIDEMIOLOGY

C. pneumoniae causes approximately 10% of all community-acquired pneumonias (Saikku 1992).

Most likely, it is primarily transmitted from human to human by the respiratory tract without any animal reservoir (Saikku *et al.* 1985; Kleemola *et al.* 1988). *C. pneumoniae* infection spreads slowly, in fact the incubation period is several weeks, which is longer than that for many other respiratory pathogens (Kuo *et al.* 1995). The time span of infection spread in families is shorter, however, ranging from 5 to 18 days (Mordhorst *et al.* 1992; Blasi *et al.* 1994). The infection appears to be most common among school-aged children (Kuo *et al.* 1995). In some areas, however, infections are already common in children aged 1 to 4 years (Saikku *et al.* 1988b; Normann *et al.* 1998). The prevalence increases dramatically after the age of 5, and by the age of 20, half of the population are estimated to have detectable antibody levels.

Thereafter, seroprevalence continues to increase in adult age, but at a slower rate, and reaches a level of approximately 75% in the elderly (Saikku 1992b; Kuo *et al.* 1995). Seroprevalence rates continue to be high despite the fact that some individuals lose their antibodies over a period of several years, suggesting that the majority of people are infected during their lifetime, and that reinfections are common (Grayston *et al.* 1990).

Seroprevalence is almost equal in both sexes up till adolescence, but higher among adult men than adult women (Saikku 1992b; Kuo *et al.* 1995). In addition to male sex, smoking has been shown to be associated with infection (Hahn *et al.* 1992; Karvonen *et al.* 1994; Laurila *et al.* 1997b; Von Hertzen *et al.* 1998; Mayr *et al.* 2000).

1.8 DIAGNOSIS

Criteria for *C. pneumoniae* diagnosis are convened by the Centre for Disease Control and Prevention (Atlanta) and the Laboratory Centre for Disease Control (Ottawa, Ontario, Canada). CDCs reviewed current diagnostic tests and provide recommendations for standardized approaches.

1.8.1 Culture

The culture is the golden standard in chlamydial diagnosis but the task of isolating and growing is more difficult. Isolation is best performed by cell culture and the most sensitive cell lines are HeLa (Kuo *et al.* 1990) and Hep- 2 (Roblin *et al.* 1992; Wong *et al.* 1992). The sensitivity of cell culture in the diagnosis of acute respiratory infection is approximately 60% compared to serology, while specificity is close to 100%.

However, isolation from the chronic stage is much more difficult. The probable reason for the difficulties of isolation in chronic stages is that deeper tissues, such as lung interstitial macrophages, arterial wall macrophages and smooth muscle cells, are not readily accessible by routine sample collection methods.

1.8.2 Serology

Serology is the most frequently used method for diagnosing infections. The best serological evidence of acute infection is a four-fold rise in IgG or IgA antibody titre between paired sera.

A positive IgM antibody titre is also considered a marker of a current infection. In primary infection, IgM antibodies are produced about 3 weeks after the onset of the illness, whereas IgG and IgA antibodies may not appear until 6-8 weeks after onset.

In reinfection, on the other hand, IgM antibodies appear only at low titres while IgG and IgA titres rise quickly, within 1 or 2 weeks, and may reach very high levels. IgM titre usually begins to fall within 2 months and disappears within 4-6 months. IgA antibodies also have a short half-life, whereas IgG persist in the body and may be detectable for more than 3 years. Especially older patients, who have probably had multiple infections, may have persistently high IgG titres. (Kuo *et al.* 1995).

Persistent production of IgA antibodies, compared to long-lasting IgG antibodies, seems to be a better marker in chronic infections (Saikku *et al.* 1992a; Laurila *et al.* 1997a; Laurila *et al.* 1997b).

The diagnosis is generally made with the microimmmunofluorescence (MIF) test, which was developed in the early 1970s. It is able to measure separately antibodies in the IgM, IgG and IgA classes and is therefore suitable for distinguishing recent from past infections and reinfections (Kuo *et al.* 1995). The test format uses purified formalinized elementary bodies from *C. pneumoniae, C. trachomatis,* and *C. psittaci* that have been fixed onto glass slides as distinct dots. Dilutions of sera are placed over the antigen dots and incubated.

When properly performed and read, this test is the most sensitive and specific method for diagnosing acute infections. However, the assay is technically complex, interpretation is subjective, and neither reagents nor diagnostic criteria have been standardized (Peeling *et al.*2000). Kits based on the MIF format are commercially available.

The antibodies may be measured not only from serum samples, but also from circulating immune complexes (IC) after precipitation (Linnanmäki *et al.* 1993) and from sputum samples (von Hertzen *et al.* 1995).

Another immonoenzymatic test used for identification of *Chalmydia* is the Enzyme immunoassay (EIA). It is also able to differentiate between the three antibody classes. EIA kits with LPS-extracted EBs or synthetic peptides as antigen are commercially available. However, problems with sensitivity and specificity have been observed. (Peeling 1999).

1.8.3 Direct antigen detection

Several commercial or in house-made monoclonal antibodies specific for the detection of EBs in various samples, have been reported. Their performance in direct fluorescent antibody (DFA) tests appears to be fairly comparable to other assays (Montalban *et al.* 1994). The sensitivity of DFA is 20 to 60% compared to culture or serology. It is somewhat higher for specimens from deep sites. Several commercial kits designed for *C. trachomatis* can be used for the detection of *C. pneumoniae*, because the capture antibody used in these kits is the genus-specific LPS (Peeling 1999).

1.8.4 Polymerase chain reaction (PCR)

Nucleic acid–based amplification techniques, such as PCR, have identified *C. pneumoniae* in clinical samples ranging from respiratory specimens (Tong *et al.* 1993; Boman *et al.* 1997; Gaydos *et al.* 1994) to samples of vascular tissue (Kuo *et al.* 1993; Campbell *et al.* 1995;

Grayston *et al.* 1995), serum (Jackson *et al.* 1997), and peripheral blood mononuclear cells (Boman *et al.* 1998). Despite significant improvements in the development of molecular methods for the detection of *C. pneumoniae,* some laboratories report consistent detection of the organism in specimens of vascular tissue (Ong *et al.* 1996), whereas others do not (Paterson *et al.* 1998) (table 1). This variation may be related to differences in means of specimen collection and processing, primer design, nucleic acid extraction, amplification product detection, or prevention and identification of false-positive and false-negative results.

Although many in house PCR methods are available for detection of *C. pneumoniae,* but more studies need to be conducted using proper controls and a large number of clinical specimens obtained from patients to compare and evaluate more adequately the usefulness of different PCR tests for the diagnosis of *C. pneumoniae* infection.

Nested PCR is, in general, more sensitive than single-step PCR because of the 2-step amplification and the use of 2 sets of primers may greatly enhance both sensitivity and specificity (Black *et al.* 1994; Boman *et al.* 1997). The disadvantages of nested PCR are the increased risk of contamination and re-amplification of the products, which makes the assay more time-consuming and expensive.

Moreover, multiplex reactions decrease sensitivity and specificity if the annealing temperatures for the individual primers are not identical. Internal or amplification controls allow the monitoring of PCR assay inhibition, which may be caused by a number of factors. These controls can have the disadvantage of competing for primers when identical primers are used for both target genes and internal controls.

Type of report or assay	Target region	Product size, bp	Method of detection	Year of study [reference]
Published reports regarding assays that meet validation criteria[a]				
S + R	Cloned *Pst*I fragment	437	AGE	1992 [43]
S	16S rRNA gene	463	AGE	1992 [44]
N + T	MOMP	Outer, 333; inner, 207	AGE	1993 [29]
S + T + HS + M	16S rRNA gene	195	AGE	2000 [45]
Published reports regarding assays				
S	16S rRNA gene	463	EIA	1993 [46]
N	16S rRNA gene	Outer, 1397; inner, 858	AGE	1994 [47]
S	53-kDa protein coding gene	499	AGE	1996 [48]
N	16S rRNA gene	Outer, 317; inner, 178	EIA	1996 [49]
N + M	16S rRNA gene	Outer, 436; inner, 221	AGE	1997 [50]
N	Cloned *Pst*I fragment	Outer, 437; inner, 128	AGE + SBH	1997 [51]
N	16S rRNA gene	Outer, 463; inner, 269	AGE	1997 [39]
S	60-kDa protein coding gene	183	EIA	1998 [52]
S	16S rRNA gene	465	EIA	1998 [53]
S + T + HS	16S rRNA gene + MOMP	165	AGE + SBH	1998 [54]
N	MOMP	Outer, 496; inner, 189	TRF	1998 [55]
S + IC	16S rRNA gene	463	EIA	1998 [56]
S + R + IC	16S rRNA gene	465	AGE	1999 [57]
N	16S rRNA gene	Outer, 492; inner, 304	AGE + DBH	1999 [58]

NOTE. AGE, agarose gel electrophoresis; DBH, dot-blot hybridization; HS, hot-start PCR; IC, internal control; M, multiplex PCR; MOMP, major outer membrane protein; N, nested PCR; R, restriction enzyme digestion; S, single-step PCR; SBH, Southern blot hybridization; T, touchdown PCR.

Table 1. PCR assays for detection of *C. pneumoniae* in clinical samples (Dowell *et al*. 2001)

In the last decade, the real-time PCR-based fluorescence assay was introduced as a new tool in PCR methods. It is an automated technology which presents many advantages like high sensitivity and specificity, lesser chances of contamination, and DNA quantization. In addition, its results can be visualized faster than gel-based PCR assays. This technology has recently been used for detecting and quantification of *C. pneumoniae* in human samples (Mygind *et al.* 2001; Ciervo *et al.* 2003). Ciervo *et al.* describe a real-time PCR based on the Light Cycler FRET using 2 probes hybridizing an internal, 128 bp region-*Pst* I fragment of *C. pneumoniae*.

The Fig. 3 shows the melting curve profile of hybridization probes. In this study the authors demonstrate that real-time PCR is specific and more sensitive than nested PCR, probably because the low copy numbers could not be visualized in agarose gel after nested PCR (Fig. 3).

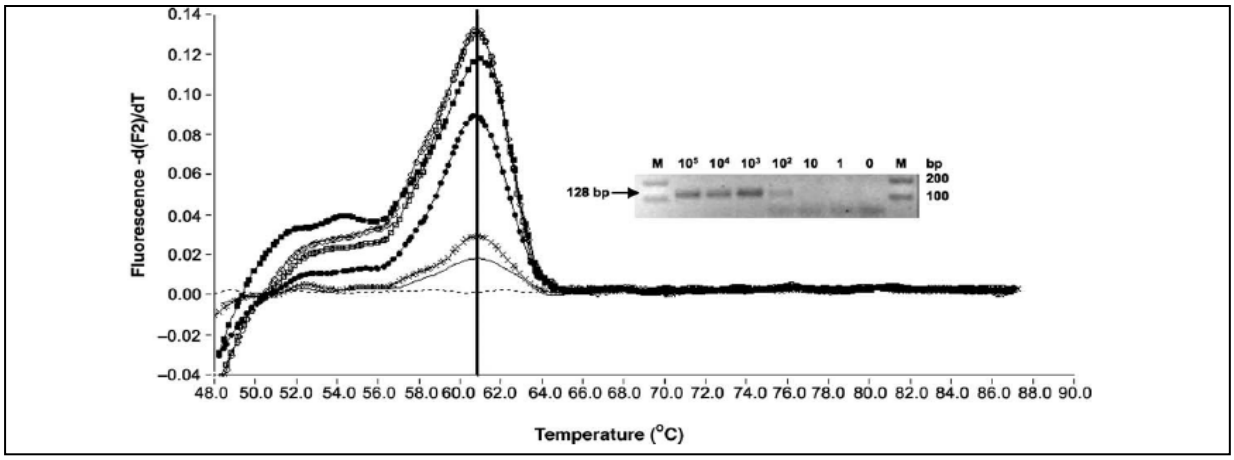


Fig. 3. Melting curve determination and gel analysis of amplicons of *C. pneumoniae* -specific *Pst*I. Serial copy number dilutions of purified *C. pneumoniae* DNA were (■) 10^5, (○) 10^4, (□) 10^3, (●) 10^2, (x) 10, (-) 1, and (- -) negative control (Ciervo *et al.* 2003).

PCR amplification assay by LightCycler typically lasted 40–60 min, while amplification by nested PCR and agarose gel detection of amplicons typically lasted 5–7 h for completion and accurate reading. PCR technology is particularly suited in this area. However, there is a lack of a gold standard for PCR detection of *C. pneumoniae* in clinical samples. Guidelines for

standardizing assays have been published in a report which summarizes many in house PCR methods, and DNA target regions used for detection of *C. pneumoniae* in various samples (Dowell *et al.* 2001), and a recent multicenter study report has emphasized the difficulties of standardizing nested PCR (Apfalter *et al.* 2002). The ability of the PCR technique to amplify small amounts of specific nucleic acid has made it an important and convenient diagnostic tool with a potential to detect rapidly and reliably. Several different targets (16S rDNA, MOMP, pmp4), primers and reaction protocols have been described for the detection of DNA.

Laser capture microdissection (LCM) has been proposed as potential application in human pathology to isolate material of interest for molecular evaluation, and it has been successfully applied in cancer and cardiovascular research.

It is possible to consider that combination of LCM with the quantitative real time PCR assay could be a potential tool to detect and quantify *C. pneumoniae* DNA in the atherosclerotic plaques (Ciervo *et al*. 2008).

PCR tests are expected to be more sensitive than culture methods. It has been estimated that PCR, in general, is at least 25% more sensitive than culture. Guidelines have been developed to minimise the risk of PCR false-positive as well as false-negative results (Boman *et al.* 1999).

1.9 TREATMENT

A number of different antibiotics have been tested in search for an appropriate treatment for infection. Azithromycin and clarithromycin are two macrolides which have shown high activity against the organism *in vitro* (Agacfidan *et al.* 1993; Welsh *et al.* 1996). Some of the new fluoroquinolones and ketolides, a new class of macrolides, have also turned out effective (Roblin *et al.* 1998; Strigl *et al.* 2000; Miyashita *et al.* 2001a; Miyashita *et al.* 2002).

The organism is not susceptible *in vitro* to sulpha drugs, and penicillin and ampicillin prevent the growth of the organism, but do not destroy it. Clinical experience has shown that the symptoms of infection frequently recur after short or conventional courses of appropriate antibiotics, and intensive long-term therapy is therefore highly recommended (Kuo *et al.* 1995). Inappropriate antibiotic treatment may lead to chronicity of the disease. The insidious nature of infection makes prevention very difficult, and the development of anti-chlamydial vaccines remains an important goal for researchers.

1.10 FOCUS: ATHEROSCLEROSIS and *C. pneumoniae* INFECTION

1.10.1 Atherosclerosis: definition

According to American Heart Association (2002), atherosclerosis is a disease of large and medium-sized arteries characterised by thickening and hardening of the vascular wall. It involves a substance called plaque in the inner lining of the arteries. Over time, this build up grows large enough to narrow the artery and significantly decrease the blood flow through it.

When atherosclerosis affects the arteries that supply blood to the heart, it ultimately restricts blood flow to the heart muscle, causing heart pain (angina), irregular heartbeat (arrhythmia) and other problems. The plaques may also become fragile.

Rupturing plaques form blood clots (thrombus) that may block the blood flow through an artery or break off and travel to another part of the body (embolus). If, either happens and occludes a blood vessel that feeds the heart can cause coronary artery diseases (CAD), CHD and AMI.

1.10.2 Atherosclerosis: risk factors

American Heart Association (2002) has identified several risk factors for CAD and CHD. Most of the risk factors can be modified, treated or controlled. High blood pressure, elevated serum LDL cholesterol level and tobacco smoke are considered the major classical risk factors for the development of CAD. Additional factors predisposing to CAD include age, gender male, heredity, race, obesity, physical inactivity, diabetes and high serum triglyceride and low HDL cholesterol levels. Other factors contributing to the heart disease risk are infections and the individual response to stress and excessive alcohol consumption.

1.10.3 Atherosclerosis: inflammatory disease

Atherosclerosis is a chronic inflammatory disorder and it is thought to begin with damage of the endothelium. According to the response to injury hypothesis of atherosclerosis by Ross (Ross 1999), endothelial cells may be injured not only by modified LDL (the modified LDL hypothesis), but also by many other factors, such as elevated plasma homocysteine concentrations, hypertension and infectious microorganisms.

Endothelial dysfunction includes increased endothelial permeability to lipoproteins and other plasma constituents, expression of adhesion molecules and elaboration of growth factors that lead to increased adherence of monocytes, macrophages and T lymphocytes. These cells may migrate through the endothelium and situate themselves within the subendothelial layer.

In the vascular wall, macrophages accumulate lipids and become large foam cells. Foam cells, in turn, release growth factors and cytokines that promote migration of smooth muscle cells and stimulate neointimal proliferation, continue to accumulate lipid and support endothelial cell dysfunction. Foam cells, T cells and smooth muscle cells eventually form the fatty streak. This step also includes platelet adherence and aggregation. As fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap that walls off the lesion from the lumen that covers a mixture of leukocytes, lipid and debris, which may form a necrotic core that represents the results of apoptosis and necrosis, increased proteolytic activity and lipid accumulation. Rupture of the fibrous cap or ulceration of the fibrous plaque may rapidly lead to thrombosis and usually occur at sites of thinning of the fibrous cap that covers the advanced lesion.

Finally, degradation of the matrix may lead to haemorrhage from plaque microvessels or from the lumen of the artery and result in thrombus formation and occlusion of the artery (Fig. 4). Each of the stages of lesion formation is potentially reversible. If the cause of injury is removed, or if the inflammatory or fibroproliferative process is reversed, lesions may regress at any stage. (Ross 1999; Libby *et al.* 2002).

Clinical and laboratory studies have shown that inflammation plays a major role in the initiation, progression and destabilisation of atheromas.

C-reactive protein (CRP) is an acute phase protein, whose concentration may increase up to 1000-fold after the onset of a stimulus. It is a sensitive, but unspecific marker of inflammation. In serious bacterial infections, such as pneumonia, meningitis or sepsis, the CRP level increases dramatically usually within 24 hours (Ablij *et al.* 2002). Functionally, in addition to providing downstream integration of overall cytokine activation, CRP has several direct effects on vascular disease progression, such as an ability to bind and activate complement, induce expression of adhesion molecules, mediate LDL uptake by endothelial macrophages and induce monocyte recruitment into the arterial wall (Libby *et al.* 2002).

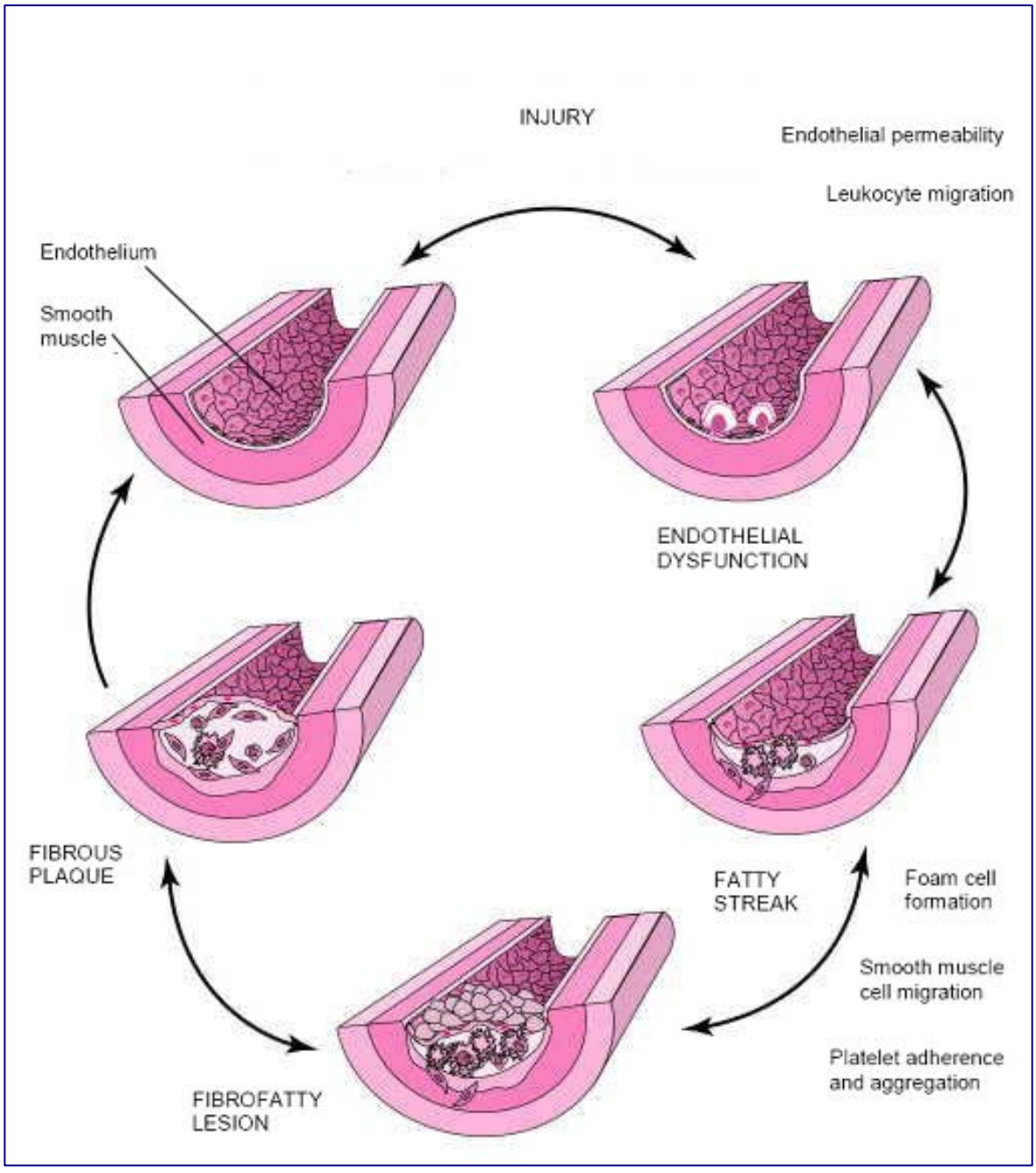


Fig. 4 The response to injury hypothesis of atherosclerosis (modified after: Ross 1999). Encyclopedia of Medical Images).

1.10.4 Atherosclerosis: **autoimmune disease**

There is evidence to suggest that the immune system plays a dominant role in atherogenesis (Libby *et al.* 1991). The putative antigen maintaining the inflammatory process in the arterial wall must be ubiquitous and present early in life, which explains the prevalence of this disease at young age already (Stary 1989).

On the other hand, chlamydial Hsp60 has been shown to induce foam cell formation by inducing oxidation of LDL in monocytes (Kalayoglu *et al.* 1999). Oxidised LDL (oxLDL) is another candidate for an autoantigen in atherosclerosis. Antibodies to oxLDL have been detected in patients with atherosclerosis, and they have been found in atherosclerotic lesions (Bergmark *et al.* 1995). OxLDL antibodies are predictive of C-reactive protein AMI (Puurunen *et al.* 1994; Wu *et al.* 1997) and the progression of carotid atherosclerosis (Salonen *et al.* 1992). T lymphocytes isolated from human atherosclerotic lesions have been shown to respond to oxLDL and to be a major autoantigen in the cellular immune response (Stemme *et al.* 1995).

1.10.5 Atherosclerosis: **infectious disease**

Infections may contribute to the development of atherosclerosis by inducing both inflammation and autoimmunity. A large number of studies have demonstrated a role of infectious agents, both viruses (cytomegalovirus, herpes simplex viruses, enteroviruses, hepatitis A) and bacteria (*H. pylori*, periodontal pathogens) in atherosclerosis (Danesh *et al.* 1997; Libby *et al.* 1997; Mattila *et al.* 1998; Epstein *et al.* 1999; Leinonen *et al.* 2002).

Recently, a new pathogen hypothesis has been proposed, suggesting that multiple infectious agents contribute to atherosclerosis, and that the risk of cardiovascular disease posed by infection is related to the number of pathogens to which an individual has been exposed (Zhu *et al.* 2000; Zhu *et al.* 2001).

Evidence of a seroepidemiological association of *C. pneumoniae* with atherosclerosis and its complications was first presented by Saikku *et al.* (Saikku *et al.* 1988a). Since then, over twenty seroepidemiological studies have confirmed these findings (reviewed by Saikku 1999). Indeed, a few prospective studies suggest that chronic infection, defined by elevated anti *C. pneumoniae* IgA antibodies, is a significant risk predictor for the development of CHD and CAD (Saikku *et al.* 1992a; Mayr *et al.* 2000; Kiechl *et al.* 2001).

The presence of specific T lymphocytes in atherosclerotic tissue specimens suggests that *C. pneumoniae* participates in the maintenance of the inflammatory response in the tissue and

may thus be involved in the progression of the disease (Halme *et al.* 1999; Curry *et al.* 2000; Mosorin *et al.* 2000).

In mouse animal models, intranasal inoculation of *C. pneumoniae* has been shown to cause a systemic spread of infection. In rabbits, intranasal infection has been found to induce inflammatory changes in the aorta and even calcified lesions containing *Chlamydia* (Fong *et al.* 1997; Laitinen *et al.* 1997). In apoE-deficient mice, infection has been shown to accelerate the development of atherosclerosis (Moazed *et al.* 1999).

C. pneumoniae infection affects lipid metabolism, and persistently elevated antibodies against the microorganism have been shown to be associated with elevated triglyceride and total cholesterol levels as well as lowered HDL cholesterol levels (Laurila *et al.* 1997b). Furthermore, it has been shown that exposure of macrophages to followed by LDL caused a marked increase in the number of foam cells and accumulation of cholesterol esters (Kalayoglu *et al.* 1998a).

The bacterium has been shown to induce foam cell formation by LPS (Kalayoglu *et al.* 1998b) and LDL oxidation by HSP60 (Kalayoglu *et al.* 1999). It has also been shown that persistent infection inhibits apoptosis *in vitro* for up to 120 h of follow-up post-infection and is restricted to the cells carrying chlamydial inclusions, suggesting that inhibition of apoptosis may be one of the pathogenetic mechanisms by which infection survives inside the host cells and thus mediates the development of chronicity (Airenne *et al.* 2002) (Fig. 5).

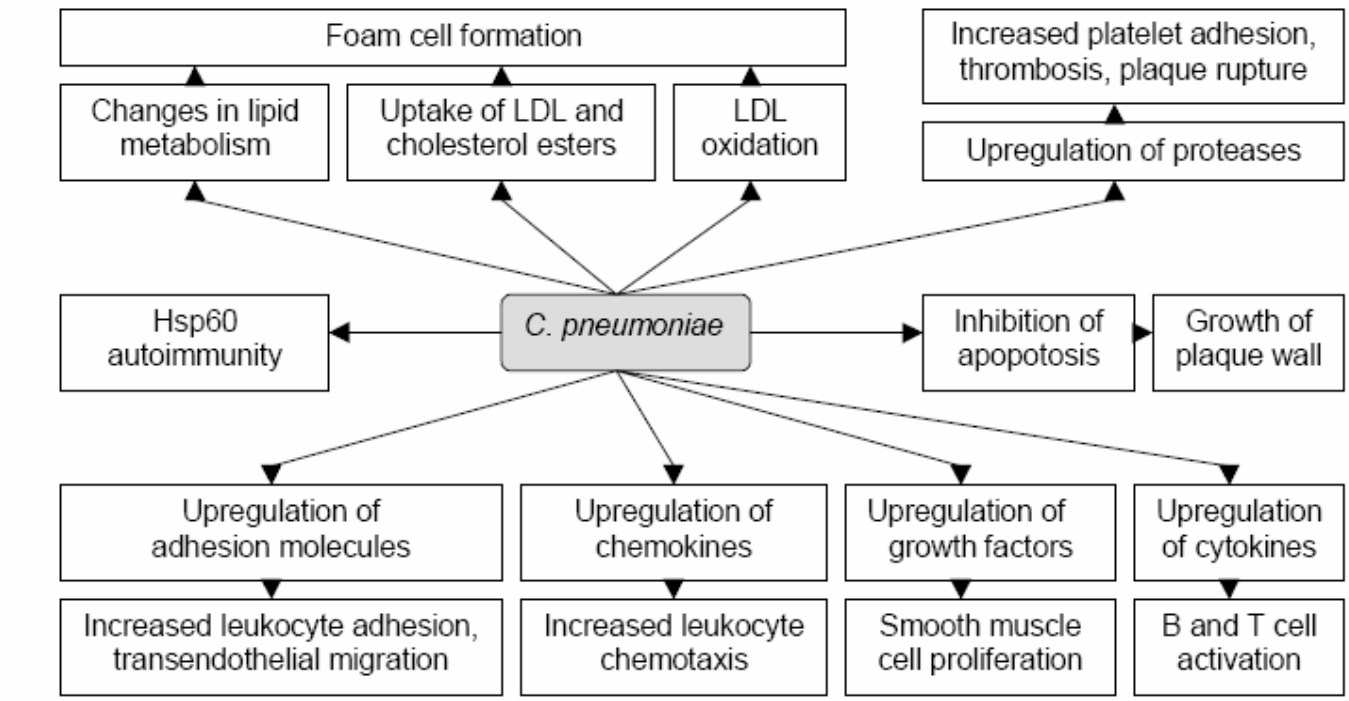


Fig. 5. Pathogenetic mechanisms through which *C. pneumomiae* could affect the development of atherosclerosis (modified after: Leinonen *et al.* 2002, Summersgill *et al.* 2000)

Preliminary antibiotic trials have suggested that patients with antibodies could benefit from antibiotic treatment. The azithromycin may lower the risk for further adverse cardiovascular events in post-AMI patients (Gupta *et al.* 1997), the roxithromycin appeared to extend the clinical benefit of preventing death and re-infarction for at least 6 months after initial treatment (Gurfinkel *et al.* 1999), and the clarithromycin appears to reduce the risk of ischaemic cardiovascular events in patients presenting with unstable angina (Sinisalo *et al.* 2002).

However, negative results have also been reported (Muhlestein *et al.* 2000), and large intervention trials are still going on.

1.11 IMPORTANCE of PHOSPHOLIPASE D PROTEIN in BIOLOGICAL PROCESS and in PATHOGENESIS

To determine a role for this infectious agent in atherosclerosis, and therefore addressing new preventive and/or immunotherapeutic approaches, it is important to clarify which pathogenic interactions of the organism with the host can induce cell alterations that are characteristic of atherosclerosis. The induction and progression of atherosclerosis is largely due to a reaction to a chronic fibroproliferative inflammatory stimulation, and to the oxidative damage of endothelial layer of the arteries (Lusis 2000). Phospholipid mediators are certainly key players in inflammation and thus in atherosclerosis. Much interest in this field has been focused on platelet activating factor, the oxidation products of phospholipids and oxLDL (Ninio 2005). The enzymes responsible of phospholipid metabolism are proteins included in phospholipase D (PLD) superfamily.

PLD activity was first discovered in carrot extracts as a phospholipid-specific phosphodiesterase activity that hydrolyzed phosphatidylcholine giving phosphatidic acid (PA) and choline (Hanahan *et al.* 1948). Over 50 years of biochemical studies culminating in the identification of PLD genes in the mid 1990s have implicated PLD activity and its product PA in a wide range of physiological processes and diseases, including inflammation, diabetes (Hammond *et al.* 1995), cytoskeletal rearrangement (Cross *et al.* 1996), vesicle trafficking and exocytosis (Siddhanta *et al.* 2000), phagocytosis (Kusner *et al.* 1996), neuronal and cardiac stimulation (Frohman and Morris 1999; Park *et al.* 2000), matrix metalloproteinase production (Reich *et al.* 1995), oncogenesis, (Welsh *et al.* 1994), pathogenic actions of bacteria and spider venom (reviewed Van Dijk *et al.* 1998), and the oxidative respiratory burst in neutrophils (Waite *et al.* 1997).

Much of this data comes from studies exploiting the unique ability of the PLD enzymes to catalyze a transphosphatidylation reaction in the presence of primary alcohols, generating phosphatidylalcohols, which are presumed to be functionally inactive.

PLD activities have also been characterized in many species of bacteria, such as *Streptococcus, Pseudomonas, Bacillus subtilis* (Hammond *et al.* 1995), *Yersinia pestis, Streptomyces antibioticus* and *Streptoverticillium cinnamoneum* (Liscovitch *et al.* 2000). A number of PLD-like sequences are also present in vaccinia virus and other pox viruses, and their human homologues (Blasco *et al.* 1991; Frohman *et al.* 1999).

PLD activity appears to be present in most cell types, and within the plasma membrane, cytosol, endoplasmic reticulum (ER), Golgi and nuclei (Meier *et al.* 1999). Several studies indicate that PLD activity and concomitant formation of PA are required for structural integrity of the Golgi. One interpretation of these data is that PLD2, which has higher intrinsic activity than PLD1, could play a "housekeeping" role providing PA for maintaining Golgi structure, while the regulated PLD isoform, PLD1, could provide stimulus coupled control of Golgi function (Chen *et al.* 1997). Activity measurements have revealed that PLD activity can be increased under stress conditions, which include wounding, osmotic challenge, and treatment with stress hormones. Several recent lines of evidence point to a role for PLD in signalling pathways that oppose apoptosis and promote cell survival.

These enzymes have broader substrate specificity than the plant enzymes, and their ability to catalyze transphosphatidylation reactions has made them useful for synthesis of unnatural lipids. The catalytic core of the eukaryotic PLD enzymes consists of four conserved regions (I-IV). Domains II and IV are particularly highly conserved and contain the invariant charged motif, HxK(x) 4D(x) 6GSxN (HKD). Duplication of the HKD motifs have led to the proposal that eukaryotic PLD genes may be the result of a gene duplication event and that PLD may be a bilobed enzyme (Ponting *et al.* 1996). The complete conservation of the HKD domains in PLDs and their relatives suggested a vital role in catalysis that was verified by mutational studies.

Evidence for the importance of the HKD motif has come from the mutation of the lysine of the second HKD motif of SPO14p, a *Saccharomyces cerevisiae* PLD, to arginine and the lost of PLD activity and the inability to compliment meiotic defects (Sung *et al.* 1997).

Two models had been proposed for the catalytic process; one postulated that each motif would function independently, while the other proposed that each would form half of the actual active site (Ponting *et al.* 1996). Evidence suggests the latter hypothesis is correct,

since mutation in either motif abolishes activity, demonstrating that the duplicated motifs cannot function independently (Sung *et al.* 1997)

The *Salmonella typhimurium* endonuclease Nuc possesses only a single HKD motif, but dimerizes to perform catalysis, each monomer lying adjacent to each other forming a single active site held together by a series of hydrogen bonds (Stuckey *et al.* 1999). The Nuc enzyme model (Gottlin *et al.* 1998) suggest that the histidine residue from one HKD motif acts as a nucleophile attacking the phosphate of the phosphodiester bond to form a phosphoenzyme intermediate, while other motif acts as a general acid protonating the leaving group. In the second half of the reaction water would hydrolyze the phosphoenzyme intermediate.

The Fig. 6 shows a model for the catalytic mechanism of the PLD enzymes highlighting the formation of a covalent enzyme substrate intermediate. Deletion studies in pathogenic bacteria suggest roles for PLD in viability and pathogenesis but the mechanisms involved are unclear (Roberts 1996).

One possibility is that bacterial PLD enzymes cause local inflammation by catalyzing the production of the lipid mediator lysophosphatidic acid (LPA) (Van Dijk *et al.* 1998).

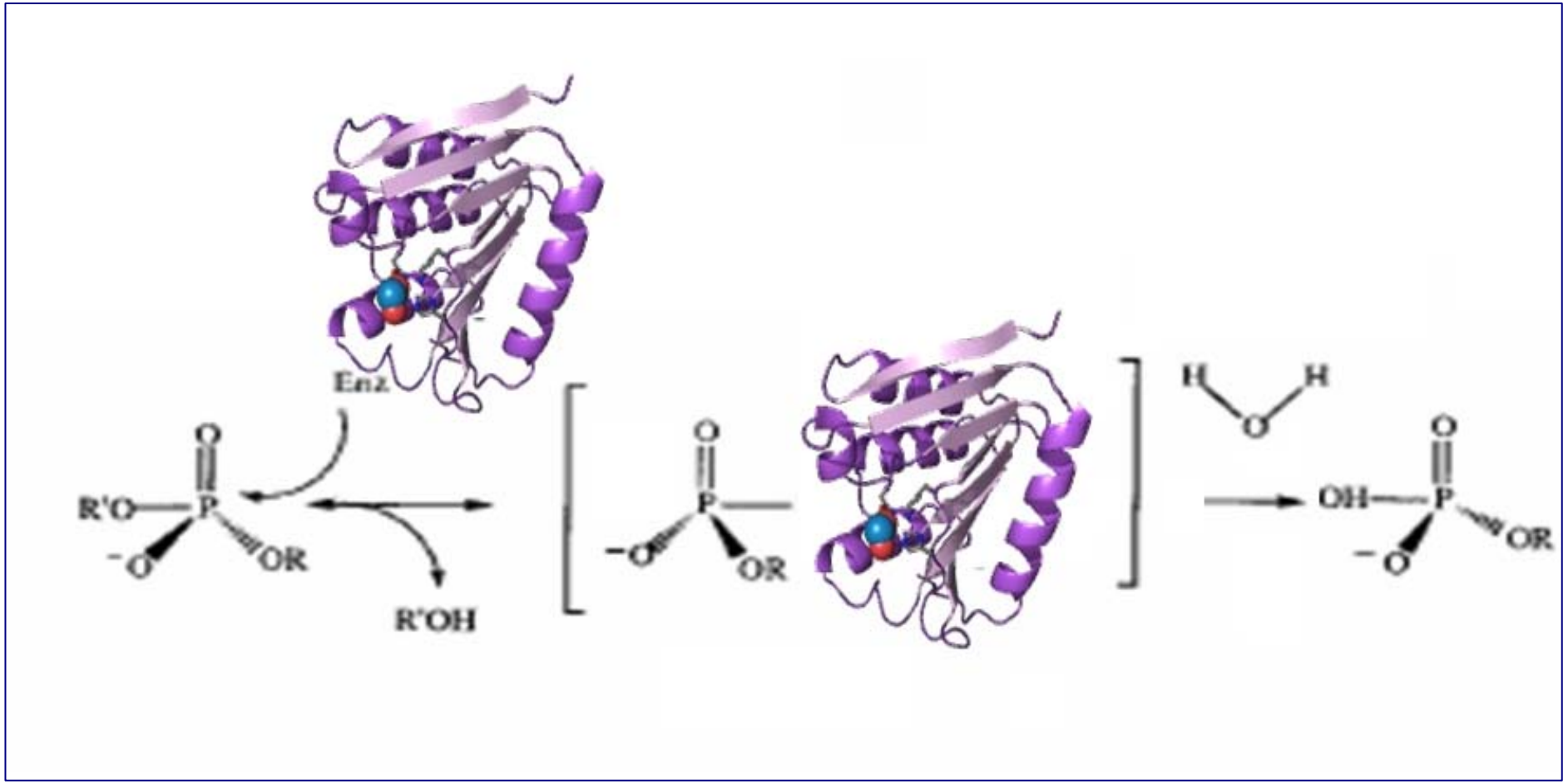


Fig. 6 The Nuc enzyme model. Histidine residue from one HKD motif acts as a nucleophile attacking the phosphate of the phosphodiester bond to form a phosphoenzyme intermediate, while other motif acts as a general acid protonating the leaving group. In the second half of the reaction water would hydrolyze the phosphoenzyme intermediate.

Interestingly the HKD motif is also found in two related bacterial enzymes, cardiolipin (CL) synthase and phosphatidyl serine synthase (Pss) (Koonin 1996; Morris *et al.* 1996; Ponting *et al.* 1996). These enzymes both mediate phospholipid synthesis, in which an alcohol (phosphatidylglycerol or serine) is conjugated to a phospholipid (phosphatidylglycerol or phosphatidic acid) through transphosphatidylation. This has led to the suggestion that PLD may have evolved from a superfamily of alcohol transphosphatidylase enzymes, and may catalyze physiologically relevant transphosphatidylation reactions, such as synthesis of bisphosphatidic acid using DAG as a substrate (Van Blitterswijk *et al.* 1993; Koonin 1996; Morris *et al.* 1996; Ponting *et al.* 1996; Sung *et al.* 1997).

CL synthase and Pss are key enzymes of phospholipid biosynthesis. The genes encoding several of these enzymes from bacteria and yeast have been cloned and sequenced. A search of a non-redundant protein sequence database (National Center for Biotechnology Information, NIH, Bethesda, MD, USA) with the *E. coli* CL synthase protein sequence using the BLASTP program revealed highly significant similarity to homologous uncharacterized proteins from several bacteria and a limited, but also statistically significant, similarity to plant phospholipases D (Fig .7).

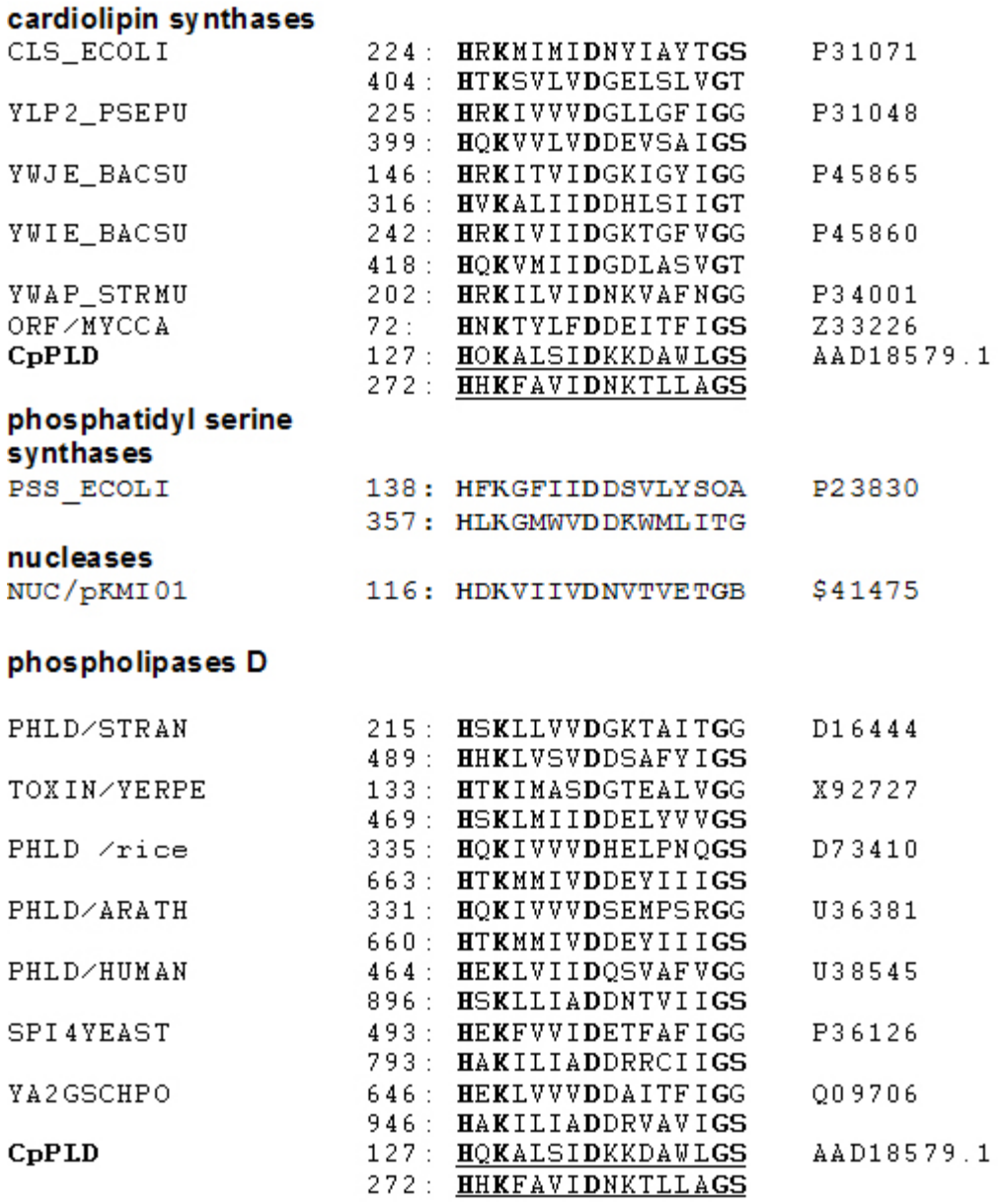


Fig.7 The consensus motif of the PLD superfamily is H(X)K(X)4D, a characteristic motif of the PLD superfamily, which includes phospholipase D, phosphatidylserine synthase (PSS), poxvirus envelope proteins and Yersinia pestis murine toxin (Ymt) but found as a single copy in the nucleases. Here is showed the HKD motif in cardiolipin synthases, phosphatidylserine synthase (PSS) and phospholipases and Nuc. Duplicated copies of the HKD motif from the same protein are grouped together. The distinct groups of proteins showing highly significant similarity to each other are shown as separate blocks. The consensus shows amino acid residues that are conserved in the majority of sequences in each of the groups.

The position of the first residue of each segment in the respective protein sequence is indicated by a number. PHLD, phospholipase D. Organism name abbreviations: PSEPU, *Pseudomonas putida;* BACSU, *Bacillus subtilis;* STRMU, *Streptococcus mutans;* MYCCA, *Mycoplasma capricolum;* STRAN, *Streptomyces antibioticus;* ARATH, *Arabidopsis thaliana;* SCHPO, *Schizosaccharomyces pombe;* YERPE, *Yersinia pestis*.

1.12 IMPORTANCE of CARDIOLIPIN (CL) and CL SYNTHASE ENZYME in BIOLOGICAL PROCESS and in PATHOGENESIS

CL, has been shown to be localized at the septal and on the polar membranes in *E. coli* cells by the CL-specific fluorescent dye 10-*N*-nonylacridine orange (NAO) (Mileykovskaya *et al.* 2000, Mileykovskaya *et al.* 2001). It has been suggested that CL plays specific roles in essential cellular processes including the initiation of DNA replication and the processes of cell division (Mileykovskaya *et al.* 2005).

In *B. subtilis* was found (Kawai *et al.* 2004) an increased NAO-fluorescence in polar septal and on forespore membranes during the sporulation phase and have shown that CL content is indeed higher in sporulation phase cells, an apparent similarity to *E. coli,* which has an increased CL content in the stationary phase (Nishijima *et al.* 1988).

One likely reason for the high CL content in sporulating cell membranes is that it is needed in the sporulation process and it probably plays a vital role in the dormant and/or the germinating state. These dates open new perspectives for our understanding of the role of CL in bacterial membranes.

In eukaryotic cells CL is localized exclusively to the mitochondria. Accumulating evidence suggests that CL may play an integral role in the generation of energy production via oxidative phosphorylation in mammalian cells since it is required for the reconstituted activity of several key mitochondrial enzymes.

Some study were done in *C. trachomatis* (Fraiz *et al.* 1988), observing that infection in HeLa cells resulted in trafficking of host cell glycerophospholipids, including CL, to the intracellular chlamydial inclusion. It also observed that the phospholipid composition of the elementary body, the infectious but dormant extracellular form of the organism, was similar to the phospholipids composition of a purified mitochondrion (Hovius *et al.* 1990), implicating mitochondria as a possible source of glycerophospholipids trafficked to *C. trachomatis* (Wylie *et al.* 1997).

The relationship between mitochondrial metabolism and CL remodelling in *C. trachomatis*-infected eukaryotic cells might be linked to an elevation in mitochondrial metabolism and the eukaryote derived phospholipids in *Chlamydia* may be direct reflection of the composition of the host donor membrane. The best characterized example of direct membrane contact as a means of phospholipid transfer in eukaryotic cells is the “mitochondrial associated membrane”, a putative membrane bridge via which lipids are transported between endoplasmic reticulum and mitochondria (Vance 1990; Rusinol *et al.* 1994). The 4-10 fold increased incorporation of fatty acids into PG and CL, two lipids closely associated with

mitochondria, in *C. trachomatis* infected HeLa cells provided evidence of alteration in glycerophospholipid remodelling in mitochondria in response to chlamydial infection.

The link between CL remodelling and elevated mitochondrial metabolism in *Chlamydia* infected cells is likely related to energy metabolism.

Chlamydia encodes an ATP/ADP translocase that allows for the exchange of host ATP for parasite ADP. The net result is that host ATP is utilized by the parasite to fuel metabolic reactions. The presence of CL in prokaryotes and eukaryotes has been used as an argument for the endosymbiotic hypothesis, according to which mitochondria were derived from prokaryotes that lived inside a eukaryotic progenitor cell (Lowry *et al.* 1951).

CL contains two 1,2-diacyl-*sn*-glycero-3-phosphoryl (also called 3-phosphatidyl groups) linked by a glycerol bridge (Fig. 8) (LeCocq *et al.*1964). The presence of two phosphate groups may give rise to two negative charges, a fact that may become important for protein cross-links and for protein interactions in general. However, in aqueous dispersions with neutral pH, CL contains a single charge only, because one proton gets trapped in a bicyclic resonance structure formed by the two phosphates and the central hydroxyl group (Fig. 8) (Kates *et al.* 1993).

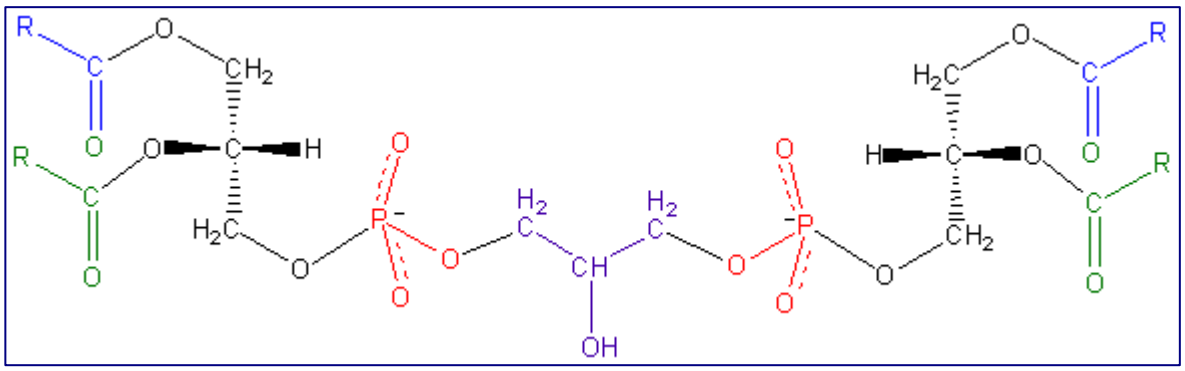


Fig. 8 CL Structure. Two phosphatidyl residues, both in *R* conformation, are linked by a central glycerol bridge.

In prokaryotes, phosphatidylglycerol receives a phosphatidyl group from another phosphatidylglycerol by transesterification, catalyzed by a phospholipase D-type enzyme (Fig. 9b).

In eukaryotes, phosphatidylglycerol receives an activated phosphatidyl group from phosphatidyl-CMP, which is catalyzed by an enzyme that falls into the category of phosphatidyltransferases (Fig. 9 a) .

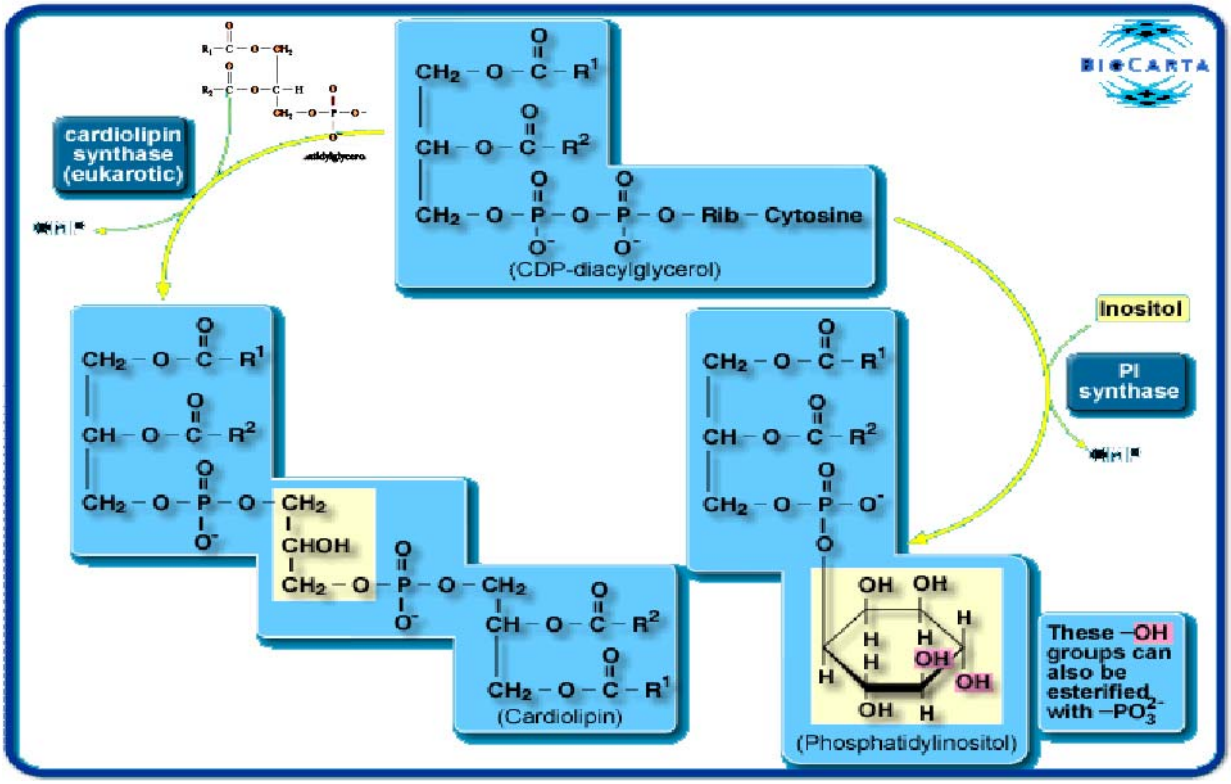


Fig. 9 a http://www.biocarta.com/pathfiles/cardiolipinPathway.asp

Enzymes of this category also produce phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol. The prokaryotic formation of CL is a near-equilibrium reaction, whereas the eukaryotic formation has a considerable negative change in free energy.

In the case of CL synthase, transphosphatidylation occurs between two phosphatidylglycerols, one acting as phosphatidyl donor, the other as phosphatidyl acceptor (Fig. 9 b). This highlights the fact that bacterial CL synthase may act in the reverse direction, which leads to the decomposition of CL.

Thus, CL formation in prokaryotes depends not only on the expression level of CL synthase but also on other factors that affect the transphosphatidylation equilibrium, such as the free energy of CL in the membrane and the local concentration of glycerol and other alcohols.

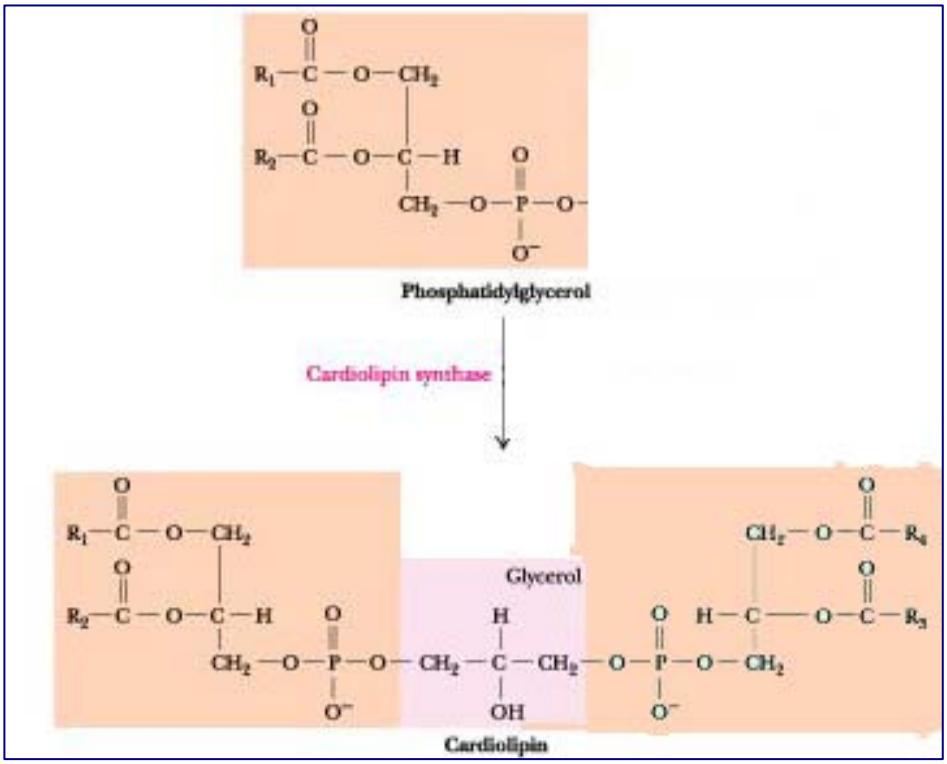


Fig. 9 b. Comparison of reaction mechanisms of phospholipase D and CL synthase. Prokaryotic CL synthase is a phospholipase D-type enzyme, in which glycerol replaces water as the phosphatidyl acceptor. Note that both steps of prokaryotic CL synthase are reversible, whereas the hydrolysis step of phospholipase D is irreversible. Eukaryotic CL synthase is a phosphatidyltransferase that catalyzes an irreversible reaction.

Bacterial membranes contain low levels of CL, which increase only during the stationary growth phase and under certain conditions of environmental stress (Shibuya 1992; Short *et al.* 1971; Koch *et al.* 1984). The increase in CL formation seems to be driven by higher expression of CL synthase (Heber *et al.* 1991; Hiraoka *et al.* 1993). The localization of CL in septal regions may help to maintain the proper spatial segregation of proteins, including the osmosensory transporter that was also shown to localize to the poles. The accumulation of CL at the bacterial poles can be explained on the basis of lipid self-organization, because CL clusters have a high intrinsic curvature and therefore have lower energy when they are located in curved membrane regions (Huang *et al.* 2006; Romantsov *et al.* 2007). Thus, the localization of CL to bacterial poles may be the result of its spontaneous tendency to form homogeneous clusters.

CL is involved in different stages of the mitochondrial apoptotic process and in mitochondrial membrane dynamics. CL alterations have been described in various pathological conditions. Patients suffering from Barth syndrome have an altered CL

homeostasis caused by a primary deficiency in CL remodelling. Alterations in CL content or composition have also been reported in more frequent diseases such as diabetes and heart failure. The decline in mitochondrial CL was considered important because it correlated with the release of the apoptotic trigger cytochrome *c* from the mitochondrial compartment (Ostrander *et al.* 2001). Cytochrome *c* is known to be attached to CL on the outer face of the inner membrane, these data suggested that the mobilization of cytochrome *c* is caused directly by CL deficiency.

The mechanism of CL degradation during apoptosis and ischemia was felt to be the result of oxidative damage. The specific formation of oxidized CL in response to apoptotic stimulation was demonstrated by mass spectrometry, and it was shown that this oxidation is catalyzed by the peroxidase activity of CL-bound cytochrome *c* (Kagan *et al.* 2005).

The CL oxidation is a critical step in apoptosis, as it sets cytochrome c free into the intermembrane space. The cytochrome *c* released into the cytosol, can trigger the caspase cascade, including mitochondrial fragmentation and outer membrane permeabilization. It is unclear whether oxidation is a requirement for the apparent redistribution of CL during apoptosis, which results in gradual mass transfer toward the mitochondrial periphery (i.e., from the inner leaflet of the inner membrane to the outer membrane) (Kaga *et al.* 2005). Furthermore, the chemical structure of oxidatively modified CL is still unknown.

2. AIMS OF THE RESEARCH

To determine a plausible role of *C. pneumoniae* in atherosclerosis, and therefore addressing new preventive and/or immunotherapeutic approaches, it is important to clarify which pathogenic interactions of the microorganism with the host can induce cell alterations that are characteristic of atherosclerosis.

Phospholipases are a heterologous group of enzymes involved in a variety of different diseases. Nonetheless, their most intriguing pathogenicity aspect is their potential to interfere with cellular signalling cascades and to modulate the host immune response.

In recognition of this putatively critical role of phospholipases and phospholipid metabolism in *C. pneumoniae* pathogenicity and atherosclerosis, the aims of the study were:

- The molecular cloning of *C. pneumoniae* PLD (*Cppld*) gene and the study of the secondary structure of CpPLD protein.
- The generation of the recombinant CpPLD protein in *E. coli* expression/purification system;
- The production of a mouse polyclonal antibody against r CpPLD;
- The *Cppld* transcription and CpPLD expression analyses during the *C. pneumoniae* life cycle in Hep-2 infected cells;
- The *in silico* structural CpPLD analysis;
- The determination of CpPLD enzymatic activity;
- The study of the immune response against CpPLD in ACS patients and in healthy subjects;
- The characterization of CpPLD immunological responses through selected peptides.

3. MATERIALS AND METHODS

3.1. CELLS and BACTERIA

Hep-2 cells were purchased from the American Type Culture Collection, Manassas, VA. The cells were routinely grown in 25 cm^2 flask with Eagle's MEM supplemented 10% foetal calf serum, gentamicin 10 mg/ml, and amphotericin B, 1 mg/ml (Gibco Life Technologies, Grand Island, NY). Strain Parola was propagated in Hep-2 cell cultures as previously described (Ciervo *et al.* 2002). Chlamydial EBs were purified by density gradient centrifugation with Renographin (Schering Japan, Osaka, Japan) (Caldwell *et al.* 1981). Purified EBs were suspended in sucrose–phosphate–glutamic acid buffer (0.2M sucrose, 3.8 mM KH_2PO_4, 6.7 mM Na_2HPO_4, 5 mM L-glutamic acid (pH 7.4)) and then stored at -70°C until used. Inclusion-forming units (IFU) of the prepared EBs were titrated by counting chlamydial inclusions in Hep-2 cell monolayers.

3.2. *C. pneumoniae "in vitro"* INFECTION

The Hep-2 cells were dispensed to 6-well culture plates and incubated in 5% CO_2 at 37° C. The confluent cell monolayer was then infected with EBs at a multiplicity of infection of 10^2 by centrifugation at 900 x g for 1 h at 37° C. After additional 30-min incubation at 37° C, fresh growth medium was added and infected cultures were incubated at 37° C under 5% CO_2 for appropriate time points (0, 8, 24, 48 and 72 h). Uninfected Hep-2 cells were also prepared as a control culture. Post-Infected (PI) cells were harvested by centrifugation (6000 x g, 4° C), and the supernatant samples for each point of PI were precipitated over night at -20° C with three volumes ice-cold acetone.

3.3. *Cppld* GENE and CLONING OF *Cppld* I and *Cppld* II DOMAINS

DNA was obtained from purified EBs by the commercial kit Nucleospin Tissue kit (Macherey-Nagel GmbH, Du¨ ren, Germany) using the standard protocol adapted for DNA extraction from bacteria. The *Cppld* gene sequence was amplified by PCR using as template the *C. pneumoniae* strain Parola DNA.

Appropriate sets of oligonucleotide primers were designed to construct rCpPLD protein full length, rCpPLD I motif (aa 1- 214) and rCpPLD II motif (aa 215 - 353).

The system used to generate the protein rCpPLD and both domains rCpPLD I and rCpPLD II was Champion™ pET Directional TOPO® Expression Kits (Invitrogen).

The primers used include the 4 base pair sequences (CACC) necessary for directional cloning to the 5′ end of the forward primer.

The primers for CpPLD full length protein are:

- primer sense 5-CACCATGAATAAAAGACAAAAAGATAA-3
- anti-sense primer 5-GGGTCAGCTAATCACGCTGCTTCTTGCTCTT-3.

CpPLD I (aa 1- 214) was produced using the

- sense primer 5-CACC ATGAATAAAAGACAAAAAGATAA-3
- anti sense primer 5-AGCAAA TCAAGCAACTTGGATGGTT-3

While the second region CpPLD II (aa 215 to 353) was cloned using

- sense primer 5-CACCATGTTTGCTCTGACCCACTC-3
- antisense primer 5-GGGTCAGCTAATCA CGCTGCTTCTTGCTCTT-3

PCR products were cloned in pET200/D-TOPO vector and the cloned vector used to transform competent *E. coli* One Shot® TOP10.

Positive transformantswere analyzed using PCR and restriction analysis and the correct recombinant plasmids were trasferered into BL21 Star™ (DE3) One Shot® *E. coli* for protein expression.

3.4. EXPRESSION and PURIFICATION of rCpPLD, rCpPLD I and rCpPLD II.

For the expression of the rCpPLD, rCpPLD I and rCpPLD II, bacteria were exponentially grown in Luria broth at 37° C and induced with 0.5 mM IPTG for 1, 2 and 3 h to set up the time course of the expression's protein. Cells were harvested by centrifugation (6000 x g, 4° C), and aliquots of bacterial pellet and 1ml of supernatant sample for each point of induction precipitated overnight at -20° C with three volumes ice-cold acetone, were analyzed by western blot.

For large scale production of protein purification, 1L of bacterial culture induced for 1 h was centrifuged and the pellet was resuspended in 20 ml of ice-cold lysis buffer (50 mM Tris-HCl, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8) in presence of protease inhibitors (complete cocktail tablets, Roche Diagnostics, GmbH, Germany) and sonicated in ice. After sonication 1% SDS was added. The bacterial lysate was gently mixed at room temperature for 1 h, and subsequently subjected to centrifugation at 15,000 x g for 30 min to remove cellular debris. The supernatant was filtered through a 0.45 μm pore size membrane and overnight dialyzed in

10mM phosphate-buffered saline (PBS), pH 7.0 with protease inhibitors at room temperature. The rCpPLDs carrying the His6 tag at their N-terminus was purified in native condition by nickel-nitrilotriacetic acid-agarose (Ni-NTA) affinity chromatography.

The dialyzed supernatant was supplemented with Ni-NTA resin and gently mixed at 25° C for 2 h. The lysate- Ni-NTA mixture was loaded into a 15-ml column and washed with 50 mM Tris–HCl, pH 8, 300 mM NaCl, 25 mM imidazole. The fusion protein was eluted from the column with 300 mM imidazole. All recombinant proteins were extensively dialyzed at 4° C in PBS, and stored at -70 °C until used. The proteins concentration was determined by the method of Bradford (Bradford 1976).

3.5. MOUSE IMMUNIZATION and IMMUNOGENICITY ASSAY USING rCpPLD

Only purified protein rCpPLD was mixed with complete Freund's adjuvant (Sigma Chemical Co.) and used to immunize CD2F1 mice (5 animals, 20 μg of protein each). At weekly intervals, three additional boosts of purified antigen (20 μg) in incomplete Freund's adjuvant (Sigma Chemical Co.) were given to each animal. After two additional weeks the animals were bled, and approximately 0.7 ml of polyclonal antiserum was obtained from each animal and stored at -20° C. Specificity of the antibody against the rCpPLD was assessed by immunoblotting.

Antibody titers were measured by an enzyme-linked immunosorbent assay (ELISA).

Briefly, polystyrene microtiter plates (Greiner Labortechnik) were coated overnight at 4° C with 150 ng of antigen dissolved in 100 ml of 0.05 M sodium carbonate (pH 9.6). After a wash in bovine serum albumin–PBS blocking solution, 100 ml of twofold dilutions in PBS-0.05% Tween 20 of serum from immunized animals was applied to the plate in triplicate and incubated at 37° C for 2 h. Pooled serum (diluted 1:2) from non-immunized mice was used as a negative control. After three washes with PBS-Tween 20, a 1:20,000 dilution in PBS of alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Sigma) as the secondary antibody was added to wells (1 h at 37° C), and the reaction was developed with p-nitrophenylphosphate (SigmaFAST tablets; Sigma Chemical Co.) as the substrate. Titers were defined as the highest dilution of mouse serum which gave an optical reading of at least twice the reading of the negative control ±3 standard deviations (SD).(A_{405}>0.07).

3.6. GEL ELECTROPHORESIS and WESTERN BLOT ANALYSIS

Purified rCpPLDs, Hep2 cells infected with *C. pneumoniae* and BL21 Star *E. coli* CpPLDs, were separated on 12% polyacrylamide gel (SDS-PAGE), while a 20 % polyacrylamide gel (SDS-PAGE) was used for rCpPLD I and rCpPLD II. Gels were stained with silver stain (BioRad Laboratories, GmbH, Germany) or transferred to a nitrocellulose membrane (Amersham).

Western blots were performed using either a commercial mouse monoclonal antibody recognizing the His6-tag epitope (1:3000; Qiagen, GmbH, Germany) for all proteins, while the mouse anti-rCpPLD polyclonal antibody (1:1000) generated in this study was used for only CpPLD.

An anti-mouse IgG preparation conjugated to alkaline phosphatase (Sigma Chemical Co.) was used as secondary antibody at a concentration 1:8000.

3.7. CONSTRUCTION of the RNA *Cppld* and *16S rRNA* STANDARD CURVES

Two different RNA standard curves were generated from the *Cppld* and *16S rRNA* genes of *C. pneumoniae*. Briefly, on the basis of the relative gene sequences of *C. pneumoniae* CWL029 (accession number AE001363 locus tag CPn0329 for *Cppld* gene and CPnr01 for *16S rRNA* gene) two set of pair primers were designed into their internal region (Table 2) and the relative PCRs were performed. The PCR products were directionally cloned in PGEM-T easy vector under the T7-promoter transcription.

The recombinant plasmids were linearized with the *Spe* I restriction enzyme and purified from agarose gel using the Nucleospin Extract (Macherey-Nagel GmbH, Duren, Germany). The in vitro transcription was performed with the commercial kit Riboprobe System T7 (Promega, Madison, WI) and the specific RNA transcripts were treated with DNase I (RNase-free; Fermentas, GmbH, Germany.) and purified with the Nucleospin RNA II (Macherey-Nagel GmbH, Du¨ ren, Germany). The concentration of RNA was spectrophotometrically quantified after incubation at 60° C for 5'.

The measurement of the RNA transcripts were performed in triplicate and then converted to the molecule number by the application of the following formula [N (molecules per µl) = (C/K) x F] where C is the concentration of RNA (µg/µl), K is the fragment size (bp) and F ($182.5x10^{13}$) is the numeric factor derived from the molecular mass and the Avogadro constant. The dilution series (10^2–10^{11}) were aliquoted and stored at –70° C until used with 40U of RNase inhibitors (Fermentas, GmbH, Germany). Transcript specificity was confirmed by quantitative real-time PCR without reverse transcription using the relative pair primer sets.

3.8. RNA ISOLATION, QUANTITATIVE REVERSE TRANSCRIPTION REAL-TIME PCR and QUANTITATIVE REAL-TIME PCR

The total RNA was extracted from non-infected and infected cells at specificity time points of *C. pneumoniae* infection, using the Nucleospin RNA II according to the manufacturer's instructions with the protocol for bacterial cells. The extracted RNAs were treated with DNase I to eliminate the contaminating DNA and purified again with the Nucleospin RNA II columns. The concentration of RNA was quantified by spectrophotometry, and RNA was stored at –70° C with 40U of RNase inhibitors until used. The resulting DNA-free RNAs were confirmed by the quantitative real-time PCR using primers for the 16S rRNA gene (Table 2).

One-step quantitative reverse transcription real-time PCR was conducted using QuantiTaq probe RT-PCR (Qiagen, GmbH, Germany). The quantitative real-time PCR were also performed in parallel for each sample to check the possible DNA contamination in the RNA preparation using QuantiTaq probe PCR (Qiagen, GmbH, Germany). The reactions were carried out according to the manufacturer's guidelines for the LightCycler instrument in a final volume of 20 µl, and the relative reaction mixture and the amplification procedure for the qrt-RT-PCR and for the qRT-PCR are listed in Table 2.

To prevent carryover contamination, an aerosol-resistant tip was used in all steps and the preparation of the PCR mixture was performed in a separate area. All reactions were performed in triplicate and sequence-specific standard curves were generated using 10-fold serial dilutions (10^2 to 10^8 copies/µl) of the specific RNA standards. The number of copies of each sample transcript was then determined with the aid of the LightCycler software. The mean of *Cppld*-mRNA copy number values were divided by the corresponding mean of the *16S rRNA* transcripts to normalized the number of chlamydial bodies present at each point of infection.

Table 2

Specific primers/Taqman-probes[a] and the quantitative reverse transcription real-time PCR (qrt-RT-PCR) and quantitative real-time PCR (qRT-PCR) amplification procedures

Gene (size bp)	Primer/probe[b] sequence 5′–3′	qrt-RT-PCR-	qRT-PCR
16S rRNA (266 bp)	Forward AAATACAGCTTTCCGCAAG Reverse ATTTGCTCCATCTCACGAT Probe CCCTTATCGTTAGTTGCCAGCA CTT	Mixture: primer/probe: 1 μM/0.5 μM 2 × QuantiTech probe RT-PCR Master Mix: 10 μl QuantiTech RT Mix: 2U RNA template: 2 μl	Mixture: primer/probe: 1 μM/0.5 μM 2 × QuantiTech probe-PCR Master Mix: 10 μl RNA template: 2 μl
CpPLD (249 bp)	Forward AAGAAAGATGCTTGGCTAGG Reverse AGCAAACATAGCAACTTGGA Probe CATTCTAGGAATGCATAGCTCG GAGC	Program One cycle at 50 °C for 30 min; one cycle at 95 °C for 15 min; 45 cycles: 94 °C for 0 s, 51 °C for 20 s and 60 °C for 40 s with the ramp of 20 °C/s for each cycle.	Program one cycle at 95 °C for 15 min; 45 cycles: 94 °C for 0 s, 51 °C for 20 s and 60 °C for 40 s with the ramp of 20 °C/s for each cycle.

[a]Primers and probes were designed on the basis of the gene sequences of *C. pneumoniae* CWL029 (accession number AE001363 locus tag CPn0329 for *pld* gene and CPnr01 for16S *rrna* gene).

[b]The 5′ end of probes was labeled with 6-FAM whereas the 3′ end was labeled with TAMRA.

3.9. IMMUNOFLUORESCENCE MICROSCOPY

Infected or uninfected Hep-2 cell monolayers were fixed in methanol for 1 h at room temperature. Commercial murine monoclonal antibody specific for major outer membrane protein (MOMP) of *C. pneumoniae* (Imagen *Chlamydia pneumoniae*, Dako Diagnostics GmbH, Germany) or rabbit anti-Chlamydia polyclonal antibody (Abacam Cambrige, UK) were used according to the manufacturer's instructions. The murine hyperimmune serum against rCpPLD was used as reported below. Fixed cells were:

(i) blocking for 2 h at 37° C with 3% BSA in PBS,

(ii) incubation with primary antibodies (1:10) for 2 h at 37° C

(iii) incubation for 1 h at 37° C with the secondary anti-mouse IgG FITC conjugated antibody (1:60; Sigma Chemical Co.) or anti-rabbit IgG TRITC conjugated antibody (1:60; Sigma Chemical Co.) and with or without 0.01% Evan's blue (Sigma Chemical Co.).

Hoechst (Blue, Sigma Chemical Co.) was used to visualize DNA. Each step was followed with three wash in PBS (10 min each) and two wash in H_2O (5 min each). After, microscopy fluorescence was performed with a Leitz Diaplan fluorescence microscopy (magnification, x1000; Leica Microsystems GmbH, Germany) connected to an Olimpus Camedia C5050 Zoom digital fotocamera.

3.10. PHOSPHOLIPASE D and CL SYNTHETASE ASSAYS FOR ENZYMATIC ACTIVITY DETECTION.

Phospholipase D assay was performed using Amplex® Red Phospholipase D Assay Kit (Invitrogen - A12219). Kit provides a sensitive method for measuring phospholipase D (PLD) activity in vitro using a fluorescence microplate reader or fluorometer.

The activity of CL synthetase of rCpPLDs, were detected as described previously by De Siervo and Salton (De Siervo *et al.* 1973) using a modified protocol. The assay mixture contained 0.3 mM -phosphatidylglycerol [1-(3-sn-Phosphatidyl)-rac-glycerol - BioChemika], 200 mM Tris-hydrochloride buffer, pH 7.0, and 0.25% Triton X-100 and 200 μg for each protein and 200 μg of Albumin from bovine serum (BSA) as negative control.

Solution and sample were added to produce a final volume of 0.2 ml. Samples were incubated at 37° C for 60 min, and then the reaction was terminated by the addition of chloroform, methanol, and water 65:25:4.

Mixtures were incubated at 4° C overnight, and later were vortexed for 10 sec to extract lipids and then subjected to a brief centrifugation. The upper phase was recovered and the

chloroform phase was evaporated. The lipids were dissolved in chloroform-methanol 10 ul (2:1, v/v) and separated by thin layer chromatography (TLC).

3.11. THIN LAYER CHROMATOGRAPHY (TLC)

The TLC of phospholipids was performed using silica gel G plates with the following solvent systems: chloroform/methanol/water (65:25:4). The plate was washed the day before with isopopanol and then dry on the air.

The plate was activated before use by heating for 40 min at 100°C and cooled. The eluent solution was chloroform, methanol and water 65:25:4 and 0.25% KCl.

The phospholipids, CL (Sigma) and phosphatidylglycerol were revealed by using molybdenum blue spray reagent (M1942 Sigma).

3.12. LINEAR B CELL EPITOPE MAPPING and SYNTHESIS of PEPTIDES

To define potential antigenic determinants of the CpPLD protein sequence it was used the BcePred Prediction Server (Hall 1999).

Potential surface oriented regions were identified by constructing aerophilicity and hydrophilicity profiles of the protein. The BcePred server is able to predict epitopes with 58.7% accuracy using flexibility, hydrophilicity, polarity, and surface properties combined at a score threshold of 2.38. Peptides were synthesized by standard methods of solid-phase multiple peptide synthesis with an Fmoc strategy at the Inbios s.r.l. (Napoli, Italy).

3.13. COMPARATIVE 3D STRUCTURAL MODELLING

Because there are no experimental structural data as yet available for CpPLD, it was searched the Protein Data Bank (PDB) for structural homologues using the BLASTp option in the NCBI website (http://www.ncbi.nlm.nih.gov). The 3D structure was obtained from the most similar X-ray structure among PLD proteins with 2 HKD catalytic motifs. The best structure found was 1F0I.pdb, displaying a BLAST alignment score of 28.1 and an E value of 3.6. This template structure is the PLD from *Streptomyces* spp. strain PMF, which belongs to the super-family of PLD enzymes.

Target and template sequences were aligned using CLUSTALW and then the amino acid alignment was manually edited to maximize positional homology using the Bioedit program version 7.0.9.0 (Savarino *et al.* 2007).

The alignment was then submitted electronically to the Swiss Model server (ExPASy Proteomics Server http://www.expasy.org), which automatically generates a homology model

based on the template structure. The sequences of interest in the protein model were highlighted using Pymol program (PyMOL Homepage http://pymol. sourceforge.net/).

3.14. DOT BLOTTING

Hand-dropped dot blot was made on nitrocellulose membranes (Hybond-C extra, Amersham). The recombinant protein CpPLD (Saha *et al.* 2005) and the commercial Glutathione S-Transferase (GST) protein (Sigma Chemical Co.) were used as positive and negative controls, respectively. Proteins and peptides were immobilized in a membrane spot at concentrations of 2 mg and 5 mg, respectively. After being blocked with 3% BSA (Sigma) in TBS-T buffer [125 mM Tris base, 300 mM NaCl, 0.1% Tween 20 (pH 8.0)] for 3 h at room temperature, the membrane was probed overnight at 4° C with the murine polyclonal anti-CpPLD antibody (29) diluted 1:300 in TBS-T.

Bound antibodies were detected by incubation with a secondary antibody anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical Co.) at a concentration of 1:8000.

3.15. HUMAN SERA and IMMUNOBLOT ANALYSIS using rCpPLD

Human sera from a biological sample collection were randomly selected and examined for anti-*C. pneumoniae* IgG antibodies with a commercial MIF assay (Labsystems Oy, Helsinki, FI), according to the manufacturer's instructions, and titers >32 were regarded as positive. A total of 20 sera, 10 MIF negative and 10 MIF positive, were tested in western blot analyses with the recombinant protein.

One microgram of rCpPLD for each serum sample was separated in SDS-PAGE, electroblotted onto 5-mm nitrocellulose strip, pre-saturated with BSA and incubated for 3 h with human serum diluted 1:50 in PBS–1% BSA buffer. Bound antibodies were visualized with 1:15,000 alkaline phosphatase-coniugated anti-human IgG (Sigma Chemical Co.) and p-nitrophenylphosphate as the substrate.

3.16. SERUM SAMPLES from ACS PATIENTS and MIF ASSAY

A total of 100 sera from ACS patients were obtained from the Institute of Cardiology, Catholic University of Rome. All sera were selected on the basis of homogeneity for conventional risk factors such as atherosclerosis, hyperlipidemia, hypertension, C-reactive protein, diabetes and smoking. All ACS patients were also positive for IgG antibodies to *C. pneumoniae* HSP60 antigen (Ciervo *et al.* 2002).

One hundred control sera from healthy volunteers with no record of recent respiratory infection were also enrolled in the study. Patients' and control sera were examined for anti-*C. pneumoniae* IgG and IgA antibodies with a commercial MIF assay (Labsystems Oy, Helsinki, FI), according to the manufacturer's instructions. IgG or IgA titers >32 were regarded as positive.

Informed consent was obtained from all human subjects.

3.17. ELISA USING rCpPLD and SYNTHETIC PEPTIDES as ANTIGENS

Indirect ELISA was carried out using NUNC Maxisorp plates. Coating was performed overnight at 4° C in 50 mM $NaHCO_3$ buffer, pH 9.6, using 0.15 μg/well (1.5 mg/ml) of the recombinant CpPLD 0.2 mg/well (2 mg/ml) for the synthetic peptides and commercial GST protein as negative control.

Plates were washed three times with wash buffer (PBS, 0.1% Tween 20) and blocked with 3% ovalbumin (grade V) in PBS plus 0.15% Tween 20, for 90 min at 37° C. The plates were washed three times and incubated overnight at 4° C with 1:50 dilution of individual patients' sera in a blocking solution (PBS, 0.05% Tween 20, 0.1% ovalbumin). After three washes, plates were incubated with a dilution of 1:4000 in blocking solution of alkaline phosphatase-conjugated goat anti-human IgG antibody (Sigma Immuno Chemical; Sigma Chemical Co., St. Louis, MO). After incubation for 1 h at 37° C and next washes, substrate p-nitrophenylphosphate (SigmaFAST tablets; Sigma Chemical Co.) was added, and colour was left to develop for 30 min at 37° C prior to addition of NaOH at 1 N final concentration.

The ELISA reaction was measured at 405 nm absorbance (A_{405}). Each sample was assayed in duplicate in CpPLD-coated wells, as well in duplicate uncoated wells, as control. A positive sample was defined as one yielding an A_{405} value that was at least 3 SD above the mean value obtained with a panel of 30 randomly selected MIF-negative sera

3.18. DATA ANALYSIS

The statistical analysis of data obtained from ELISA absorbance measurements was performed by using the nonparametric ManneWhitney U test. Differences between proportions were calculated by the two-tailed χ^2 test. Correlation between variables was assessed with the two-tailed Spearman test. The statistical significance was set at $P < 0.05$.

4. RESULTS

4.1. SEQUENCE OF *C. pneumoniae* PLD (*Cppld*) STRAIN PAROLA

The sequence of *Cppld* gene cloned from *C. pneumoniae* strain Parola was found to be identical to the PLDs of *C. pneumoniae* CWL029 (accession number AAD18478) and *C. pneumoniae* J138 (accession number BAA98539) genes, while the identity was 99% with *C. pneumoniae* TW183 (accession number AAP98272) and *C. pneumoniae* AR39 (accession number AAF73666) genes.

As shown in Fig. 10, the CpPLD protein presents a 30aa putative signal peptide (underlined in the figure), suggesting the existence of an immature and a mature form of CpPLD proteins with a estimate molecular weight-isoelectric point of 40.35281 Da-9.75 and 36.98757 Da-9.27, respectively (expasy proteomics server: http://www.expasy.org).

Fig. 10 also shows the predicted secondary structure of the protein and 2 HKD motifs (boxed) which are conserved in all members of the PLD superfamily and are critical for biochemical activity (Ponting *et al.* 1996).

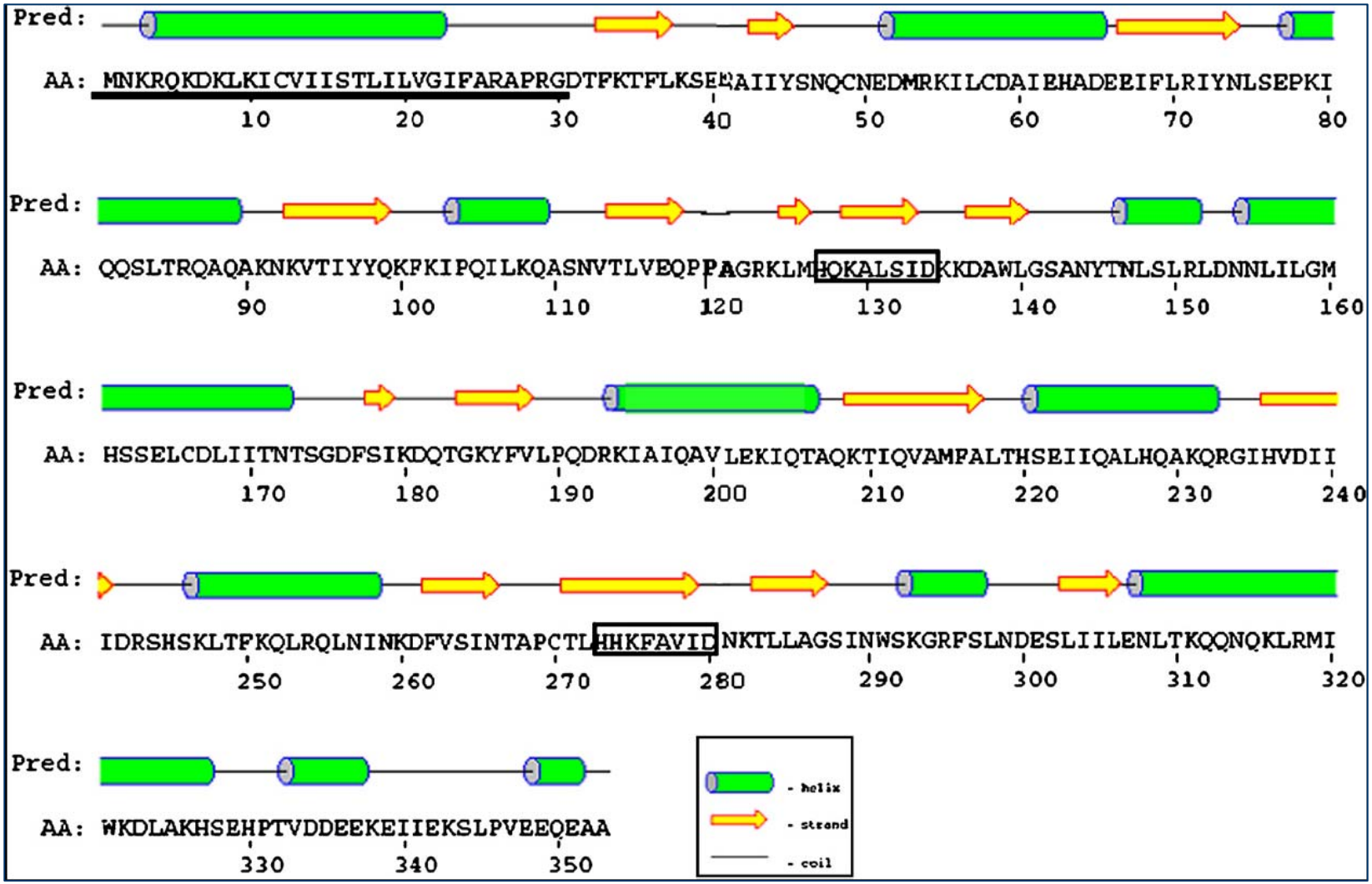


Fig. 10. Predicted secondary structure of the phospholipase D protein of *C. pneumoniae*. The Expasy proteomics server (http://www.expasy.org) was utilized to search the putative signal peptide (underlined) and the 2 HKD motifs (boxed).

4.2. EXPRESSION, PURIFICATION of RECOMBINANT rCpPLD, rCpPLD I and rCpPLD II PROTEINS.

A time course of the recombinant proteins expression in *E. coli*, at 1, 2 and 3 h after induction with 0.5 mM IPTG, was assessed by western blot analysis of the cell lysate, by using a commercial antibody recognizing the six histidine tail (His6-tag epitope) of the recombinant products. The proteins were seen to be mostly expressed during the first two hours of induction (data not shown).

Purification of the rCpPLD by nickel affinity chromatography generated two protein bands of 40 and 36 kDa (Fig. 11A), corresponding to the intact and the proteolytically digested protein, which are probably associated under native condition. As expected from the His6-tag epitope position, the immunoblot analysis with the anti-His6-tag antibody recognized only the intact form (Fig. 11B). On the contrary, recombinant epitopes rCpPLD I and rCpPLD II showed only one protein band for each domain of 26 kDa and 18 kDa respectively.

The dialyzed rCpPLD was used to immunize CD2F1 mice. The immunized animals produced elevated IgG levels, with a serum titer of 1:10,000 for all products.

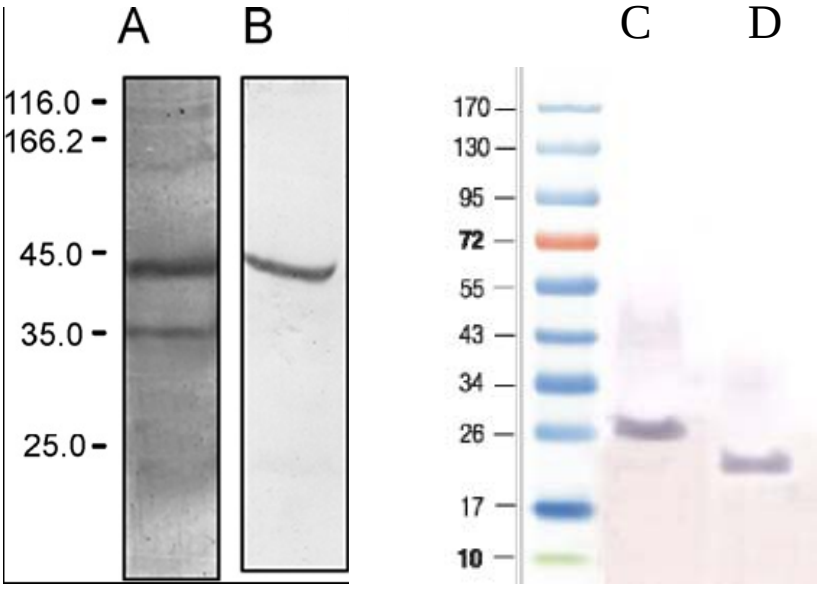


Fig. 11 Purification and Western blot analysis of rCpPLD. SDS-PAGE of the purified protein products (A). Immunoblot analysis of the purified recombinant protein with anti-His6 monoclonal antibody (B). Immunoblot analysis of the purified rCpPLD I (C) and rCpPLD II (D) with anti-His6 monoclonal antibody. The molecular weight protein standards expressed in kDa are shown on the left.

4.3. *Cppld* GENE TRANSCRIPTION and EXPRESSION in *C. pneumoniae*-INFECTED CELLS

To quantitative *Cppld* expression in *C. pneumoniae* infected Hep-2 cells, the reverse transcription real time PCR analysis was used. First, standard curves for *Cppld* and *16S rRNA* for copy numbers normalization were established, and serial dilutions (10^2–10^8 transcripts) of the respective RNAs obtained by *in vitro* transcription and checked for DNA contamination by quantitative real-time PCR were used. A strong linear relationship with a high regression coefficient was found in all experiments between log-copy numbers and the fractional cycle numbers (Fig 12 bis). Therefore, gene expression level was investigated by harvesting the cells at 8, 24, 48, and 72 h PI. The *Cppld* RNA transcripts were measured by quantitative reverse transcription real-time PCR in comparison with the standard curve in three independent runs and in three different experiments. The triplicate mean of RNA copy numbers was divided by *16S rRNA* copies to normalize against the number of chlamydial particles. As shown in Fig. 12 A the level of specific transcripts had a peculiar cyclic behaviour in as much as it increased from 8 to 24 h, decreased between 24 and 48 h and again increased from 48 to 72 h, meaning that the highest expression of *Cppld* gene occurs in the early and late chlamydial developmental cycle (Fig. 12).

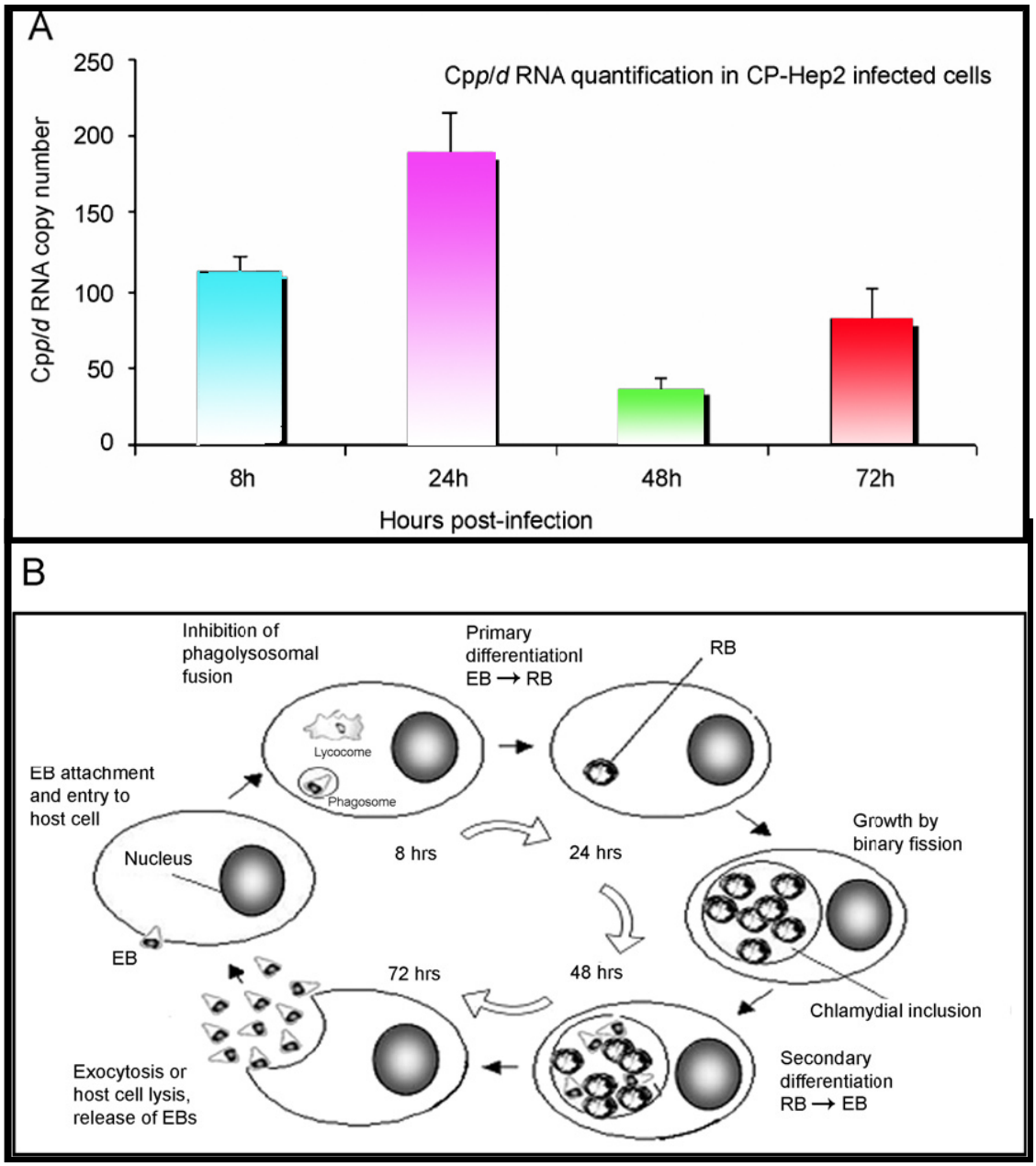
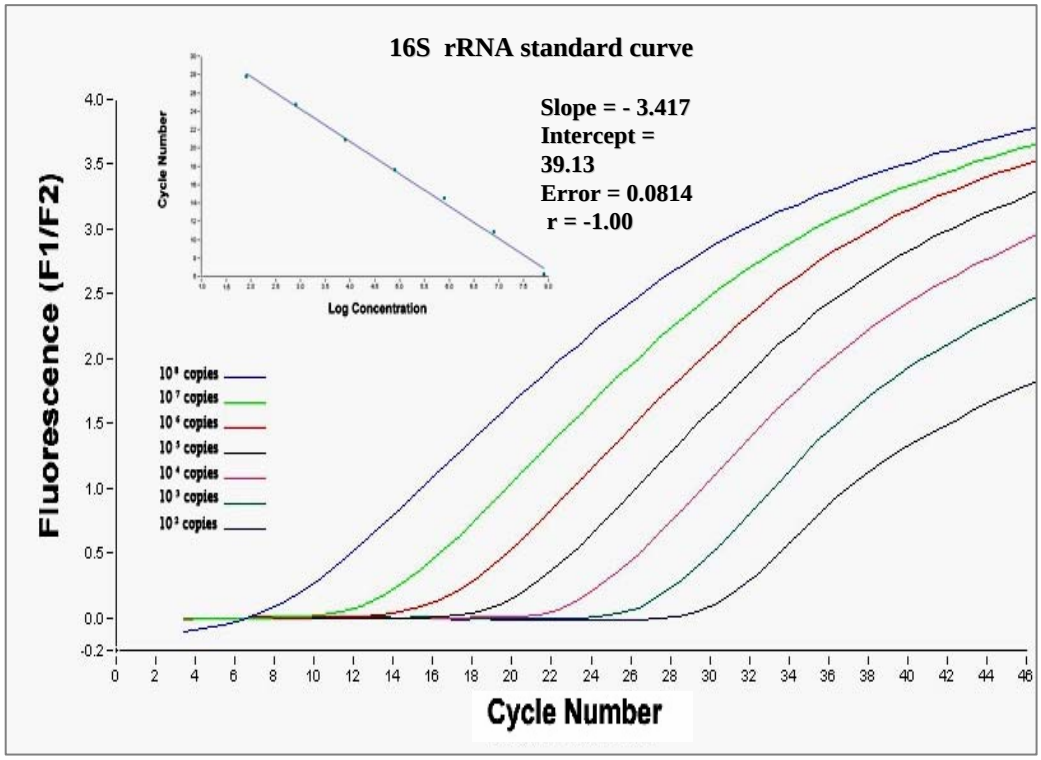


Fig. 12 Gene expression level in real-time, reverse transcription PCR. (A) Sample were taken at 8, 24, 48, and 72 h PI and the triplicate mean of RNA copy number values were divided by those for 16S rRNA to normalize for the number of chlamydial particles. The error bars are shown and indicate the standard deviation of transcript copy numbers.
(B) Representation of *C. pneumoniae* life cycle.



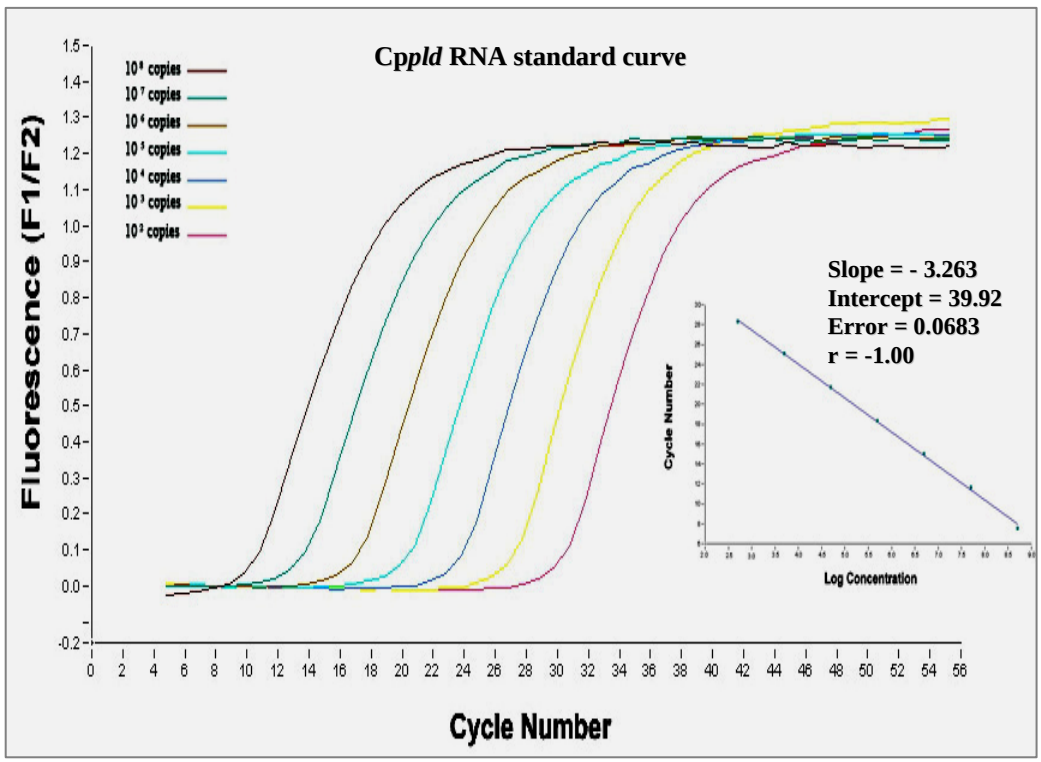


Fig. 12 bis Real-time, reverse transcription PCR. *16S rRNA* and *Cppld* RNA standard curve

Protein expression was also investigated by western blots of Hep-2 cell lysates with the anti- CpPLD antibody. Equivalent protein amounts of cell lysates, at each time point PI were loaded and blotted. This was compared with correspondent western blots of cell culture supernatants, which were previously centrifuged at 30,000 x g and precipitated in acetone. As shown in Fig. 13A the full-length 40 kDa and the truncated 35 kDa proteins were detected in all lysates, at each time point (Fig. 13 A, lanes 1, 2, 3, 4). In contrast, the 35 kDa of CpPLD protein was found in the corresponding supernatants (Fig. 13 B, lanes 1, 2, 3, 4), demonstrating that only the proteolytically-digested, mature form of the protein is secreted from the infected cells.

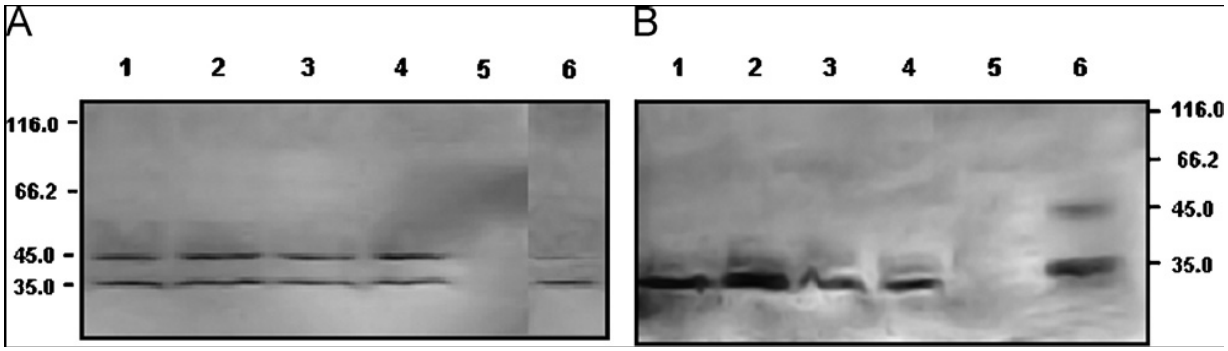


Fig. 13 Western blot analysis of the CpPLD protein expressed during the CP infection in Hep-2 cells. Samples were taken at 8, 24, 48, and 72 h PI and were probed with anti-rCpPLD polyclonal antibody. Hep-2 infected cells (A) and the respective supernatant culture precipitated in acetone (B) at 8 h (lane 1), 24 h (lane 2), 48 h (lane 3), 72 h (lane 4), the negative control-Hep-2 cells (lane 5) and the positive control-CP elementary bodies (lane 6). The molecular weight protein standards expressed in kDa are shown on the right and on the left.

4.4. CpPLD LOCALIZATION DURING INFECTION

Since *Cppld* gene transcription and immunoblot assays demonstrated that the CpPLD protein is expressed, it was attempted to establish possible differential localization of native CpPLD in Hep-2 infected cells, also in new of the above described time-dependent CpPLD protein expression throughout the developmental cycle of *C. pneumoniae* infection. Thus, Hep-2 infected cells at each time PI were fixed, treated with the anti-rCpPLD antibody, and observed by the fluorescence microscope. Aliquots of the cells were also treated with a commercial anti-MOMP monoclonal antibody as control. Coherently with the results shown above, the CpPLD protein was detected by immunofluorescence at all stages of infection. However, it was present within the inclusion bodies from 8 to 48 h while at 72 h it was mainly detected at the margins of the chlamydial inclusion (Fig. 14 A and C). The intracellular growth of the microorganism was monitored with the commercial anti-MOMP antibody.

As shown in Fig. 14 B, the size of inclusion increased until 72 h of infection, where many distorted and ruptured inclusions were observed.

4.5. ENZYMATIC ACTIVITY of the rCpPLD, rCpPLD I and rCpPLD II

Purified rCpPLD, rCpPLD I and rCpPLD II were analyzed with the phospholipase D kit, but any specific enzymatic activity was detected (data not show). The CL synthase assay was carried out using PG as a substrate.

As shown in Fig. 15 the whole protein and the two domains, show CL synthase-specific enzymatic activity because they catalyzed the reaction synthesis of CL from 2 molecules of PG (lane 3, 4, 5).

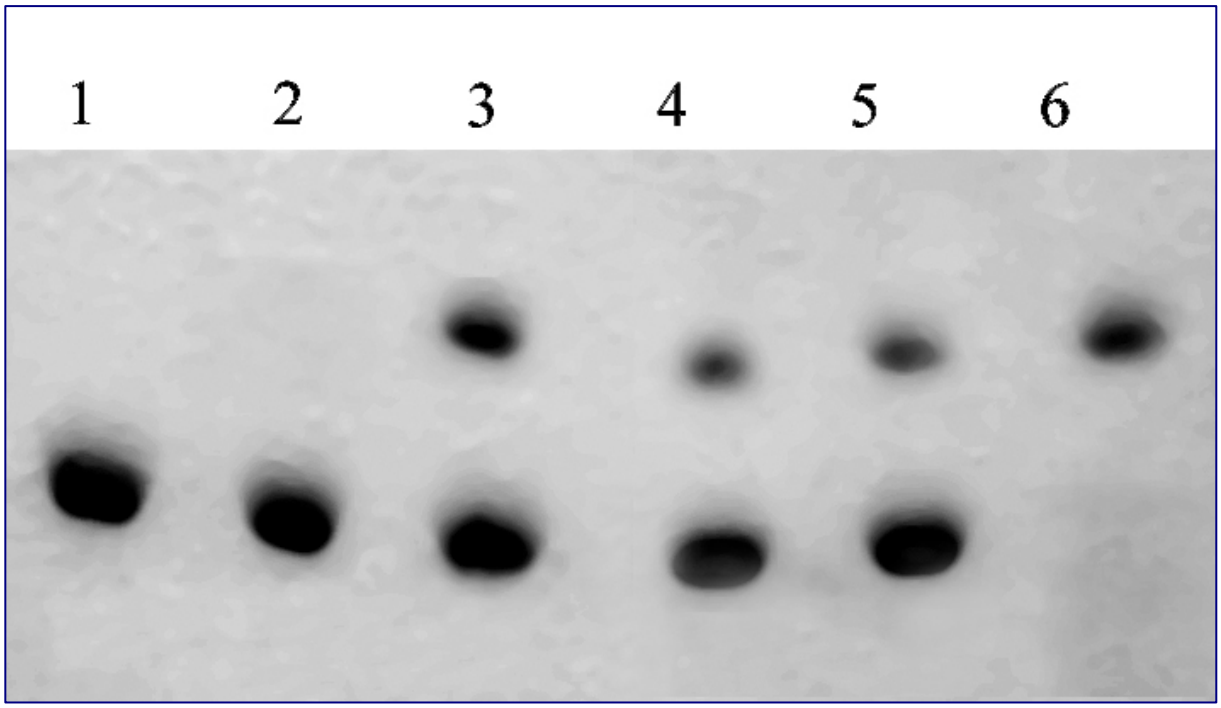


Fig. 15 CL synthetase enzymatic assay and TLC. The phospholipids, CL and phosphatidylglycerol (PG) were revealed by using molybdenum blue spray reagent. line 1 PG; line 2 BSA; line 3 rCpPLD; line 4 rCpPLD I; line 5 rCpPLD II; lines 6 CL.

4.6. CpPLD EPITOPE PREDICTION and 3D MODEL

Based on the bioinformatic algorithm described in Methods, seven linear epitopes with the maximum score of physico-chemical properties for serum immuno-assays were selected (Table 3).

Table 3. Amino acid sequences of synthetic peptides

Name	Length / Position	Sequence
P1	**14 aa / 20-33**	**LVGIFARAPRGDTF**
P2	**14 aa / 95-108**	**TIYYQKFKIPQILK**
P3	**14 aa / 143-156**	**ANYTNLSLRLDNNL**
P4	**20 aa / 187-206**	**FVLPQDRKIAIQAVLEKIQT**
P5	**20 aa / 233-252**	**RGIHVDIIIDRSHSKLTFKQ**
P6	**14 aa / 298-311**	**LNDESLIILENLTK**
P7	**15 aa / 324-338**	**LAKHSEHPTVDDEEK**

It was also calculated a protein model derived by comparative modelling using the Swiss Model Server. The root mean square deviation (RMSD) between a carbon atoms of the two aligned sequences was 0.40 A° with 1160 atoms involved (0.32 A° with 290 atoms). In this hypothetical 3D structure the seven epitopes are distributed throughout the whole protein surface and all are to some extent exposed and accessible to antibodies (Fig. 16 A and B, and Fig. 17).

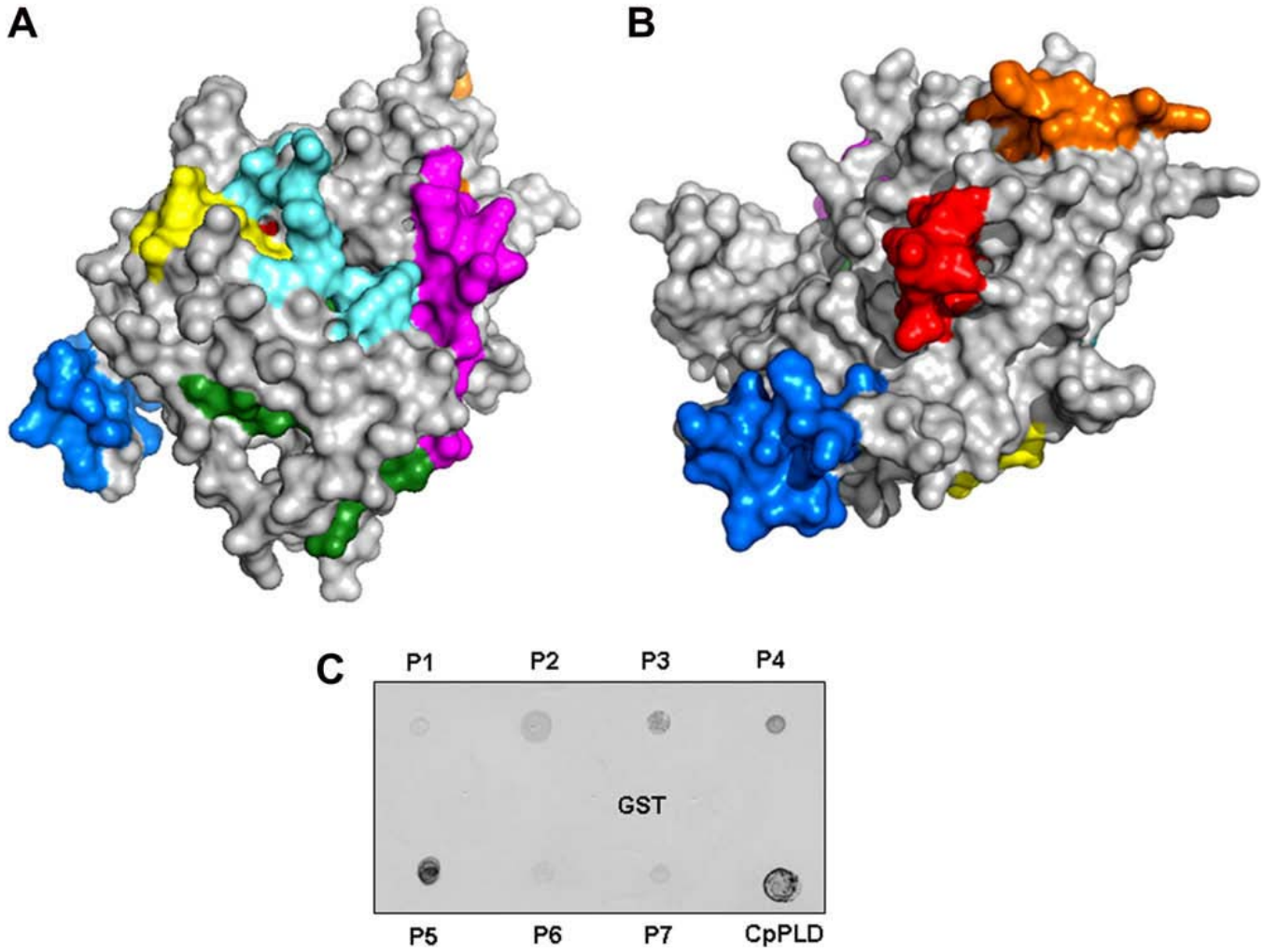


Fig. 16 Putative 3D structure molecular surface of CpPLD and epitope mapping of the peptides used in this study. A) P1 (cyan), P4 (pink), P5 (green), P6 (yellow), P7 (blue); B) P2 (orange), P3 (red), P7 (blue). C) Dot blot reactivity analysis of peptides probed against the polyclonal mouse antibody anti- CpPLD. The CpPLD recombinant protein and GST (in the center of the nitrocellulose membrane) were used as positive and negative controls, respectively.

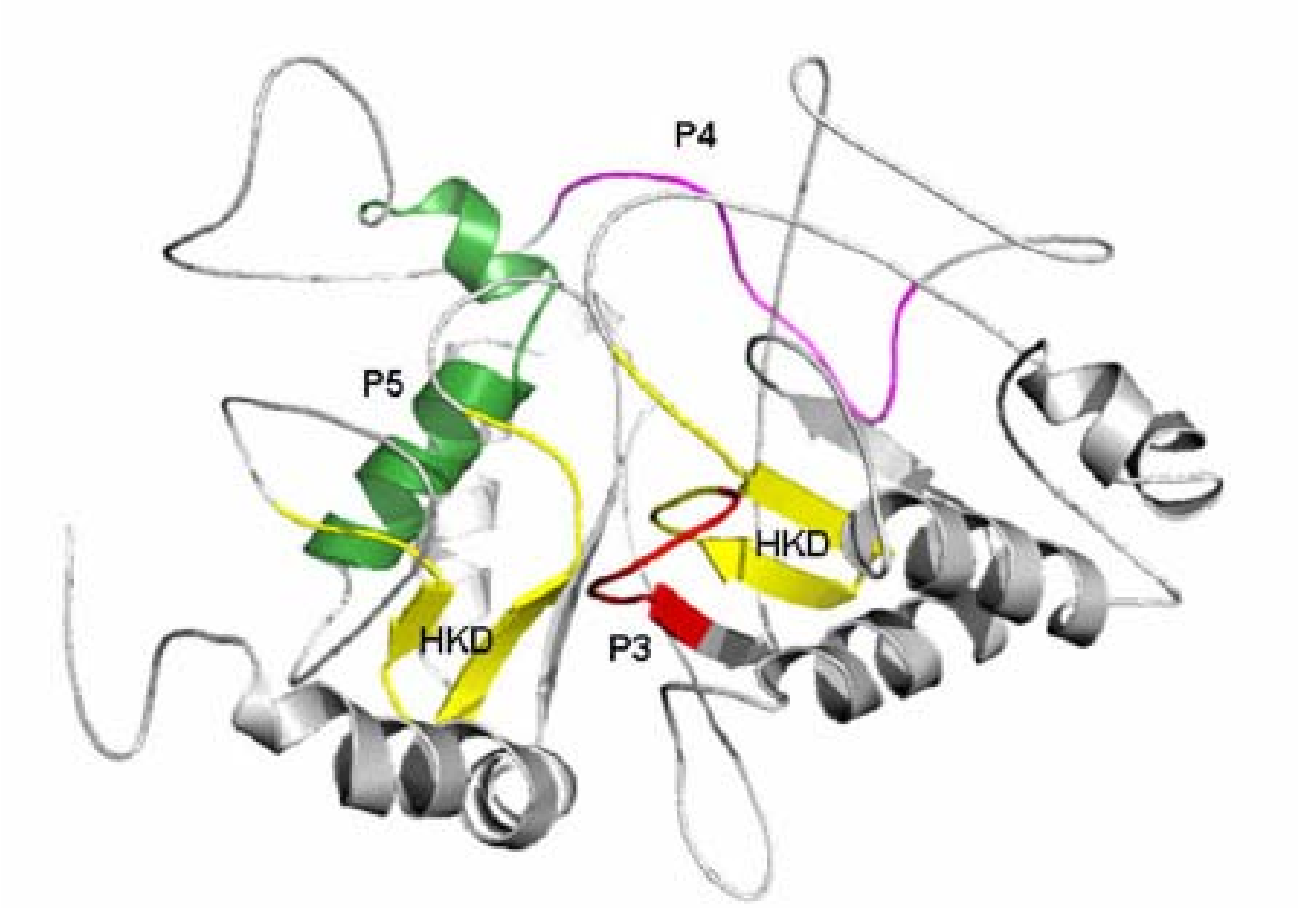


Fig. 17 CpPLD 3D model showing the two regions (yellow) containing the conserved catalytic triad residues (HKD). Helices are shown as spirals and b-strands as arrows. P3 (red), P4 (pink) and P5 (green).

4.7. DOT BLOT

To verify the surface display of our epitopes, we performed a dot blot where the peptides were spotted on a nitrocellulose membrane and probed with the previously described, anti-CpPLD mouse immune-serum. As shown in Fig. 16 C peptides P2, P3, P4 and P5 were well recognized by the specific antibody whereas peptides P1, P6 and P7 were weakly or not recognized at all (signals were detected with GST protein).

4.8. IMMUNOBLOT ANALYSIS with rCpPLD in HUMAN SERA from BIOLOGICAL COLLECTION

Western blot analyses were performed with randomly selected *C. pneumoniae* IgG-MIF-negative and positive human sera using the rCpPLD as antigen. All IgG-MIF negative sera were negative in western blot (data not shown), while 7 out of 10 Cp IgG-MIF positive sera recognized the 2 protein forms (Fig. 18). As a rule, the apparent intensity of the reaction of the individual human sera with the CpPLD protein was not associated with elevated IgG seroreactivity in MIF test, suggesting that the anti- CpPLD antibodies are not part of the antibody repertoire detected by MIF assay.

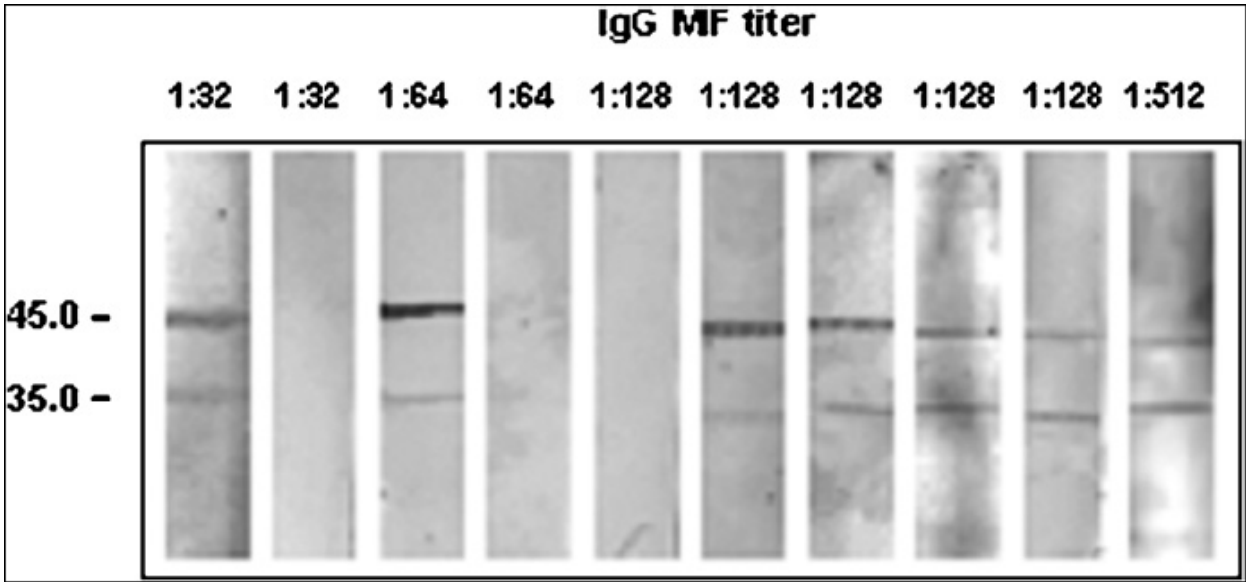


Fig. 18 Immunoblot for the detection of IgG antibodies to rCpPLD protein in CP-MIF positive sera patients. The IgG MIF titer for each sample and the molecular weight protein standards expressed in kDa are shown on the top and on the left of the figure, respectively.

4.9. ANTIBODIES AGAINST rCpPLD in ACS PATIENTS and in HEALTHY CONTROLS

Anti-*C. pneumoniae* and anti-CpPLD antibodies were assessed by MIF and ELISA, respectively, in sera of ACS patients and healthy controls. MIF positivity was more frequent in patients than in controls (82% vs. 40%). Among MIF-positive subjects, IgG plus IgA were frequent in patients (53 subjects over 82) and absent in controls.

Concerning anti-CpPLD antibodies, assuming a cut-off value of the ELISA reaction as A405 > 0.2 (see Methods), no antibodies were found in any MIF-negative sera (data not shown), whereas antibodies were detected in MIF-positive sera (15% in controls and 65% in ACS patients; Table 4).

Table 4 MIF and ELISA data in healthy controls and ACS patients.

SUBJECT	SEROLOGICAL ASSAY			
	MIF -	MIF +		ELISA (CpPLD IgG)
		IgG	IgG + IgA	
HEALTHY CONTROLS	60/100	40/40	0/40	15/100
ACS	18/100*	82/82	53/82*	65/100*

MIF-positive serum sample was defined as having a titer ≥ 32. An ELISA positive sample was defined as one yielding an A_{405} value that was at least 3 standard deviations (SD) above the mean value obtained with a panel of 30randomly selected MIF-negative sera.
+ = ¼ positives; - = ¼ negatives; MIF ¼ microimmunofluorescence; *P < 0.001 determined by χ^2 test (ACS vs. healthy controls).

Notably, the percentage of ACS individuals with anti- CpPLD antibodies was higher in sera of subjects with MIF IgG + IgA than in patients with only MIF IgG (72% vs. 28%).

All sera from control subjects, irrespective of being MIF negative or -positive, contained very low quantity of anti-CpPLD antibodies, whereas higher levels of anti-CpPLD IgG were found in patients' sera. In fact, the median ELISA OD values and the interquartile range (IQR) were 0.3 (0.23-0.41) for controls and 0.5 (0.3-0.72) for patients with ACS. As shown in Table 5, the ACS patients with MIF IgG + IgA displayed an appreciable higher absorbance median titer in comparison to the same group with only MIF IgG (IQR 0.7 (0.4-1.1)).

Furthermore, a highly significant correlation was detected between IgG MIF assay and ELISA ($P < 0.0001$ with $r_s = 0.7$).

Table 5 Comparison of IgG ELISA CpPLD titers between ELISA positives and *C. pneumoniae* IgG or IgG + IgA MIF

GROUPS	ELISA + (*n*)	Median value ELISA titer (IQR)
HEALTHY CONTROL		
MIF IgG +	40	0.3 (0.23 – 0.41)
MIF IgG and IgA +	0	0
ACS		
MIF IgG +	18	0.5 (0.3 – 0.72)*
MIF IgG and IgA +	47	0.7 (0.4 – 1.1)*

+ = ¼ positives; MIF ¼ microimmunofluorescence; IQR ¼ interquartile range; *P < 0.001 vs. control groups.

4.10. REACTIVITY of ACS PATIENT SERA to SYNTHETIC PEPTIDES

Synthetic peptides were tested as antigens by ELISA against individual sera from randomly selected 20 patients with ACS and 20 healthy controls. Patients' sera were divided into 10 MIF + with IgG only and an equal number which were MIF + with IgG and IgA.

Individual serum samples were allowed to react with each peptide and the median ELISA values are shown in Fig. 19.

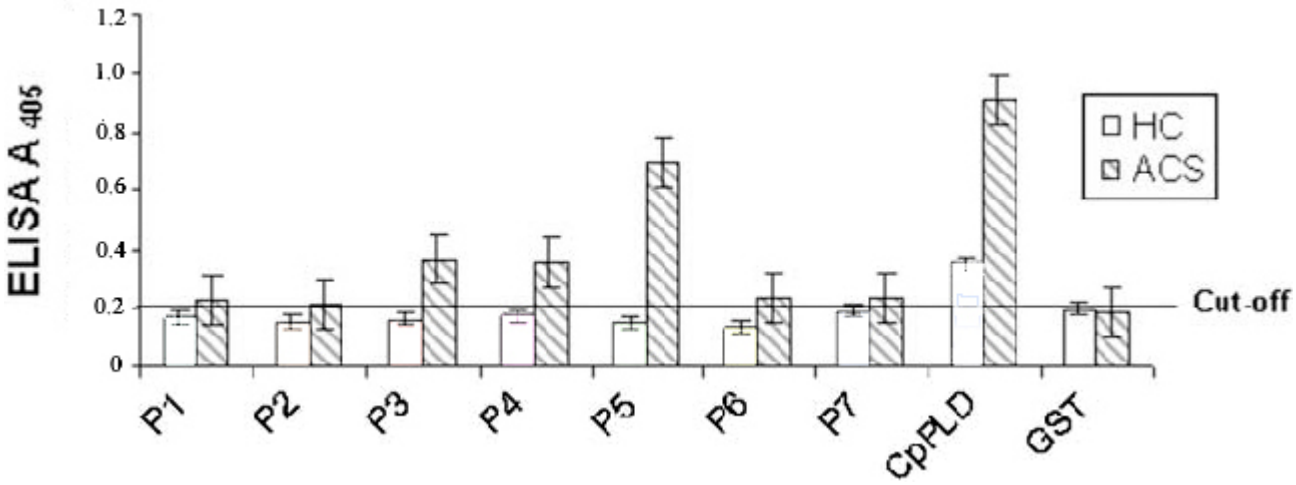


Fig. 19 IgG reactivity of patients and control group sera against the seven synthetic peptides. The absorbance median value in ELISA test of human sera against each peptide is represented and the cut off value is also shown. HC are the healthy controls and ACS are subjects with Acute Coronary Syndromes. The GST protein is the negative control. The error bars are shown and indicate the standard deviation of the absorbance.

Peptides P1, P2, P6 and P7 showed median OD values hardly exceeding the cut-off of 0.2 both in patient and control sera. Peptides P3, P4 and, mostly, P5 gave positive reactivity (median values 0.31, 0.33 and 0.68, respectively) with ACS but not with control sera. Among the ACS subjects, no significant difference in any anti-peptide reactivity was found between MIF + IgG and MIF + IgG plus IgA subjects.

5. DISCUSSION

Many bacterial PLDs have been studied such as the toxin of *Clostridium perfringens* which has been reported to be a major pathogenic factor in the development of gas gangrene (Sakurai 1995), the b toxin of *Staphylococcus aureus* (Doery *et al.* 1963) and other PLCs or PLDs from *Corynebacterium pseudotuberculosis* (Yozwiak ML *et al.* 1993), *Helicobacter pylori* (Slomiany *et al.* 1992), *Listeria monocytogenes* (Smith *et al.* 1995), as well as PLA and PLD from rickettsiae (Winkler *et al.* 1980; Renesto *et al.* 2003).

Sequence comparisons among PLD family members have identified two highly conserved duplicated regions contain the variant motif HxKxxxxD (Morris *et al.* 1997) (Fig.7).

All chlamydiae sequenced until now maintain at least one ancestral chromosomal PLD (Horn *et al.* 2004). Nevertheless, *C. trachomatis* (Carlson *et al.* 2005) and *C. muridarum* (Read *et al.* 2000) encode multiple PLD orthologs within the hypervariable region of the genome named the ‘‘plasticity zone (PZ)’’, which is involved in the pathogenesis and in the specific tissue tropism (Nelson *et al.* 2005). The PZ region is absent from *C. pneumoniae, C. caviae* and *parachlamydiae* (Read *et al.* 2003).

Several lines of evidence suggest that bacterial PLD may be involved in pathogenesis. Overall chlamydial PLDs could play a critical role during the persistent infection, and in particularly in those infections where lipid metabolism is affected and lipids are exchanged and modified with host cells (Nelson *et al.* 2006).

From this point of view, the CpPLD protein might have a role in the progression of the atherosclerosis and in the interaction/development of foam cells (Ninio *et al.* 2005).

To address the issue of the possible role of CpPLD in pathogenesis of chlamydial infection and its chronic persistent sequelae, it was considered as a priority to generate a recombinant form of PLD to use as a novel biochemical and immunological tool, and the study of PLD gene expression and localization throughout *C. pneumoniae* developmental cycle.

The primary structure of our recombinant protein showed a putative signal peptide of 30 aa and 2 HKD motifs that are required for the catalytic activity.

In *E. coli*, the rCpPLD is expressed at 1 h post induction with IPTG, and the full-length and the truncated forms were recognized by the anti-rCpPLD polyclonal antibody. However, after 2 h post induction the totality of rCpPLD is processed because only proteolitically digest form was detected.

On the other hand, in the natural system of *C. pneumoniae* Hep-2 infected cells, the two molecular forms were simultaneously present during the whole life cycle, probably because the two proteins need to be associated other for expression of enzymatic activity (Stuckey *et al.* 1999; Renesto *et al.* 2003; Yang *et al.* 2004; Ciervo *et al.* 2007). It was also shown that CpPLD is secreted in the medium, with a kinetic totally compatible with intracellular expression (Fig. 13).

It was also investigated quantitative *Cppld* gene expression by quantitative reverse transcription Real-Time PCR in culture of *C. pneumoniae* infected cells. The highest quantitative expression was observed in the early and late developmental cycle, respectively corresponding to the clamydial entry/EB to RB transition and the chlamydial exit or RB maturation into infectious EB (Fig. 12).

Similarly, the CpPLD protein was detected by immunofluorescence at all stages of infection. It was present in the inclusion bodies from 8 to 48 h while at 72 h the protein was distributed around the margins of the chlamydial inclusion (Fig. 14).

Overall, there is an apparent similarity with the localization of plasticity zone (PZ) phospholipase D of *C. trachomatis* recently reported by Nelson *et al.* in HeLa 229 cell line. In this latter study, a role for the PZ PLD family enzymes of *C. trachomatis* in pathogenesis has also been proposed (Nelson *et al.* 2006). Interestingly, at variance with *C. trachomatis* PLD which was expressed in the mid-to-late developmental cycle of life, with an increasing transcription profile starting 16 h post infection, the *Cppld* gene was expressed and the protein detected during the whole developmental cycle, suggesting that the protein might have partially different role in the two chlamydial species. The differential chlamydial PLD gene expression may reflect its putative role in lipid metabolism and the sophisticated lipid exchange between chlamydiae and host cells suggesting that PLD may mediate these interactions.

Another important aspect of CpPLD was the characterization of the enzymatic activity. This aspect may be important for the evaluation of the strain-specificity of chlamydial disease and the ability of chlamydiae to persist within the host, eliciting potent and chronic inflammatory responses, which are thought to be essential for pathogenesis (Hackstadt *et al.* 1995; Carabeo *et al.* 2003).

In general, classical PLDs are implicated in the acquisition of host ribonucleosides and processing of host lipids. It is notorious that chlamydiae incorporate a number of lipids from the host cell pool, including phosphatidylcholine, cardiolipin, and cholesterol (Nelson *et al.* 2006; Wylie *et al.* 1997; Kalman *et al.* 1999).

Sequence comparisons reveal a marked similarity between the CpPLD with others cardiolipin synthase CLs and in all these proteins the histidine residue within in the conserved HKD sequence is very important for the catalytic activity.

The enzymatic activity studies showed that the rCpPLD and in particular rCpPLD I and rCpPLD II products were able to synthesize CL from PG substrate and unable of the phospholipase degradation (Fig. 15).

Previous studies put evidence for the importance of both 2 HKD motif for CL synthesis. Here it was demonstrate that each one single HKD motif, in PLD I or PLD II, has an independent CL synthetase activity, but obviously a HKD omodimer formation for both PLD portions cannot be exclude.

Recent studies demonstrated that in spore membranes of *B. subtilis,* CL plays a role in an early step of germination, involving the functioning of nutrient receptors in the inner spore membranes (Hudson *et al.* 2001), and is indispensable in the exponential phase and during vegetative growth (Kawai *et al.* 2004).

This data may encourage the hypothesis that CL could be an important constituent for chlamydial aberrant forms, typical of the chronic infection. In addition, Byrne has proposed that inclusion membrane of Chlamydia may be a direct reflection of host mitochondrial membrane (Byrne 1988). In this study, it was been showed that HeLa cells infected with *C. trachomatis* provided evidence of alteration in glycerophospholipid, PG and CL, increasing the synthesis ex novo of PG an CL in mitochondrial remodelling membrane, also linked to an elevation in mitochondrial metabolism (Paradies *et al.* 1988).

These findings could have some relevance for host-parasite relationship, as also inferred by the elevated immunogenicity of the rCpPLD in mouse immunization experiments. More important was the CpPLD recognition by immune sera of the majority of subjects who had been infected by, or exposed to *C. pneumoniae*. In the context, it was investigated the anti-CpPLD immune response in healthy people previously screened by *C. pneumoniae* MIF serology (Ciervo *et al.* 2007).

The two PLD forms have always been simultaneously detected in the positive human sera indicating that the pattern of CpPLD secretion in humans, following infection or exposure, is consistent and comparable to the pattern of secretion from Hep-2 cultured cells in vitro.

While no apparent quantitative relationship was found between MIF IgG titer and immunoreactivity of human sera with CpPLD. In fact, 3 of 10 MIF-positive sera of healthy control group had no detectable antibodies against rCpPLD (Fig. 18). Besides possible erratic serum reactivity with our recombinant protein, all this suggests that CpPLD may be differentially expressed in the distinct pathologies caused by *C. pneumoniae*.

In the present study it was also constructed a plausible structural model of CpPLD and linear epitopes of this molecule were analyzed. The putative conformational peptides exposed to the surface were selected for their antigenic specificity, and a computational model was used to examine the three dimensional location of the epitopes (Fig. 16 and Fig.17).

Theoretical antigenicity and positions were experimentally verified by dot blot analysis and peptides P2, P3, P4 and P5 were effectively exposed, while P1, P6 and P7 were probably only partially accessible (Fig. 16). Establishing the surface accessibility of epitopes represents a first step toward validating predicted models of CpPLD tertiary structure (Mancini *et al.* 2009). The present study confirms the potential usefulness of such models for guiding future experimental design (Savarino *et al.* 2007).

For further immunological investigations, serum samples of ACS patients were analyzed against rCpPLD protein and its selected peptides (Fig.19) (Mancini *et al.* 2009). No anti-CpPLD antibodies were found in MIF negative subjects, indicate the specificity of the serological reactivity. It was also found that the median value of anti- CpPLD antibody titers were higher in patients with IgG + IgA positive MIF than in those with IgG only positive MIF (Table 5).

One possible explanation for the difference in the number of positive (and titer) of CpPLD ELISA in ACS patients with MIF IgG + IgA could be that the MIF IgA response occurs only in patients with a chronic *C. pneumoniae* infection, which is not always the case with the sole MIF IgG. On the other hand, in MIF positive patients with IgG + IgA, *C. pneumoniae* chronic or recurrent infections are a concrete possibility, that may lead to continuous stimulation of immune responses to CpPLD.

As expected, some (40/100) of our healthy controls had MIF-positive IgG, but a low or no anti- CpPLD titer, suggesting that past exposure or non-chronic infection does not consistently elicit persistent anti- CpPLD antibodies (Table 4).

Nonetheless, it would be unwise to speculate about the clinical significance of the CpPLD antigen as sensitivity marker in ACS patients chronically infected with *C. pneumoniae*, because the serum samples were too few to draw any solid conclusion on this aspect, particularly no distinction could be made between the different ACS subjects (stable or unstable angina and infarction) (Mancini *et al* 2009). Clearly, the determination of anti-CpPLD antibody prevalence warrants further large investigations in well-characterized categories of subjects affected by *C. pneumoniae* infection.

The peptide antigenicity was also investigated by a peptide-ELISA aimed at analyzing individual ACS serum samples. It was observed that peptide P5 reacted with the higher titer, and P3 and P4 responded moderately, whereas P1, P2, P6 and P7 showed low reactivity (Fig. 19). As mentioned before, peptide reactivity was supported from 3D modelling. As shown in Fig. 17, P5 and P4 are located on the same side of the protein while P3 is positioned in the putative catalytic site between the two HKD domains, thus being theoretically poorly accessible to antibodies.

Different hypotheses can be proposed to explain the observed response to peptides;

- A different protein folding, divergent from the 3D model proposed.
-Post-translational modifications of CpPLD may play a role in this phenomenon.
-Some peptide regions may not activate B cell responses.
-The CpPLD forms complexes (with CpPLD itself or other molecules) and that this could virtually mask the epitope(s) recognized by the specific antibodies.

This last event was clearly prospected in a previous work (Ciervo *et al.* 2007).

It was shown that during *C. pneumoniae* infection in Hep-2 cells, the two molecular forms of CpPLD (full length and proteolytically cleaved protein) were simultaneously present during the whole chlamydial life cycle, probably because the two proteins need to be associated for expression of enzymatic activity.

Another important immunological finding was that in ACS patients there were no significant differences between MIF and anti- CpPLD antibodies suggesting that our CpPLD ELISA has the potential, to reproduce the same results as those of the MIF assay. Because of this, the rCpPLD could be a promising antigen for future investigations aimed at establishing

a possible use in an ELISA for the diagnosis of the *C. pneumoniae* chronic infections in ACS patients.

This also applies to the possible use of one or more CpPLD peptides as specific diagnostic tool, in particular, the immuno dominant epitope P5 which virtually reproduces the same results obtained with the full length protein.

Overall, much more work is needed to elucidate the biological proprieties of CpPLD protein in the chlamydial life cycle and its possible pathogenetic role with host interaction, including the immune modulatory activity, and its potential deleterious effects on the vessel wall.

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