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New insights into the pathogenesis and treatment of chlamydial infections

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"*I feel that the greatest reward for doing is the opportunity to do more*"

Jonas Salk, 23 April 1956

ABSTRACT

Chlamydiae are obligate intracellular Gram-negative bacteria with a unique biphasic developmental cycle, alternating between infectious elementary bodies and replicative reticulate bodies. However, when exposed to stressful conditions such as iron deprivation and IFN-γ exposure, they fail to complete their developmental cycle generating morphologically aberrant reticulate bodies called persistent forms, which remain viable but non-infectious inside the host-cell for a long time and are difficult to eradicate with antibiotics. Chlamydiae cause a broad spectrum of diseases. *Chlamydia pneumoniae* causes community-acquired pneumonia and other respiratory tract infections, while *Chlamydia trachomatis* is the leading cause of sexually transmitted diseases all over the world and of trachoma in developing countries. More importantly, these pathogens may cause chronic sequelae. In fact, *C. pneumoniae* infections may be associated to atherosclerosis, whereas *C. trachomatis* infections lead to ectopic pregnancy, obstructive infertility and reactive arthritis. These sequelae could result from the inflammatory state induced by the persistent forms.

In our research, we studied *in vitro* different aspects of chlamydial pathogenesis and treatment in *C. pneumoniae* and *C. trachomatis*, achieving the following results. In *C. pneumoniae*, in order to investigate the atherogenic process, we set up a model of foam cell induction by means of macrophages infection. In this model, we highlighted for the first time the *C. pneumoniae*-dependent production of IL-17A, a cytokine recently reported as proatherogenic. Moreover, we investigated the protective effects of resveratrol, a natural polyphenol known to exert antioxidant, cholesterol-lowering and anti-inflammatory effects. Resveratrol is able to avoid foam cell formation in macrophages exposed to high levels of lipoproteins, with possible applications in the prevention of atherosclerosis. In *C. trachomatis*, we assessed the antichlamydial activity of the essential oil of *Mentha suaveolens*, in an effort to find out new means to prevent sexually transmitted diseases.

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1 INTRODUCTION

1.1 A brief history of Chlamydiae

Chlamydiae are obligate intracellular microorganisms, non-motile, non-spore forming, unable to live in the extracellular environment because they cannot produce and store energy (as adenosine triphosphate, ATP). The cellular structure of Chlamydiae is very similar to that of Gram-negative bacteria and although these microorganisms multiply by binary scission like other bacteria, they undergo a characteristic development cycle alternating two functionally and morphologically distinct forms: the elementary body and the reticulate body.

The name chlamydia derives from the Greek word klamis, which means mantle, and it is due to the presence of a thick vacuolar membrane, which surrounds chlamydial microcolonies in the host cell cytoplasm. Initially, Chlamydiae were considered as distinct and unrelated microorganisms rather than a single family because of the different medical cases, anatomical localisations, clinical symptoms and transmission routes that characterise them.

In 1890, sporadic cases of pneumonia were reported in traders and owners of exotic birds and, later, in 1907 the etiology of trachoma, which is a serious conjunctival infection known since ancient times (ancient Egyptians knew it since XVI century BC and even before Chinese had already known it from XXVII century BC), was attributed to Chlamydiae. In 1911, were observed cases of "inclusion conjunctivitis" associated with genital tract infections. In the years 1929-1930, following an outbreak of atypical pneumonia, studies focused on this disease began so that it was recognised as infectious. The causative agent was called *Rickettsia psittaci* and psittacosis the disease. Later, it was distinct from rickettsial agents, bacteria which live as obligate

intracellular parasites, for the fact of not being parasitic of invertebrates and therefore to not be transmitted to animals or humans through a vector.

Their obligate intracellular parasitism, their small size, their inability to grow on common culture media are features, which initially lead to the misidentification of Chlamydiae as unusually large viruses. The thirties and forties were a milestone in the history of Chlamydiae classification, because in those years began the first studies on trachoma and other diseases caused by these microorganisms (Eichmann, 1988). In this way, in 1966 it was possible to classify Chlamydiae as belonging to a single genus based on morphological and reproductive similarities that exist between the various species (Moulder, 1966; Page, 1966). Later, Storz and Page (1971) introduced the order Chlamydiales and two species: *Chlamydia trachomatis* and *Chlamydia psittaci*. In the following years have been recognised new families, genera and species.

In particular, information about the existence of strains of *Chlamydia pneumoniae* date back to 1943, when Smadel and colleagues described cases of pneumonia through the complement fixation test for psittacosis, in patients who had never been in contact with birds. It was initially thought to a strain of *C. psittaci* transmitted to humans. Later, in 1958 and 1960 were recorded cases of epidemic ornithosis in Finland (Allegra, 1995). In 1965 was isolated in Taiwan, from the conjunctiva of a child during the trachoma vaccine study, an unusual strain of Chlamydia called TW-183 (Grayston *et al*., 1986). Two years later, in Iran was isolated, from the conjunctiva of children, a strain of Chlamydia referred as IOL-207 (Dwyer *et al*., 1972). In 1978 in Finland, during an outbreak of pneumonia, was highlighted the presence of IgM antibodies directed against TW-183 (Saikku *et al*., 1985). From 1983 to 1986 in Seattle (USA) were isolated from throat swabs of patients with acute respiratory diseases, 12 strains of Chlamydia designated AR and LR

(Grayston *et al*., 1986) and after the union of the two acronyms, TW and AR, the strains were called TWAR (Taiwan Acute Respiratory).

Later, in 1989, after ultrastructural analysis and investigations about sequence homology of their genome, it was shown that TWAR strains have characteristics such as to be classified as a new species named *Chlamydia pneumoniae*, initially assigned to the genus Chlamydia and more recently to the new genus Chlamydophila (Grayston *et al*., 1989; Everett *et al*., 1999a) together with *C. psittaci*. However, the establishment of this new genus has not been universally accepted and both names are currently in use.

1.2 Classification

Until 1999, the classification of Chlamydiae was mainly based on morphological and phenotypic traits. The classification has recently undergone major changes on the basis of results obtained by the sequence analysis of the genes coding for ribosomal RNA (Grayston *et al*., 1989; Everett *et al*., 1999a; Bush & Everett, 2001). According to the previous classification, Chlamydiae belonged (Table I) to the order Chlamydiales, the family Chlamydiaceae and the genus Chlamydia which included four species: *Chlamydia psittaci*, *Chlamydia trachomatis*, *Chlamydia pneumoniae* and *Chlamydia pecorum*.

According to the current classification (Table I), four families were assigned to the order Chlamydiales: Chlamydiaceae, Parachlamydiaceae, Waddliaceae and Simkaniaceae but more recently have been proposed additional families. Currently, two distinct genera belong to the family of Chlamydiaceae: Chlamydia and Chlamydophila.

TABLE I. The order Chlamydiales: the recent taxonomic revision compared to the original classification (freely adapted from Bush & Everett, 2001).

			Typical host		
		Chlamydophila		**Chlamydia**	
		C. abortus	mammals		
		C. psittaci	birds	*C. psittaci*	
		C. felis	cats		
		C. caviae	Guinea pigs		
		C. pecorum	mammals	*C. pecorum*	
	Chlamydiaceae	*C. pneumoniae*	humans	*C. pneumoniae*	Chlamydiales Chlamydiaceae
Chlamydiales		**Chlamydia**			
		C. trachomatis	humans	*C. trachomatis*	
		C. suis	swine		
		C. muridarum	mice, hamsters		
	Parachlamydiaceae				
	Waddliaceae				
	Simkaniaceae				
New classification				**Old classification**	

The genus Chlamydia includes three species: *Chlamydia trachomatis*, *Chlamydia muridarum* and *Chlamydia suis*. *C. suis* has been isolated only from pigs, in which it causes conjunctivitis, purulent rhinitis, enteritis, pneumonia and bronchopneumonia. *C. muridarum* is the name given to two strains of bacteria isolated from mice and hamsters respectively. *C. trachomatis*, unlike the other two species of Chlamydia, is a strictly human pathogen, infects predominantly the conjunctival epithelium and the genital tract, thus causing infections in different apparatuses.

The genus Chlamydophila brings together the current species *Chlamydia pecorum*, *Chlamydia pneumoniae* and *Chlamydia psittaci* respectively called *Chlamydophila pecorum*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci* along with three new species, separated from the original *C. psittaci* and called *Chlamydophila caviae*, *Chlamydophila abortus* and *Chlamydophila felis* (Garrity *et al*., 2001; Grayston *et al*., 1989, Everett *et al*., 1999a; Bush & Everett, 2001). *C. pneumoniae* is an important agent of pneumonia in humans, and it has no known

reservoir in other mammals or birds, therefore, it is a strictly human pathogen like *C. trachomatis* and unlike the other species of Chlamydophila.

1.3 Morphology and cellular organisation

Chlamydiae present a cellular organisation similar to that of Gram-negative bacteria which differs mainly due to the lack of muramic acid and consequently of peptidoglycan in their cell wall (Ghuysen & Goffin, 1999; McCoy & Maurelli, 2006). An outer membrane and an inner membrane, delimiting a periplasmic space (Figure 1), constitute the cell wall of both elementary and reticulate bodies.

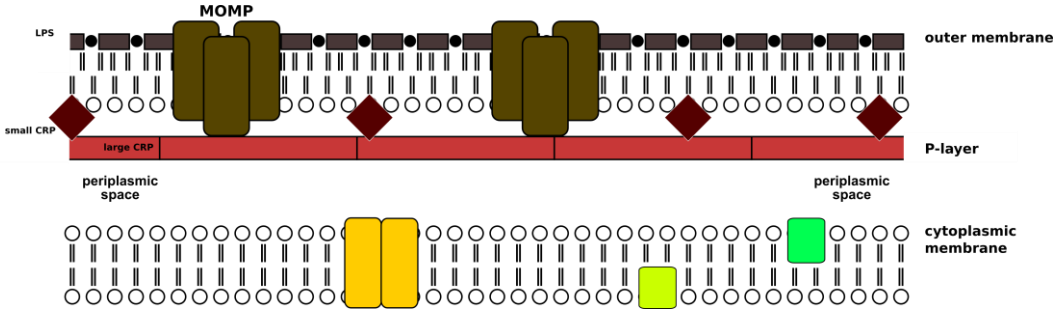

FIGURE 1. The current model of the envelope of chlamydial EBs (freely adapted from Hatch, 1996) showing only MOMP, large and small CRPs in the outer membrane and the periplasm, even though many other proteins are probably located there. The presence of a P layer, presumably consisting entirely of cross-linked large CRPs, has been confirmed by electron microscopy.

The outer membrane is composed of phospholipids (oriented in the inner wall to form a hydrophobic layer), lipopolysaccharide (oriented in the outer wall to form a hydrophilic layer) and lipoproteins. The lipopolysaccharide (LPS) is the major constituent of the outer membrane and appears approximately 30 hours after infection, in conjunction with the maturation of the inclusions, persists for the duration of the replicative cycle and is able to be embedded in the host cell membrane (Hatch, 1996; Brade, 1999).

LPS consists of an oligosaccharide core, the hydrophilic portion, known as keto-deoxyoctulosonate (KDO) region, from which originate antigenic polysaccharide chains and a lipid component, the hydrophobic portion, referred as lipid A. KDO region is essential for the survival of the bacterium, while lipid A represents the effective endotoxin. In particular, lipid A of *C. pneumoniae* possesses three epitopes similar to those identified in the lipopolysaccharide of other gram-negative bacteria, while the composition in terms of fatty acids is much more similar to that of *C. trachomatis* and *C. psittaci*. However, it differs from other Chlamydiales for the presence of long-chain fatty acids ranging from 22 to up 26 carbon atoms (Hatch, 1996).

The outer membrane proteins (OMPs) together constitute the so-called complex of the outer membrane, which includes the following components: major outer membrane protein (MOMP), cysteine-rich proteins (CRPs) and heat-shock proteins (HSPs).

MOMP, which has a molecular weight of 39.5 kDa (ranging from 38 to 42 kDA), represents about 60% by weight of the proteins in the outer membrane (Caldwell *et al*., 1981; Caldwell & Schachter, 1982). MOMP is a transmembrane protein containing four variable protein domains. This protein provides for the maintenance of the structural integrity of the membrane. In fact, each molecule of MOMP possesses at least three cysteine residues that, through disulfide bonds, are linked to other MOMP molecules and two CRPs to form aggregates (Newhall, 1987). In this regard, it had been initially proposed a model with a hexagonal structure that consisted of six MOMP dimers arranged around a central core (Chang *et al*., 1982). Moreover, MOMP has the function of a porin (with an exclusion limit of 850-2250 Da) allowing the passive diffusion of hydrophilic compounds according to their concentration gradients (Bavoil *et al*., 1984). Indeed, freeze-fracture studies have

highlighted the presence of a transmembrane channel (Louis *et al*., 1980) which has been proposed to be responsible for the porin function. Overall, the best model available to date is that of a trimeric porin unit constituted predominatly of a β-sheet structure (Sun *et al*., 2007). However, the actual structure of chlamydial MOMP remains elusive since the attempts to obtain X-ray crystals with native MOMP proteins have been unsuccessful, so the current knowledge comes from indirect studies and computational modelling (Feher *et al*., 2013).

CRPs with a molecular weight of 12 kDa (OMP3) and 60 kDa (OMP2), are responsible for the rigidity of the membrane due to the high number of intramolecular disulfide bonds which are able to form (Hatch, 1996). OMP3 (also called OmcA or EnvA) and OMP2 (also called OmcB or EnvB) are proteins that are common to all the species belonging to the family Chlamydiaceae (Everett & Hatch, 1995). In particular, OMP3 is a cysteine-rich lipoprotein anchored to the outer membrane through its lipophilic portion, while the hydrophilic segment extends to the periplasm (Hatch, 1996). OMP3 probably interacts with the host cell in the process of adhesion, by means of an amino acid sequence exposed on its hydrophilic surface.

HSPs represent a family of proteins synthesized 8-10 hours after chlamydial infection, and are involved in the preparation and assembly of proteins into multimeric complexes (Kuo *et al*., 1995). These proteins are expressed throughout the lifetime of the replicative cycle, at least for *C. pneumoniae* (Kuo *et al*., 1995). The most studied is the 60 kDa HSP (HSP60), which is responsible for hypersensitivity reactions, cross-reactivity and immunopathogenesis in chlamydial infections (Kuo *et al*., 1995). While, HSP75 is synthesized later during the replicative cycle and is a cytoplasmic protein.

Furthermore, in the membrane of the inclusions is present a group of membrane-associated proteins, called Inc (Subtil *et al*., 2001). These proteins may be

involved in the vesicular interactions with the host cell, the development of the inclusion, the avoiding of the lysosomal fusion, the acquisition of nutrients and signals associated with the differentiation of reticulate bodies into elementary bodies and vice versa (Scidmore *et al*., 1996; Wyrick, 2000; Subtil *et al*., 2001).

1.4 Antigenic structure

The antigenic structure of the Chlamydiaceae is characterised by the presence of various antigens, which are either genus-, species- or serotype-specific. The genus-specific antigen, common to all Chlamydiae, is of polysaccharide nature and thermolabile, is associated to the cell wall of both elementary and reticulate bodies, and more specifically is represented by LPS (Brade *et al*., 1987).

The polysaccharide portion of LPS presents two epitopes, one similar to that of Enterobacteriacea and the other specific to the family Chlamydiaceae, while the lipid portion presents three epitopes similar to those located on LPS of other bacteria (Schramek *et al*., 1980; Halme & Surcel, 1997). The most important antigenic group of LPS is represented by 2-keto-3-dioxaoctanoic acid (Subtil *et al*., 2001; Nurminen *et al*., 1983). But in addition to LPS were highlighted other genus-specific antigens in *C. pneumoniae* such as proteins with molecular weight of 12 and 26 kDa, and those ranging from 46 to 49 and from 65 to 70 kDa (Campbell *et al*., 1990).

MOMP presents species-specific epitopes and, in fact, the sequence homology between the different species belonging to the family Chlamydiaceae is rather low (Kuo *et al*., 1995), moreover several studies have shown that *C. pneumoniae* MOMP is less immunogenic than that of the other species. The low immunogenic power of *C. pneumoniae* MOMP, associated with a limited cross-reactivity with MOMPs of the other species, suggests that MOMP should present at least some genus-specific

antigenic determinants (Kuo *et al*., 1995). However, unlike the case of *C. trachomatis* where the genetic variability of MOMP leads to a clear division into serogroups, the ompA gene in *C. pneumoniae* appears to be highly conserved (Jantos *et al*., 1997).

In fact, *C. trachomatis* is subdivided into three biological variants (biovars): trachoma, lymphogranuloma venereum (LGV) and mouse pneumonitis (MoPn) agents. The first two biovars have been further separated into 15 serological variants (serovars) according to Wang and Grayston (1970): L1, L2 and L3 which cause LGV; A, B, Ba and C which are associated with trachoma whereas serovars D through K are commonly associated with sexually transmitted infections. However, the reliability of this distinction appears to be limited because, as highlighted by Byrne (2010), there is evidence that MOMP serovars fail to show relationships to virulence thus it is critical to extend studies beyond this paradigm.

In addition to MOMP, have been associated to the complex of the outer membrane also CRPs with a molecular weight of 98, 60 and 15.5 kDa (Kuo *et al*., 1995). The latter two proteins are similar in molecular weight and structure, to the proteins of the other Chlamydiaceae, while the 98 kDa protein is present exclusively in the complex of the outer membrane of *C. pneumoniae* (Campbell *et al*., 1990; Iijima *et al*., 1994; Kuo *et al*., 1995).

Many other antigens have been described so far, they are mainly species-specific proteic antigens or serotype-specific thermolabile antigens associated with MOMP, which are able to induce an immunogenic response.

In any case, even though Chlamydiae have many antigens in common, there are differences between the antigens of elementary bodies and those of reticulate bodies.

1.5 Development cycle

Chlamydiae have a quite limited metabolic capacity of energy production. This feature forces the bacterium to intracellular parasitism, while being capable of synthesizing macromolecules such as RNA and DNA autonomously. Chlamydiae take from the host cell not only ATP and other triphosphate cofactors (eg.: nicotinamide nucleotide, coenzyme A, etc.) necessary for the metabolism and replication but also amino acids, nucleotides and other precursors (Hackstdat, 1997; Greub *et al*., 2003).

However, it has been recently suggested that Chlamydiae are also capable of independently producing energy, since they possess a nearly complete tricarboxylic acid (TCA), glycolysis and pentose phosphate pathways for glucose catabolism (Vandahl *et al*., 2001; Maurer *et al*., 2007; Saka *et al*., 2011).

The characteristic that distinguishes Chlamydiae from all other bacteria is the mode of development, which occurs within the host cell where the microorganism is capable of alternating between two functionally and morphologically distinct forms (Figure 2): the elementary body (EB) and the reticulate body (RB) (Matsumoto, 1988; Rockey & Matsumoto, 2000). The main stages of the development cycle are as follows: adhesion, penetration, inhibition of lysosomal fusion and release of chlamydial progeny in the cellular environment (Rockey & Matsumoto, 2000) by either cell lysis or even a sort of budding.

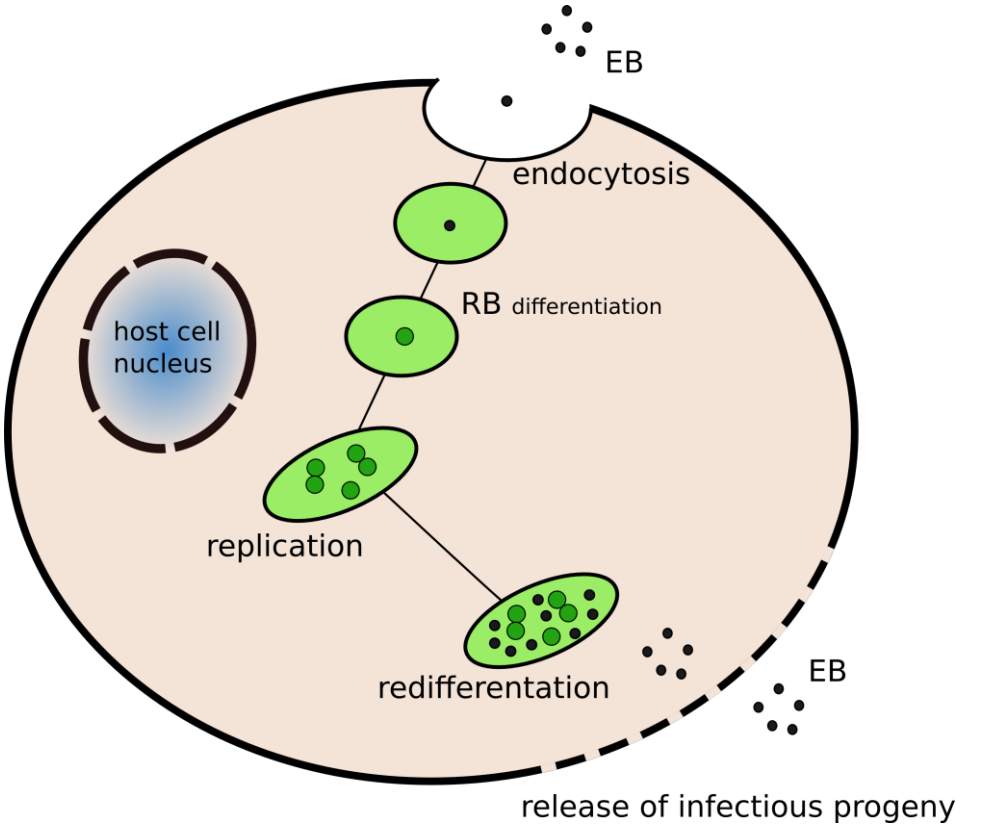

FIGURE 2. Schematic representation of the chlamydial developmental cycle. Infectious but metabolically inactive EBs enter the host-cell membrane via endocytosis; EBs transform into the replicative and metabolically active RBs; RBs multiply and then redifferentiate into EBs, which are released by the host-cell mostly via lysis.

EBs, about 300 nm in diameter, are found outside of the host cell and are adapted to extracellular survival, they are also metabolically inactive because their DNA is highly condensed and surrounded by basic histone-like proteins (Hc-1 and Hc-2). EB in fact represents the infectious form, which is able to begin a new replicative cycle (Dautry-Varsat *et al*., 2005).

The replication cycle begins when an EB adheres to a host cell. The adhesion to the host cell can be divided into two phases, the first one reversible and the second irreversible (Moulder, 1991; Dautry-Varsat *et al*., 2005). While in many microorganisms, the reversible phase is generally mediated by interactions with

glycosaminoglycans on the host cell surface, Chlamydiae appear to use a unique mode of attachment in which a glycosaminoglycan (specifically heparan-sulfate) is also located on the surface of the bacterium thus working as a bridge (Zhang & Stephens, 1992). Nevertheless, Chlamydiae should be able to utilise multiple means of entry since treatments to inactive the glycosaminonglycans on the chlamydial surface do not lead to a complete attachment inhibition (Rasmussen-Lathrop *et al*., 2000; Wupperman *et al*., 2001). In any case, the interaction between EBs and the cytoplasmic membrane is not casual, but occurs in correspondence of specific areas or lipid microdomains called rafts (Stuart *et al*., 2003). The rafts have a high content of cholesterol and glycosphingolipids, but contain a limited number of proteins.

If we consider the pear-shaped EBs of *C. pneumoniae* as a model, they are able to facilitate the adhesion by creating contact points on the host cell and favouring the formation of protrusions of its cell wall (Miyashita *et al*., 1993). The infectious particle is then internalised into the host cell, by a process of phagocytosis (endocytosis) in a specialised vacuole called phagosome, which in turn is formed by an invagination of the cytoplasmic membrane (Rockey & Matsumoto, 2000).

In the course of the intracellular cycle, EBs are confined within a phagosome, and then they are transported in a perinuclear location where they aggregate by means of a Ca^{2+}-dependent mechanism. In this way, a series of events occur that are fundamental to the survival of the bacterium such as the inhibition of phagosomal membrane fusion and remodelling of the inclusion membrane in order to favour the passage of nutrients, lipids and metabolites from the host cell cytoplasm. The inhibition of phagosomal membrane fusion and the control of the processes described above require *de novo* protein synthesis by Chlamydiae (Hackstadt *et al*., 1997). EBs, therefore, are able to evade the primary defence mechanism of the cell, because they

inhibit the phagosome-lysosome fusion by mechanisms not yet understood (Kaukoranta-Tolvanen, 1996; Hogan *et al*., 2004).

After about 10-12 hours after infection, EBs initiate the synthesis of nucleic acids and proteins, gradually increasing in diameter (from 0.6 μm to 1 μm) and transforming rapidly into larger forms called RBs. RBs are responsible for the intracellular replication (Kaukoranta-Tolvanen, 1996). The transformation of EBs into RBs involves a reduction in the number of disulfide bonds existing in the complex of the outer membrane, decondensation of the genome and beginning of the synthesis of mRNA and proteins.

RBs, metabolically active, are transported via a dynein-dependent mechanism in the proximity of the Golgi apparatus and replicate by binary fission. Some RBs after a certain number of divisions are redifferentiated into EBs. As the inclusion size increases, the number of EBs increases as well, and they localise generally near the edge of the inclusion. In this way, the inclusion contains three different types of bodies: reticulate, intermediate (a form of transition) and elementary. It could happen that within the same infected cell, there may be multiple inclusions arranged around the nucleus, similar to a string of pearls, but this is a peculiar characteristic of *C. pneumoniae*.

The replication cycle is completed when a new generation of EBs is released. The release of new EBs is generally accompanied by the host cell lysis. The duration of the replicative cycle depends on the nature of the host cell, but generally is about 48-72 hours (Kaukoranta-Tolvanen, 1996).

1.6 The chlamydial persistence

As previously mentioned, Chlamydiae have a unique replication cycle wherein the microorganism alternates two functionally and morphologically distinct forms: EB and RB (Matsumoto, 1988; Rockey & Matsumoto, 2000). However, it has been demonstrated that, during the replicative cycle, Chlamydiae can give rise to persistent forms (Figure 3) that are non-infectious, non-replicating and metabolically inactive (Beatty *et al*., 1994; Wolf *et al*., 2000; Hogan *et al*., 2004; Peters *et al*., 2005).

Ultrastructural analysis showed that the replicative cycle is stopped to the state of RB prior to its reorganisation into EB. Therefore, there is no host cell lysis and formation of new infectious progeny (Beatty *et al*., 1994; Mehta *et al*., 1998; Wiedeman *et al*., 2005).

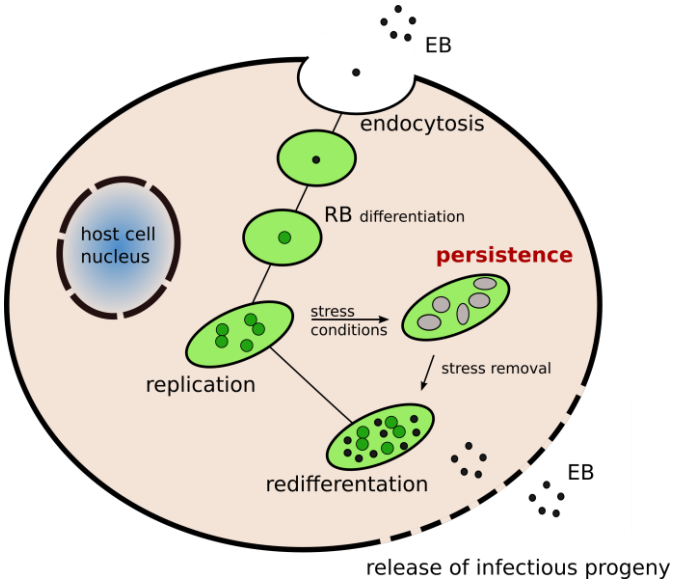

FIGURE 3. Schematic representation of the chlamydial developmental cycle showing that in the presence of IFN-γ, penicillin G or other stressful conditions, Chlamydiae give rise to non-infectious persistent forms, which may be reactivated following stress removal (Di Pietro *et al*., 2013e).

At a morphological level, the observation by light microscopy with direct immunofluorescence methods, showed smaller inclusions (Beatty *et al*., 1994; Wolf *et al*., 2000; Gieffers *et al*., 2004) which may have a lower fluorescence intensity compared to the replicative form (Goellner *et al*., 2006), although other authors reported larger pleomorphic inclusions (Gussmann *et al*., 2008; Kokab *et al*., 2010). Several ultrastructural studies, carried out by electron microscopy (Figure 4), showed that the inclusions of the persistent form contain, with respect to the replicative form, fewer RBs. These RBs, however, appear to be of a greater size (even up to 10 times), with a pleomorphic shape and a less electron-dense cytoplasm (Beatty *et al*., 1994; Mehta *et al*., 1998; Wolf *et al*., 2000). In some cases, these RBs, which are usually defined "aberrant", arbour electron-dense vesicles of uncertain origin and function, located mostly close to their plasma membrane (Wolf *et al*., 2000).

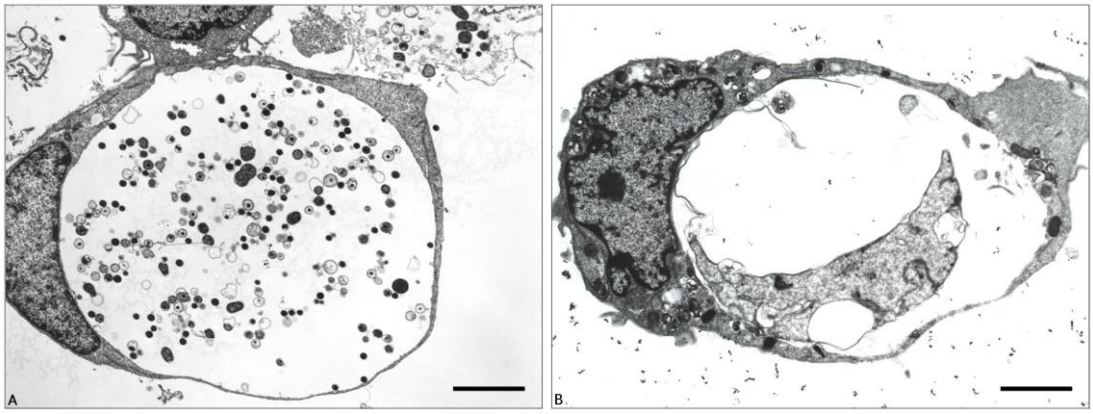

FIGURE 4. Electron micrographs of *C. trachomatis*-infected cells showing: (A) a typical inclusion containing EBs and RBs; (B) an inclusion containing enlarged, atypical RB forms resulting from penicillin G treatment (Skilton *et al*., 2009 licensed under the terms of the Creative Commons Attribution License). The scale bar represents 4 μm.

Several *in vitro* studies have reported a wide range of factors that can induce the formation of a persistent form (Beatty *et al*., 1994; Hogan *et al*., 2004). In particular, it has been shown that Chlamydiae may give rise to persistent forms as a result of depletion of essential nutrients such as iron, or following treatment with

antibiotics such as penicillin G or even after treatment with certain cytokines such as interferon-gamma (IFN-γ). It has also been highlighted the presence of persistent forms of *C. pneumoniae* after the establishment of continuously infected epithelial cell lines (a so-called model of spontaneous persistence). Furthermore, Chlamydiae in monocyte and macrophage cultures has the features of a persistent infection (Arienne *et al*., 1999; Mannonen *et al*., 2004; Beagley *et al*., 2009; Bellmann-Weiler *et al*., 2010). The latter evidence is particularly intriguing, because Chlamydiae are thought to use monocytes as a means of transport from the initial site of infection to distant anatomical districts.

In addition, it has been shown for the first time by Deka and co-workers (2006) that even coinfection conditions may give rise to persistence, when *C. trachomatis* infected cells were coinfected with HSV-2. More interestingly, it has been demonstrated later that defective viral particles are enough to trigger chlamydial persistence (Deka *et al*., 2007), and it is estimated that HSV-2 generates 50-200 defective particles per plaque forming unit.

The persistent forms, regardless of their inducing factor, retain a number of common features such as an aberrant morphology, a loss of infectivity, an altered gene expression pattern, a lesser susceptibility to antibiotics and finally a spontaneous reactivation after removal of the inducer (Gieffers *et al*., 2001; Wiedeman *et al*., 2005).

Regarding the gene expression pattern of persistent forms, the first molecular studies carried out prior to the sequencing of the whole genome of *C. trachomatis*, were restricted to the expression of MOMP and HSP60. These studies have shown that exposure to IFN-γ of *C. trachomatis* resulted in a down-regulation of MOMP and an up-regulation of HSP60 (Hogan *et al*., 2004).

Since then, several studies have investigated the transcriptional profile of the persistent chlamydial form in different *in vitro* persistence models, showing a complex and inconsistent situation, with only a few genes having a relatively uniform expression pattern (Hogan *et al*., 2004; Goellner *et al*., 2006; Maurer *et al*., 2007; Klos *et al*., 2009; Timms *et al*., 2009; Di Pietro *et al*., 2012).

Table II reports the current state of the art about the transcriptional profile of *in vitro* persistent Chlamydiae. Therefore, without a unique transcriptional profile, it is unlikely that the persistent form could be a genetically-defined state. It has been proposed that it is rather a mid-cycle arrest during the development (Maurer *et al*., 2007). Thus, it could be a complex and flexible metabolic strategy designed to favour a long-time survival in the host cell by evading the immune response, since several genes involved into the energy metabolism are up-regulated during the persistent state and some studies have suggested that Chlamydiae are capable of independently producing energy (Di Pietro *et al*., 2013c).

TABLE II. Comparison of Chlamydiae gene expression patterns during persistence (Di Pietro *et al*., 2013c). * proteomic analysis ** reactivated culture † up-regulated at 12 h p.i.

			Persistence	Genes (+ up regulation; – down regulation; = unchanged)										
Authors	Strain	Cells	Inducer	omp	omc	hctA	hctB	polA	dna	mut	ftsK	ftsW	groE	htrA
Byrne *et al.*, 2001	*C. pneumoniae* TW-183	HEp-2	INF-γ					=	=	=	–	–		
Mathews *et al.*, 2001	*C. pneumoniae* IOL-207	HEp-2	INF-γ	+	=								=	
Nicholson *et al.*, 2002	*C. trachomatis* D	n/a	Penicillin G	=			–		=	+	+	=	=	+
Belland *et al.*, 2003	*C. trachomatis* D	HeLa	INF-γ (24 h)	–	–	–	–	–	–	–	=	–	=	–
Belland *et al.*, 2003	*C. trachomatis* D	HeLa	INF-γ (48 h**)	+	=	+	+	=	–	–	=	–	=	=
Hogan *et al.*, 2003	*C. pneumoniae* TW-183	HEp-2	Continuous infection	+	+						=		=	
Slepenkin *et al.*, 2003	*C. pneumoniae* CM-1	HEp-2	INF-γ	+	+						–		+	
Goellner *et al.*, 2006	*C. psittaci*	HEp-2	INF-γ	–	–†	–						–	–	
Goellner *et al.*, 2006	*C. psittaci*	HEp-2	Iron depletion	=	–	–						–	=	
Goellner *et al.*, 2006	*C. psittaci*	HEp-2	Penicillin G	+	–	–						–	=	
Mukhopadhyay *et al.*, 2006	*C. pneumoniae* A-03	HEp-2	INF-γ*	+	–								+	+
Mukhopadhyay *et al.*, 2006	*C. pneumoniae* A-03	HEp-2	Iron depletion*	+	=								+	+
Polkinghorne *et al.*, 2006	*C. pneumoniae* A-03	HEp-2	INF-γ		+						–	+	–	+
Maurer *et al.*, 2007	*C. pneumoniae* CWL-029	HEp-2	Iron depletion	=	–	=	–	=	=		=	=	=	+
Huston *et al.*, 2008	*C. trachomatis* L2	HEp-2	INF-γ*											–
Huston *et al.*, 2008	*C. trachomatis* L2	HEp-2	Penicillin G*											+
Klos *et al.*, 2009	*C. pneumoniae* CWL-029	HeLa	INF-γ					=			–	–	+	
Klos *et al.*, 2009	*C. pneumoniae* CWL-029	HeLa	Iron depletion					–			=	–	–	
Klos *et al.*, 2009	*C. pneumoniae* CWL-029	HeLa	Penicillin G					–			–	–	–	
Timms *et al.*, 2009	*C. pneumoniae* A-03	HEp-2	INF-γ	–	–		–				–		–	+
Timms *et al.*, 2009	*C. pneumoniae* A-03	HEp-2	Iron depletion	–	–		–				–		–	+
Kokab *et al.*, 2010	*C. trachomatis* E	HEp-2	INF-γ	=							–	+		
Di Pietro *et al.*, 2012	*C. pneumoniae* AR-39	HEp-2	Penicillin G	–		–	–						–	+

Moreover, another unanswered question about chlamydial persistence is whether it could be responsible for the latent chronic chlamydial infections reported *in vivo*. Although, the aberrant persistent forms have been identified even *in vivo*, the link between them and the physiopathology of chronic inflammatory infections is still controversial (Beatty *et al*., 1994; Hogan *et al*., 2004; Wyrick, 2010) mainly because the limitations of current diagnostic methods. In fact, we do not have any effective

methods to quantise persistence forms *in vivo* so we cannot correlate them to the inflammatory response. This is because quantitative real-time PCR (qRT-PCR) cannot discriminate between DNA from replicative and persistent chlamydial forms, and if we wanted to look for mRNA instead we could not have any reliable persistence marker. Immunological methods are useless and transmission electron microscopy (TEM), the gold standard for persistent forms identification, is clearly not suitable for quantisation. However, if it was the case, the immunopathogenic antigen HSP60 could certainly contribute to the chronic inflammation which characterises the latent chronic chlamydial infections and their sequelae.

1.7 The paradox of peptidoglycan

As previously stated, Chlamydiae lack detectable peptidoglycan (PG) and the structural integrity of the cell envelope appears to be provided by a network of disulfide cross-linked proteins associated with the outer membrane. Nevertheless, many *in vitro* studies have described the developmental of chlamydial persistent forms after exposure to beta-lactam antibiotics such as penicillin G, thus suggesting a predominant role of PG in RB development. Indeed, it is well documented that penicillin typically acts by blocking PG biosynthesis through interaction with penicillin-binding proteins.

Although Chlamydiae do not synthesize PG, genome sequencing has revealed that these pathogens appear to contain a nearly complete set of genes encoding the enzymes for PG biosynthesis (McCoy & Maurelli, 2006; Pavelka, 2007), several of which are known to be expressed during the growth cycle. However, the PG biosynthetic pathway does not seem to be complete and some enzymes such as glycosyltransferases, required for the construction of the linear glycan chains (Ghuysen & Goffin, 1999), are missing.

To date several hypothesis regarding the presence of an unusual cell wall in Chlamydiae have been proposed such as that Chlamydiae possess a unique penicillin-sensitive PG that has escaped detection because it contains a carboxylated sugar other than muramic acid, or the chlamydial penicillin-binding proteins have a vital function in chlamydial replication unrelated to the transpeptidation (Moulder, 1993). Two of these hypotheses have gained consensus and support over the years, at least to a certain extent.

The first proposes that Chlamydiae synthesize PG strictly during cell division. In support of this idea, Brown and Rockey (2000) isolated antibodies, directed against a non proteinaceous antigen, called SEP (septum), that recognised an apparent division septum of Chlamydiae. In particular, these antibodies localise to a ring-like structure within or between developing RBs, reminiscent of FtsZ rings observed in most bacteria, whereas in *Escherichia coli* they localise on the entire cell wall, suggesting a structural affinity to the usual PG-containing cell wall.

The second hypothesis even more intriguing suggests that fragments of PG, called muropeptides, are delivered into the host cell cytosol and recognised by the host innate immune system. Consistent with this hypothesis, some studies (Opitz *et al*., 2005; Welter-Stahl *et al*., 2006) have demonstrated the recognition of intracellular Chlamydiae by Nod1 (nucleotide binding oligomerization domain protein), a cytosolic receptor belonging to the PRR family (Pattern Recognition Receptor) of the innate immune system. Nod1 has the ability to detect fragments of PG containing *meso*-diaminopimelic acid (*m*-DAP), and then activate the secretion of pro-inflammatory cytokines.

Chlamydiae, like certain pathogenic bacteria, may employ the type III secretion system to translocate muropeptides directly into host cell cytosol and trigger an

inflammatory response. Regarding *m*-DAP, it is located at the third position of the pentapeptide in the PG of all Gram-negative and some Gram-positive bacteria and it plays a pivotal role in PG biosynthesis by cross-linking PG glycan chains to provide strength and rigidity. Interestingly, a recent study has revealed that Chlamydiae would be able to synthesize *m*-DAP (McCoy *et al*., 2006) through an alternative metabolic pathway characterised initially only in plants and in cyanobacteria (Hudson *et al*., 2006; Hudson *et al*., 2008; Liu Y *et al*., 2010) where *m*-DAP is used for lysine biosynthesis (instead Chlamydiae obtain lysine from the host cell). In particular, the ct390 gene from *C. trachomatis* was shown to encode an L,L-diaminopimelate aminotransferase (L,L-DAP-AT), that is the main enzyme of this pathway because it converts tetrahydrodipicolinate to L,L-diaminopimelic acid, the precursor of *m*-DAP. In addition, the biochemical characterization and crystal structure of *C. trachomatis* L,L-DAP-AT have been recently reported (Watanabe *et al*., 2011). Moreover, the presence of such a gene in Chlamydiae should not surprise because the ancestral Chlamydiae may have participated in the events that led to the formation of the plant lineages, thus they might be related to the cyanobacterium-chloroplast lineage (Everett *et al*., 1999b; Brinkman *et al*., 2002).

In this context, we obtained a relevant result while studying the expression profile of relevant genes for chlamydial metabolism in order to find a reliable marker of persistence in *C. pneumoniae* (Di Pietro *et al*., 2012). We used the *in vitro* model of penicillin G mediated persistence since this persistence model is directly linked to the PG biosynthesis and in addition, it may simulate treatment failure of chronic infections *in vivo*. Notably the cp0259 gene of *C. pneumoniae*, homologous of the ct390 gene of *C. trachomatis,* is up-regulated (Figure 5) during penicillin-induced persistence (Di Pietro *et al*., 2013b), whereas some authors did not find significant up-regulation with other persistence inducers such as iron depletion and interferon γ (Belland *et al*., 2003;

Maurer *et al*., 2007). Thus, it is clear that the up-regulation of the gene encoding L,L-DAP-AT, probably aimed to counteract the effects of the penicillin G, remarks the physiological importance of the synthesis of peptidoglycan or peptidoglycan-like molecules in Chlamydiae.

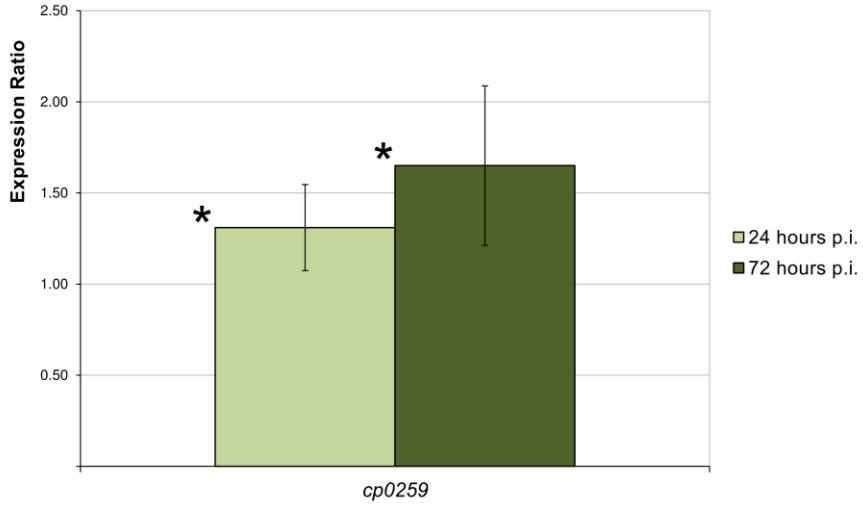

FIGURE 5. *cp0259* expression levels of *C. pneumoniae* AR 39 (ATCC 53592) determined by quantitative reverse transcription PCR (RT-qPCR) at 24 and 72 hours post infection, in cell monolayers (HEp-2 ATCC CCL 23) grown in 24-well culture plates (4.0×10^5 cells/well) infected at MOI 30 and treated with penicillin G 500 U/mL (Di Pietro *et al*., 2013b). The relative expression ratios were normalised with the rRNA 16S gene (Pfaffl's algorithm) and the results are shown as treated / untreated ratio. The sequence of primers used to amplify *cp0259* were as follow (5'-3'): forward ACATTGCCCACATTACAGGAATCCGCG and reverse GCAAGGCGCATGCCAGTAAAGCC (amplicon size 340 bp). * $p < 0.05$

1.8 *Chlamydia*-associated pathologies

To date, three species of Chlamydiae are of medical interest: *C. trachomatis*, *C. pneumoniae* and *C. psittaci*. Notably, despite the relevant set of common features discussed previously, Chlamydiae are actually able to cause a broad spectrum of diseases. *C. trachomatis* serovars A to C lead to trachoma, the leading cause of preventable blindness in developing countries, while serovars D to K are responsible for the majority of sexually transmitted diseases (STDs) all over the world. *C. trachomatis* serovars L1 to L3 of the biovar LGV, have the exclusive ability to pass

through epithelia and then to disseminate to and destroy lymphatic tissue by causing lymphogranuloma venereum (Mabey & Peeling, 2002). *C. pneumoniae* is a widespread respiratory pathogen that causes sinusitis, pharyngitis and pneumonia. The infections are often asymptomatic and the exposure to *C. pneumoniae* is extremely common; epidemiological studies indicate that anti-*C. pneumoniae* antibody prevalence is 50% by the age of 20 and increases with increasing age (Grayston, 2000). *C. psittaci* is the causative agent of psittacosis, a severe zoonotic pneumonia that can be considered as an occupational disease.

More importantly, these pathogens may cause chronic sequelae that according to several authors are due to *in vivo* persistent infections and the consequently induction of a chronic inflammatory state. Indeed, *C. trachomatis* infections may lead to ectopic pregnancy, obstructive infertility, reactive arthritis (Reiter's syndrome) and its involvement has been recently suggested in the pathogenesis of chronic intestinal diseases (Dlugosz *et al*., 2011). *C. pneumoniae* infection may lead to atherosclerosis, although relevant and suggestive this correlation is still a matter of debate (Deniset & Pierce, 2010; Rosenfeld & Campbell, 2011). *C. pneumonaie* has been associated, even though in a weaker manner, also with the exacerbation of chronic obstructive pulmonary disease (COPD) (Papaetis *et al*., 2009), multiple sclerosis (Pawate & Sriram, 2010) and Alzheimer's disease (Shima *et al*., 2010). More recently, *C. pneumoniae* has been linked to the osteoporosis related bone loss (Di Pietro *et al*., 2013a). Lastly, *C. psittaci* infection has been associated with ocular adnexal mucosa-associated lymphoid tissue (MALT) (Collina *et al*., 2012).

Chlamydiae are susceptible to antibiotics that interfere with DNA and protein synthesis, including tetracyclines (such as tetracycline and doxycycline), macrolides (such as erythromycin and azithromycin) and fluoroquinolones (such as ciprofloxacin, ofloxacin and levofloxacin), which are the compounds that have been most extensively

used for treatment of human infections (Hammerschlag & Kohlhof, 2012). Instead, the entire class of beta-lactams is not suitable for Chlamydiae treatment. Nevertheless, as previously discussed, it is not appropriate to say that Chlamydiae are not sensitive to these antibiotics because they are able to induce persistence.

However, despite the treatments available, therapeutic failures have been reported, presumably due to subinhibitory drug concentrations or reduced chlamydial sensitivity. The reduced chlamydial sensitivity has been linked to two phenomena, the chlamydial persistence and the emergence of bacterial resistance mediated by different types of mutations (Sandoz & Rockey, 2010). Thus, it is important to find out new compounds effective against Chlamydiae.

To this regard, medicinal plants represent a potential reservoir of several antimicrobial molecules. In fact, a number of studies, mainly concerning *C. pneumoniae*, have demonstrated antichlamydial activity for a few natural polyphenolic compounds (Yamazaki *et al*., 2003; Alvesalo *et al*., 2006; Salin *et al*., 2010; Salin *et al*., 2011).

1.9 The involvement of *C. pneumoniae* in atherosclerosis

Atherosclerosis, responsible for about 50% of deaths in developed countries, is a disease that affects the intima and the media of large arteries and is characterised by the presence of atheromatous plaques (Ross, 1993). It has been shown that atherosclerosis is a chronic multifactorial inflammatory disease, determined by the interaction between different cell types and effectors such as endothelial cells, smooth muscle cells, monocytes / macrophages and inflammatory mediators. The process of atherogenesis begins in response to the vessel wall damage (according to the response-to-injury hypothesis) by endothelial dysfunction and progresses with the formation of

a plaque, which in turn reduces, in part or completely, the lumen of the artery (Figure 6). This can lead to ischemia of the heart, brain or extremities, thus resulting in infarction (Ross, 1999).

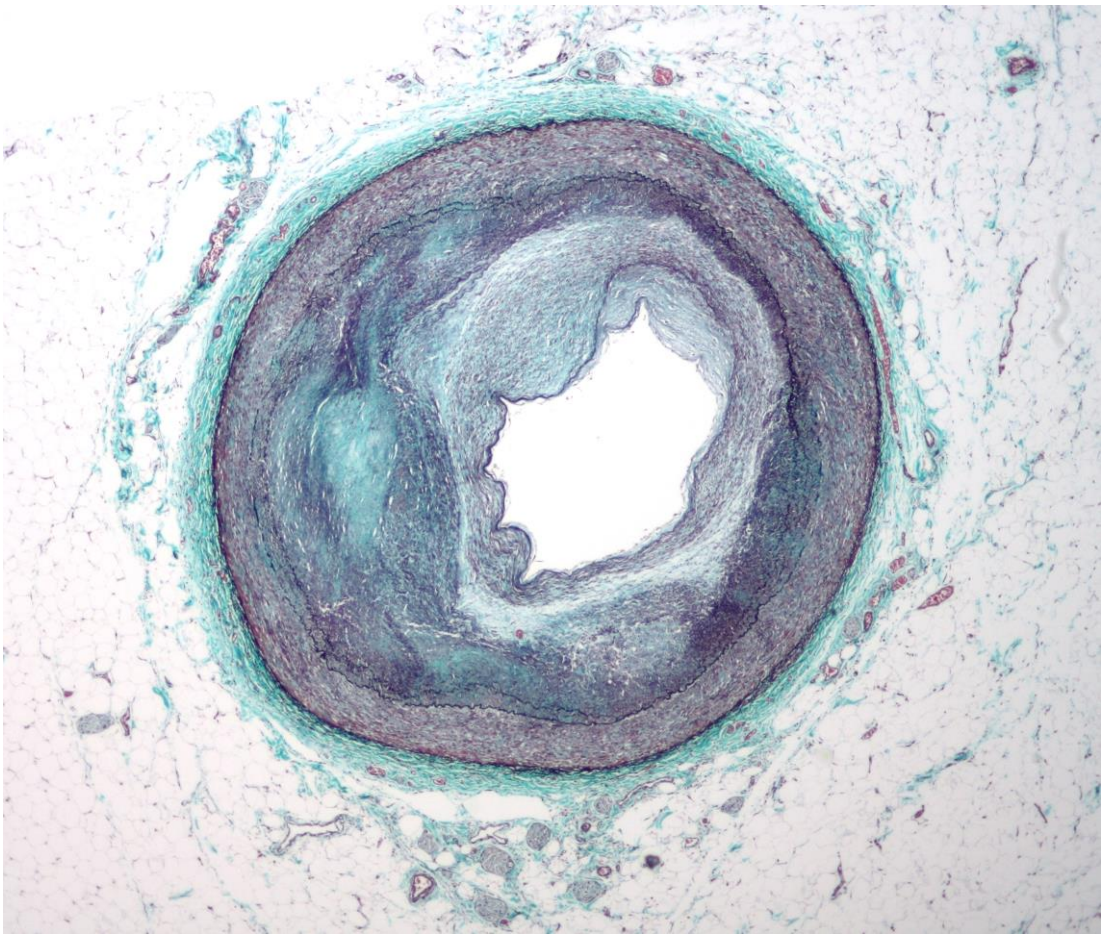

FIGURE 6. Micrograph of an artery (Masson's trichrome stain) that supplies the heart with significant atherosclerosis and marked luminal narrowing (photo by Nephron published on Wikipedia, licensed under the terms of the Creative Commons Attribution License).

The classical risk factors for atherosclerosis, which may account to the endothelial dysfunction, such as dyslipidemia, hypertension, diabetes, tobacco smoking and a sedentary lifestyle, can explain only about 40-50% of the cardiovascular events. For several years, the focus has turned to the possible link

between infection and atherosclerosis, since different experimental evidences have shown that the inflammation characterising the pathogenesis of atherosclerosis has much in common with that induced by microbial chronic infections (Ross, 1999).

For this reason, in addition to the traditional cardiovascular risk factors, in the last three decades, several infectious agents such as cytomegalovirus, *Helicobacter pylori*, periodontal pathogens and *C. pneumoniae* have been implicated in the pathogenesis of atherosclerosis (Campbell & Kuo, 2004; Rosenfeld & Campbell, 2011). C. *pneumoniae* has been considered as the most plausible additional risk factor for atherosclerosis, since it has been isolated in a viable form in the atherosclerotic plaque, and is able to multiply and persist within vascular cells and to induce the chronic inflammatory state underlying atherosclerosis (Schoborg, 2011).

The first suggestion that *C. pneumoniae* may be associated with atherosclerotic cardiovascular diseases (CVDs) was proposed in 1988 by Saikku and colleagues. They showed that patients with acute myocardial infarction and chronic heart disease had more frequently anti-*C. pneumoniae* antibodies than controls. Since then, several studies (cross-sectional, case-control, or retrospective) have confirmed the association between serological evidence of *C. pneumoniae* infection and CVDs although others (prospective studies) have failed to demonstrate such association (Boman & Hammerschlag, 2002; Jha *et al*., 2008; Swierszcz *et al*., 2012; Joshi *et al*., 2013). The main limitation of these studies is the difficulty to identify differences in seropositivity between patients and controls, since a large part of the population has pre-existing IgG antibodies from previous exposure (Dowell *et al*., 2001).

Further evidence that *C. pneumoniae* might play a role in atherosclerosis came from studies by the direct detection of antigens and nucleic acids (Watson & Alp, 2008). In particular, *C. pneumoniae* has been isolated from atherosclerotic plaques in

a viable form (Ramirez, 1996; Jackson *et al*., 1997) and it has been found by several investigators in PBMCs of patients with atherosclerotic cardiovascular diseases (Watson & Alp, 2008) so that *C. pneumoniae* in PBMCs is considered as a marker of current persistent infection. Moreover, *C. pneumoniae* is able to disseminate through the host via the infection of monocytes and macrophages, in which is also able to establish a persistent infection (Arienne *et al*., 1999; Bellmann-Weiler *et al*., 2010). In fact, it has been recently demonstrated that *C. pneumoniae* hides inside apoptotic neutrophils to infect macrophages, so it can propagate further (Rupp *et al*., 2009).

Therefore, in recent years, four large-scale randomised clinical trials (WIZARD, ACES, PROVE IT-TIMI and CLARICOR) have been undertaken to demonstrate long-term benefits in patients with cardiovascular diseases and undergoing antichlamydial antibiotic treatment (O'Connor *et al*., 2003; Cannon *et al*., 2005; Grayston *et al*., 2005; Jespersen *et al*., 2006), but none of them showed a reduction in cardiovascular events. However, the failure of the secondary prevention trials is not enough to rule out a role of *C. pneumoniae* in the pathogenesis of atherosclerosis. In fact, the results of these studies may have been affected by several factors including the refractoriness of chlamydial chronic infections to antibiotic treatment and the enrolment of patients with advanced coronary artery diseases (Yamaguchi *et al*., 2003; Deniset & Pierce, 2010; Rosenfeld & Campbell, 2011). On the other hand, a very recent clinical trial has showed a positive association between the anti-*C. pneumoniae* therapy and the secondary prevention of cardiovascular events (Dogra, 2012).

Nevertheless, we cannot ignore that the association between *C. pneumoniae* and atherosclerosis is also supported by numerous *in vivo* and *in vitro* studies (Belland *et al*., 2004; Watson & Alp, 2008; Sessa *et al*., 2009). Firstly, most of the animal-model studies have demonstrated that *C. pneumoniae* infection could initiate (Fong

et al., 1997) or accelerate the progression of the atherosclerotic lesion (Blessing *et al*., 2001). Moreover, the atherogenic effect appears to be restricted to *C. pneumoniae* because other microorganisms are not able to induce a similar effect (Kalayoglu *et al*., 2002). Secondly, several *in vitro* studies have shown that *C. pneumoniae* infection, as well as promoting the production of pro-inflammatory cytokines and other pro-atherogenic factors, may play a role in all the stages of the atherosclerotic lesion development, from the initial lesion to the plaque rupture and the thrombus formation, which is responsible for the acute cardiovascular event (Watson & Alp, 2008).

In particular, it has been demonstrated that *C. pneumoniae* is able to promote the accumulation of low-density lipoproteins (LDL) into macrophages, at least in part, by affecting the Peroxisome Proliferator-Activated Receptors (PPARs), which are fundamental regulators of macrophage lipid homeostasis (Mei *et al*., 2009; Liu W *et al*., 2010). Furthermore, *C. pneumoniae* has been shown to induce LDL oxidation (oxLDL) by stimulating the production of reactive oxygen species (ROS) (Deby-Dupont *et al*., 2005) so that oxLDL were internalised by macrophage scavenger receptors leading to foam cell formation (Kalayoglu *et al*., 1999).

Infection of monocytes with *C. pneumoniae* increases also the adherence of infected monocytes to endothelial cells. Furthermore, the multiplication of *C. pneumoniae* inside monocytes and macrophages triggers the production of pro-inflammatory cytokines such as IL-1α, IL-6, tumor necrosis factor (TNF)-α and IL-12. Infection of endothelial cells with *C. pneumoniae* promotes the expression of adhesion molecules such as endothelial leukocyte adhesion molecule (ELAM)-1, intercellular cell adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1, chemokines such as monocyte chemoattractant protein (MCP)-1 and cytokines (IL-1, IL-8, TNF-α). It also triggers vascular smooth muscle cells (VSMCs) proliferation through induction of human HSP60 and platelet-derived growth factor (PDGF). There

is also evidence that *C. pneumoniae* stimulates endothelial pro-coagulant activity through IL-6, IL-8 and plasminogen activator inhibitor. Lastly, the stimulation of matrix metalloproteinases (MMPs) may both contribute to the VSMCs migration and to the plaque rupture.

Taken together, the cellular events on vascular wall (as deduced from *in vitro* studies) are intricate and involve several regulatory proteins and enzymes (Munzel *et al*., 2010; Chen & Keany, 2012). These events are summarised in Figure 7 (Di Pietro *et al*., 2013e).

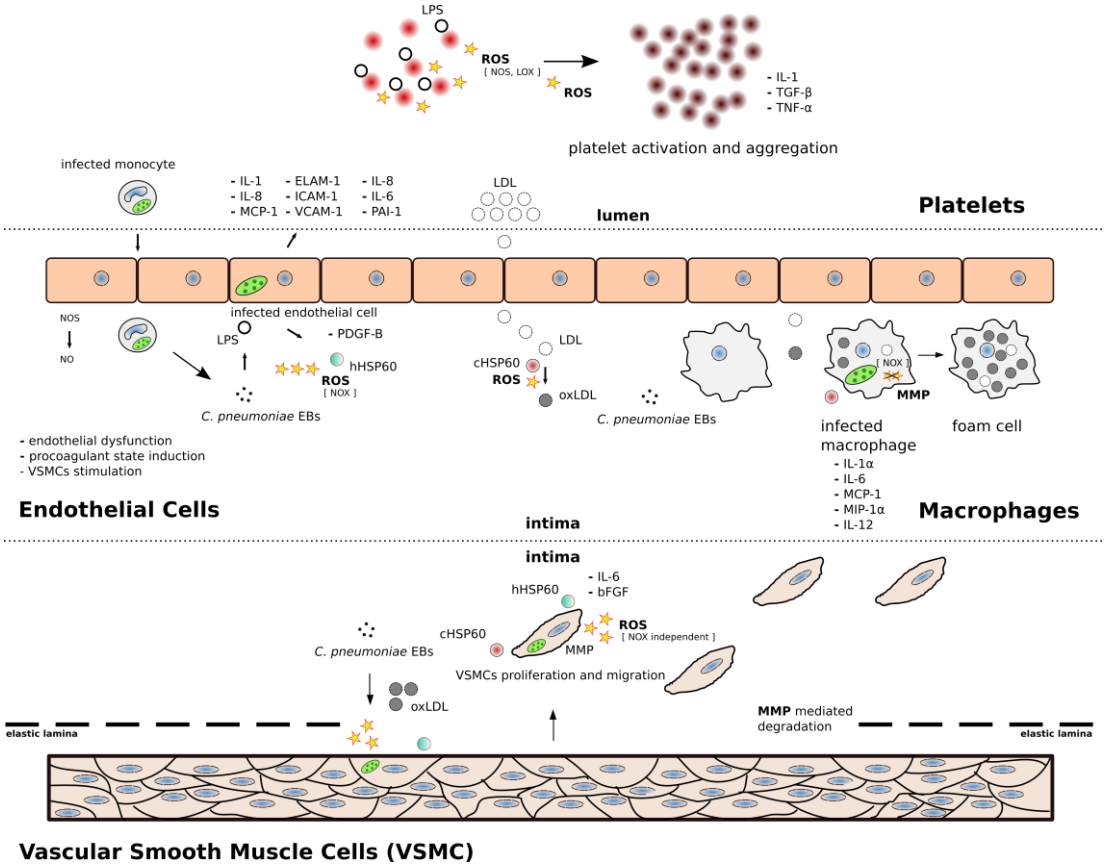

FIGURE 7. Schematic representation of the cellular events linking *C. pneumoniae* infection to the atherosclerotic lesion development (Di Pietro *et al*., 2013e). In platelets, *C. pneumoniae* contributes to platelet activation and aggregation. In endothelial cells, *C. pneumoniae* leads to endothelial dysfunction and induces an increased surface expression of human HSP60 (hHSP60). In macrophages, *C. pneumoniae* induces the oxidation of LDL and the uptake of oxidised LDL (oxLDL), leading to foam cell

formation. Moreover, chlamydial HSP60 (cHSP60) stimulates macrophages to synthetize matrix metalloproteinases (MMPs) which degrade the internal elastic lamina of the endothelial wall. In vascular smooth muscle cells (VSMCs), *C. pneumoniae* enhances cell proliferation and migration, which is consequently facilitated by a compromised internal elastic lamina.

However, we must point out that the first report on the complex profile of acute phase reactant proteins induced by acute respiratory infection of mice with *C. pneumoniae* (Campbell *et al*., 2010) revealed a contradictory situation in which both pro- and anti-inflammatory cytokines are produced. In particular, they evidenced increased plasma levels of IL-2, IL-5, IL-6, IL-10, IL-12 and IFN-γ but they did not find elevated levels of TNF-α, IL-1β and IL-4. In fact, IL-10 has an anti-inflammatory effect that may have inhibited the production of TNF-α. Thus, the actual contribution of the responses to the chlamydial infection to both acute and chronic inflammatory processes remains to be elucidated.

In any case, given the importance of the foam cells in the evolution of the atherosclerotic lesion, researchers have recently focused their attention to antioxidant and anti-inflammatory effectors, such as resveratrol and statins. Resveratrol is a natural polyphenol, more precisely a stilbenoid, produced by several plants. It is found in the skin of red grapes and various fruits but especially in the roots of the Japanese knotweed, from which it is extracted commercially. Statins are a class of drugs commonly used to lower cholesterol levels by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, which plays a pivotal role in the production of cholesterol. These compounds can help the maintenance of lipid homeostasis, as well as counteract the oxidative process involved in the pathogenesis of cardiovascular diseases (Agarwal *et al*., 2012; Xu & Si, 2012; Li *et al*., 2013).

2 AIM OF THE STUDY

Chlamydiae may cause chronic sequelae that according to several authors may be related to *in vivo* persistent forms and the consequently induction of a chronic inflammatory state. *Chlamydia*-associated sequelae are of a great importance for the public health since *C. pneumoniae* infection may be linked to atherosclerosis whereas *C. trachomatis* infection could lead to ectopic pregnancy, obstructive infertility and reactive arthritis. Moreover, chlamydial persistent forms are even difficult to treat with antibiotics. Thus, we investigated *in vitro* different aspects of the pathogenesis of *Chlamydia*-associated chronic diseases, including possible treatments. More specifically, in *C. pneumoniae*, we examined the atherogenic process by setting up a model of foam cell induction by means of macrophages infection. In this model, we studied the production of IL-17A, a cytokine recently reported as proatherogenic (Chen *et al*., 2010), and the protective effects of resveratrol (Di Pietro *et al*., 2013d), a natural polyphenol known to exert antioxidant, cholesterol-lowering and anti-inflammatory effects.

In *C. trachomatis*, we assessed the antichlamydial properties of the essential oil extracted from *Mentha suaveolens* (EOMS) (De Santis *et al*., 2012), a well-known officinal plant, in an effort to find new means for prevention and treatment of urogenital and sexually transmitted infections.

3 MATERIALS AND METHODS

3.1 Chemicals

Resveratrol, Low Density Lipoprotein (LDL), Oil Red O, PPAR-γ antagonist (GW9662), Thiazolyl Blue Tetrazolium Bromide (MTT reagent), Thiobarbituric Acid (TBA) reagent and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Fluorescein isothiocyanate (FITC)-conjugated specific antichlamydial LPS monoclonal antibody (Pathfinder Chlamydia Culture Confirmation System) was from Bio-Rad (Hercules, CA, USA) whereas the antichlamydial MOMP (MicroTrak *Chlamydia trachomatis* Culture Confirmation Test) was from Trinity Biotech (Bray, Co. Wicklow, Ireland). IL-17A ELISA kit was from R&D Systems (Minneapolis, MN, USA).

Mentha suaveolens essential oil (EOMS) was kindly provided by the Department of Chemistry and Drug Technologies, "Sapienza" University, Rome, Italy. It was obtained from wild-type plants grown in Tarquinia forests located around 100 kilometres from Rome. The oil was extracted as previously described (Angiolella *et al*., 2010), then analysed for chemical composition by gas chromatography and mass spectroscopy. The chemical composition analysis revealed that piperitenone oxide (PEO) constitutes 77% of EOMS. Alpha cubebene and alpha pharnesene were also present, among other minor constituents as reported in Table III.

TABLE III. The chemical constituents of the EOMS

Chemical constituents of EOMS			
eucalyptol	4.90%	borneol	0.30%
3-octanol	2.10%	alpha-cubebene	6.00%
para-cymene	1.03%	p-cymenol	0.50%
alpha-pharnesene	6.10%	piperitenone oxide	77.0%
beta-myrcene	0.86%	demelverine	0.60%

3.2 Cell culture, growth and titration of Chlamydiae

C. pneumoniae strain AR-39 (ATCC 53592) was propagated in Hep-2 cells (ATCC CCL-23) in presence of 2 μg/mL cycloheximide. *C. trachomatis* L2 (ATCC-VR-902B) was propagated in HeLa cells (ATCC CCL-2) in presence of 1 μg/mL cycloheximide. Hep-2 and HeLa epithelial cell lines as well as J774A.1 murine macrophages (ATCC TIB-67), were grown in Dulbecco's Modified Eagle's medium (DMEM) with 4.5 g/L glucose supplemented with 2-10% heat-inactivated fetal calf serum (FCS).

Infected cells were harvested after an incubation of either 72 h (*C. pneumoniae*) or 48 h (*C. trachomatis*) at 37 °C and 5% CO_2 by using cell scrapers and vortexed with sterile glass beads for 2 min. After the removal of cell debris by centrifugation at 250 × g for 10 min, the supernatant was centrifugated at 20,000 × g and 4 °C for 20 min and the pellet was resuspended in sucrose-phosphate-glutamate

buffer (SPG buffer) (0.2 M sucrose, 3.8 mM KH_2PO_4, 6.7 mM Na_2HPO_4, 5.5 mM glutamic acid at pH 7.4) and stored at -70 °C in small aliquots until use.

The infectious titre (Inclusion-Forming Units [IFU] per mL) was assessed by immunofluorescence assay. Briefly, fresh cell monolayers grown in 24-well culture plates on glass coverslips, were infected with tenfold serial dilutions of bacterial stock, incubated for 48/72 h, washed twice with phosphate-buffered saline (PBS), fixed for 10 min with methanol, and stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against either chlamydial LPS or *C. trachomatis* MOMP. The total number of IFU was enumerated by counting ten microscope fields using a fluorescence microscope (1000X magnification).

3.3 Cytotoxicity assays

The cytotoxic effect of resveratrol and EOMS was determined by the MTT method (Thiazolyl Blue Tetrazolium Bromide) which measures the activity of living cells via mitochondrial dehydrogenases. Macrophages (1.0×10^4 cells per well) were incubated with resveratrol at various concentrations (0, 25, 50 and 100 µM) for 48 h. HeLa cells (1.0×10^4 cells per well) were incubated with twofold serial dilutions of EOMS (0.5 mg/mL; 0.25 mg/mL; 0.125 mg/mL; 64 µg/mL; 32 µg/mL; 16 µg/mL; 8 µg/mL; 4 µg/mL; 2 µg/mL; 1 µg/mL). 10% in volume of MTT reagent (5 mg/mL) was added to each well and incubated for additional 2 h. Afterwards, the medium was removed and MTT crystals were dissolved with the MTT Solubilisation Solution (0.1 N HCl in isopropanol). The amount of formazan produced was detected by measuring the absorbance at 570 nm (ELISA Reader). The cytotoxic effect of resveratrol and EOMS on cells were expressed as percent cell viability compared to the control cells.

3.4 *C. pneumoniae*, foam cells and resveratrol

3.4.1 Anti-chlamydial assay of resveratrol

To evaluate the antichlamydial effect of resveratrol, J774A.1 murine macrophages (1.0 x 10^5 cells per well) were infected with *C. pneumoniae* at a multiplicity of infection (MOI) of 2 as described above. *C. pneumoniae*-infected macrophages were incubated for 72 h in the presence or absence of 25 μM resveratrol. Then, macrophages were fixed and stained with FITC-conjugated monoclonal antibody against chlamydial LPS as previously described. The percentage of infected cells per coverslip was used to evaluate the infectivity of bacteria.

3.4.2 C. pneumoniae-induced foam cell formation

J774A.1 murine macrophages (1.0 x 10^5 cells per well) were infected with *C. pneumoniae* (MOI = 2), then washed with PBS and cultured for 48 h in the presence or absence of human LDL (100 μg/mL). Foam cell formation was evaluated by Oil Red O staining.

3.4.3 Effects of resveratrol on C. pneumoniae-induced foam cell formation

J774A.1 murine macrophages (1.0 x 10^5 cells per well) were pretreated with 25 μM resveratrol for 1 h at 37 °C and 5% CO_2. Next, cells were washed with PBS, infected with *C. pneumoniae* (MOI = 2), and incubated with LDL (100 μg/mL) in the presence or absence of resveratrol (25 μM) for additional 48 h. Control samples were also provided with uninfected cells and cells treated only with resveratrol.

For experiments with the PPAR-γ antagonist, macrophages were pretreated with GW9662 (10 μM) at 37 °C and 5% CO_2 for 2 h before resveratrol treatment

(25 μM). Macrophages were then infected with *C. pneumoniae* (MOI = 2) and incubated with resveratrol (25 μM), GW9662 (10 μM) and LDL (100 μg/mL) for additional 48 h.

3.4.4 Oil Red O staining

Macrophages were washed twice with PBS, fixed with 10% paraformaldehyde for 10 min, stained with 1% Oil Red O solution (60% isopropanol) for 10 min and then counterstained with hematoxylin for two min. Cells were washed three times with tap water and examined by light microscopy (1000X magnification). The intracellular lipid droplets were stained red whereas cell nuclei were stained blue.

Foam cells, defined as cells with ten Oil Red O-positive lipid droplets or more, were quantified and expressed as percentage of positive Oil Red O cells to total cells.

3.4.5 Lipoprotein oxidation assay

LDL oxidation was quantified by measuring the levels of malondialdehyde (MDA) present as thiobarbituric acid reactive substances (TBARS).

After 48 h hours of incubation at 37 °C and 5% CO_2, cells were collected into microfuge tubes and then centrifugated at 1500 × g for 10 min. 400 μL of supernatant were incubated with 800 μL of TBARS reagent (0.67% thiobarbituric acid in 0.1 M NaOH-trichloroacetic acid, 1:1 ratio), at 95 °C for 30 min. The mixture was cooled and centrifuged at room temperature for 10 min at 500 × g, and the absorbance of the supernatants was measured at 532 nm in a spectrophotometer. Concentrations of LDL oxidation products were determined from a standard curve constructed using malondialdehyde (MDA), which reacts with TBA to yield fluorimetric MDA-TBA adducts. Oxidation values were expressed as nmol MDA/mL.

3.4.6 IL-17A measurement

IL-17A levels in the supernatants stored at -20 °C were measured by using a commercially available ELISA kit according to the manufacturer's instructions.

3.5 Antimicrobial activity of EOMS against *C. trachomatis*

3.5.1 Susceptibility testing after EOMS exposure

Semi-confluent HeLa monolayers (2.0 x 10^5 cells), grown in 24-well (with glass coverslips) culture plates, were infected with *C. trachomatis* at a multiplicity of infection (MOI) of 0.05 by centrifugation at 900 × g at 37 °C for 1 h. The inoculum was then aspired and fresh medium (the infection-medium), with or without EOMS, was added. Infected HeLa monolayers were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. The cell monolayers were stained with a FITC-conjugated monoclonal antibody and the inclusions were counted as described above.

3.5.2 Infectivity yield testing after EOMS exposure

To assess the infectivity of chlamydial elementary bodies after 48 h exposure to EOMS, infected HeLa monolayers grown on 24-well culture plates were disrupted as described above and repassaged onto fresh confluent HeLa monolayers, grown on glass coverslips. The infected cells were incubated at 37 °C for 48 h. The cell monolayers were stained with a FITC-conjugated monoclonal antibody and the inclusions were counted as described above.

3.5.3 Infectivity yield testing after treatment with EOMS during different stages

EOMS was added during one of the following stages in different samples: EBs suspension pretreatment for 2 h at 37 °C before *C. trachomatis* inoculation; EBs suspension pretreatment for 2 h and *C. trachomatis* inoculation for 1 h; *C. trachomatis* inoculation for 1 h; *C. trachomatis* inoculation for 1 h and cell monolayers post-incubation for 48 h; only cell monolayers post-incubation for 48 h. Infected HeLa monolayers were disrupted, repassaged and stained with a FITC-conjugated monoclonal antibody as described above.

3.5.4 Time killing of C. trachomatis EBs by EOMS pretreatment

EBs suspensions of *C. trachomatis* were incubated (pretreatment) for 30, 60 or 120 min at 37 °C in the presence or absence of EOMS at different concentrations before inoculating semi-confluent HeLa monolayers grown in 24-well (with glass coverslips) culture plates, at a MOI of 0.05 as described above. Infected HeLa monolayers were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. The cell monolayers were stained with a FITC-conjugated monoclonal antibody and the inclusions were counted as described above.

3.6 Statistical analysis

Two independent series of experiments were performed in triplicate. The data was given as mean ± standard deviation and the P-value was calculated using the Student's *t* test ($p < 0.05$ was considered significant).

4 RESULTS

4.1 *C. pneumoniae*-induced foam cell formation and the effects of resveratrol

First of all, we performed experiments to examine the cytotoxic effect of this polyphenol on the cells used. Thus, macrophages were incubated for 48 h with resveratrol at various concentrations and cell viability was determined by the MTT test. No significant reduction of cell viability or morphological changes were observed when macrophages were treated with resveratrol at 25 µM, as reported in Figure 8.

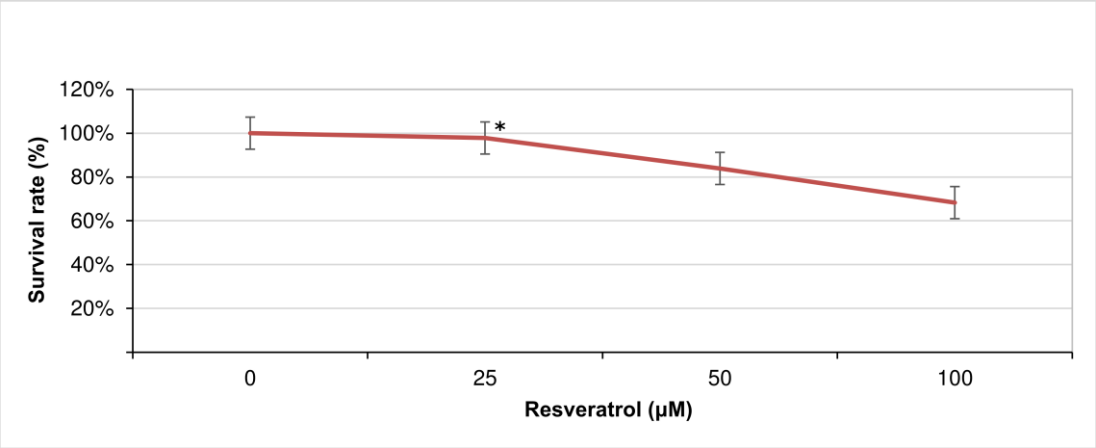

FIGURE 8. Cytotoxicity of resveratrol on J774A.1 murine macrophages. The cell monolayers were treated with different concentrations of resveratrol for 48 h. Cytotoxicity was tested by the MTT method. Results were expressed as percentage of survival. * p >> 0.05

As shown in Figure 9, *C. pneumoniae* infection induced macrophages-derived foam cell formation in the presence of high levels of LDL (100 µg/mL). Compared to uninfected and LDL-treated macrophages, *C. pneumoniae* infection significantly increased the number of Oil Red O-stained lipid droplets ($p = 0.006$). By contrast, the

treatment with 25 µM resveratrol markedly reduced *C. pneumoniae*-induced foam cell formation ($p = 0.03$).

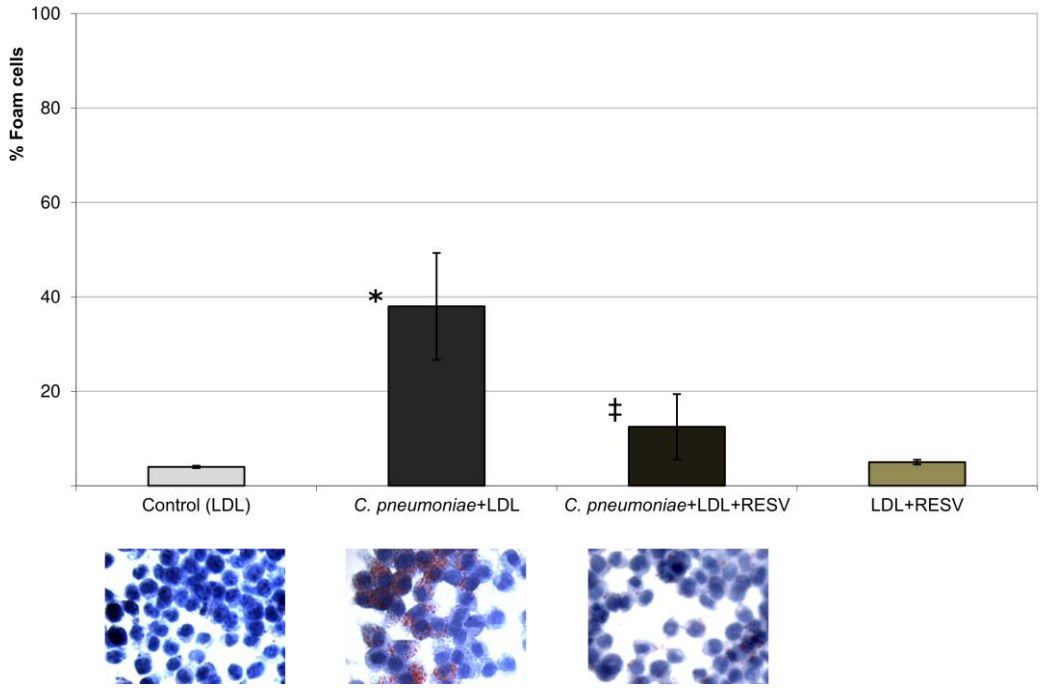

FIGURE 9. Resveratrol reduced *C. pneumoniae*-induced foam cell formation. Foam cells expressed as percentage of positive Oil Red O cells compared to total macrophages. Macrophages stained with Oil Red O to detect lipid content, cellular nuclei counterstained with haematoxylin (400X magnification). * $p < 0.05$ vs control; ‡ $p < 0.05$ vs *C. pneumoniae*/LDL

To determine whether the reduction in the number of foam cells were due to an antichlamydial activity of resveratrol, *C. pneumoniae* infected macrophages were incubated for 72 h in the presence of this polyphenol (25 µM) and the subsequent number of infected cells was determined. There was no significant reduction in the number of infected macrophages treated with resveratrol (75% ± 2.3) as compared to untreated cells (69% ± 3.1).

As previously reported, *C. pneumoniae* induces foam cell formation partly by stimulating LDL oxidation (Kalayoglu *et al*., 1999). As shown in Figure 10, a significantly increased production of TBARS ($p < 0.001$), expressed as MDA, was observed in infected macrophages as compared to uninfected cells. In contrast, in presence of resveratrol, the LDL oxidation was significantly inhibited ($p = 0.008$) as shown by the reduction of MDA levels when compared to untreated cells.

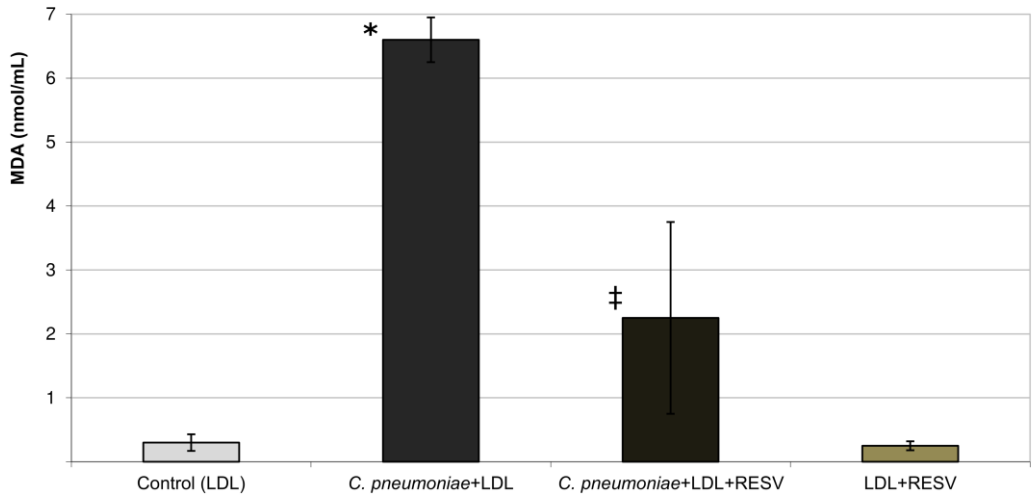

FIGURE 10. Resveratrol (25 µM) reduced LDL oxidation induced by *C. pneumoniae* infection. TBARS produced in culture medium, expressed as nmol MDA/mL, after 48 h incubation with LDL (100 µg/mL), *C. pneumoniae*/LDL, *C. pneumoniae*/LDL/RESV or LDL/RESV. * $p < 0.001$ vs control; ‡ $p < 0.01$ vs *C. pneumoniae*/LDL

It has been also reported, that *C. pneumoniae* induces foam cell formation via a PPAR-γ-dependent pathway (Mei *et al*., 2009). Therefore, we examined whether the inhibitory effects of resveratrol on *C. pneumoniae* induced foam cell formation were dependent upon PPAR-γ. The treatment with the PPAR-γ-specific antagonist, GW9662 (10 µM), significantly diminished ($p < 0.01$) the effect of resveratrol increasing the intracellular lipid accumulation and therefore the foam cell formation (Figure 11).

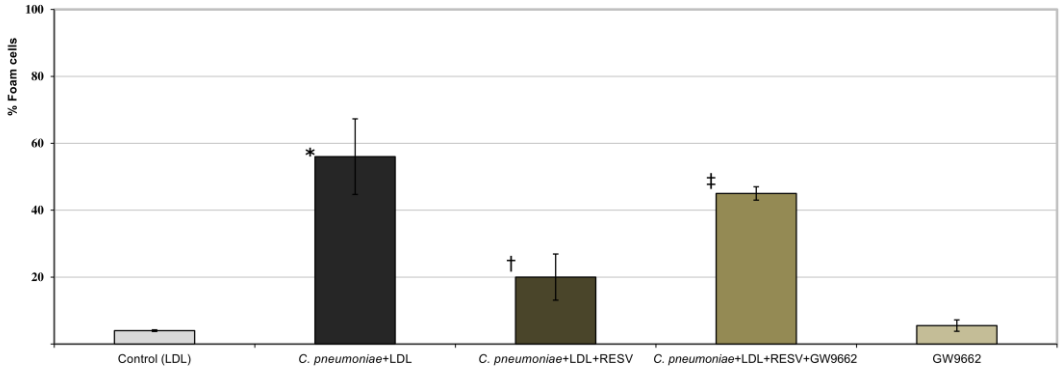

FIGURE 11. Effect of PPAR-y antagonist on resveratrol-mediated inhibition of foam cell formation. Macrophages were pre-treated for 2 h with the PPAR-γ antagonist GW9662 (10 µM), before the addition of resveratrol (25 µM), and then further incubated for 48 h with resveratrol, GW9662 and LDL (100 µg/mL) in the presence or absence of *C. pneumoniae*. Foam cells expressed as percentage of positive Oil Red O cells compared to total macrophages. * $p < 0.01$ vs control; † $p < 0.01$ vs *C. pneumoniae*/LDL; ‡ $p < 0.05$ vs *C. pneumoniae*/LDL/RESV

Moreover, compared to uninfected and LDL-treated macrophages, *C. pneumoniae* infection significantly increased IL-17A production ($p < 0.001$). On the contrary, IL-17A levels were markedly lower in infected macrophages treated with resveratrol ($p < 0.01$) as compared to untreated cells (Figure 12).

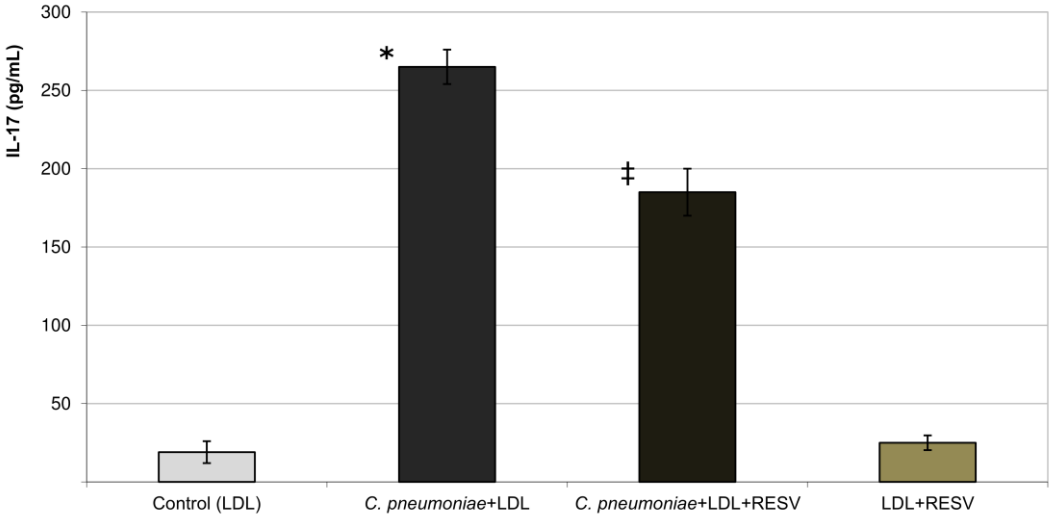

FIGURE 12. Effect of resveratrol (25 µM) on *C. pneumoniae*-induced IL-17A production. IL-17A levels in supernatants of macrophages incubated with LDL (100 µg/mL), *C. pneumoniae*/LDL, *C. pneumoniae*/RESV/LDL or LDL/RESV for 48 h were measured by ELISA assay. * $p < 0.001$ vs control; ‡ $p < 0.01$ vs *C. pneumoniae*/LDL

4.2 Antimicrobial activity of the essential oil of *Mentha suaveolens* against *C. trachomatis*

We started by assaying the inhibitory properties of the essential oil of *Mentha suaveolens* (EOMS) against *C. trachomatis*, by using different concentrations of EOMS (up to 64 µg/mL) according to the results of our cytotoxicity assays (Figure 13).

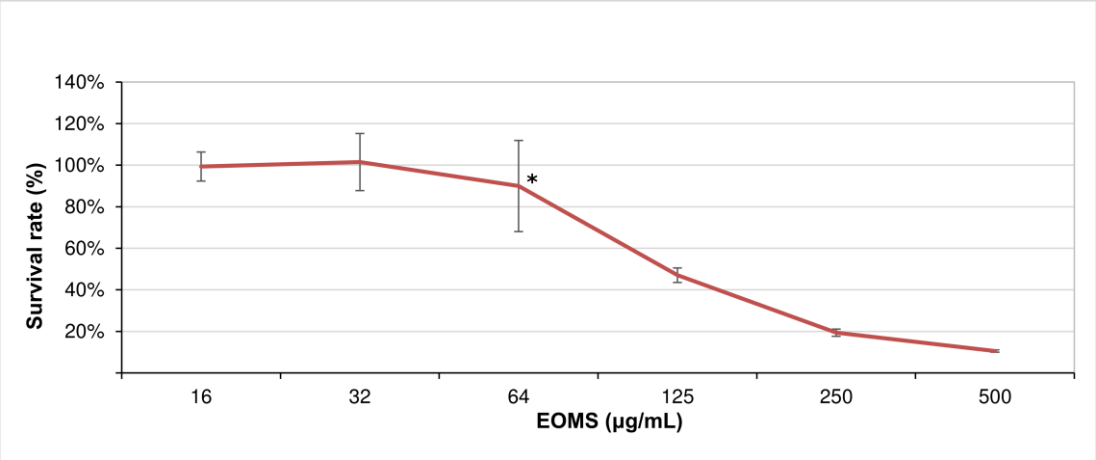

FIGURE 13. Cytotoxicity of EOMS on HeLa cells. The cell monolayers were treated with different concentrations of EOMS for 48 h. Cytotoxicity was tested by the MTT method. Results were expressed as percentage of survival. * p = 0.36

We observed inclusions even at the highest concentration tested (64 µg/mL), so we were not able to determine a MIC value as defined as the lowest concentration at which no inclusion can be seen. However, we obtained a dose-dependent decrease of the average size of the inclusions (Figure 14), without a significant reduction of the number of inclusions (data not shown).

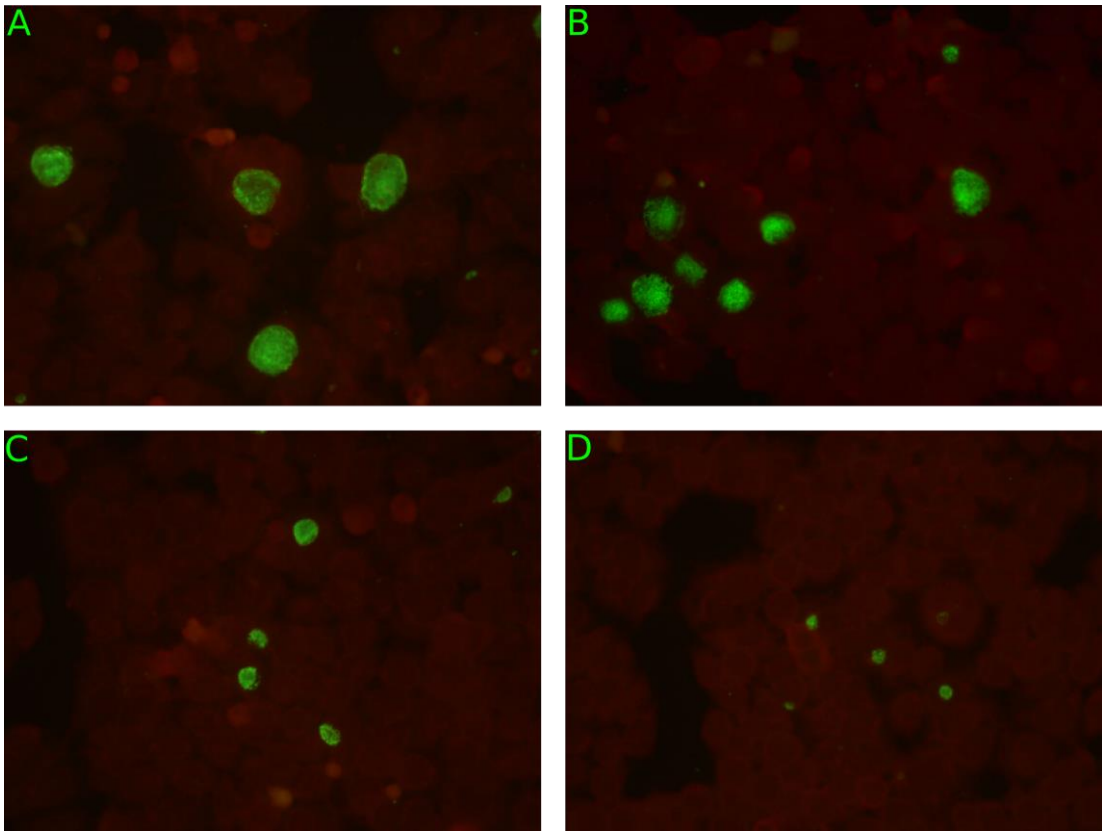

FIGURE 14. FITC-staining of *C. trachomatis* infected HeLa cell monolayers (MOI = 0.05) treated for 48 h with different concentrations of EOMS: (A) untreated, (B) 16 µg/mL, (C) 32 µg/mL, (D) 64 µg/mL (400X magnification).

In order to determine if the EOMS exposure affects the chlamydial infectivity, infected cell monolayers were incubated with different concentrations of EOMS for 48 hours and were repassaged onto fresh cell monolayers. As shown in Figure 15, a statistically significant reduction of infectious EBs was observed in the infected cells treated with 64 µg/mL EOMS as compared to untreated cells ($p = 0.01$).

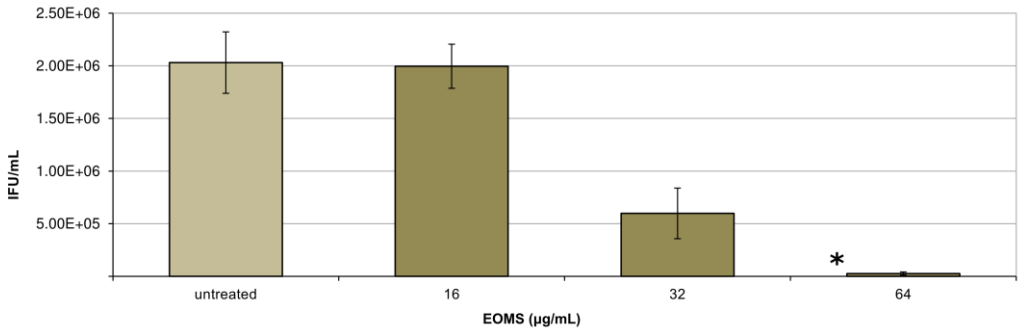

FIGURE 15. Infectivity yield of *C. trachomatis*-infected HeLa cell monolayers (MOI = 0.05) treated with different concentrations of EOMS added during the post-incubation for 48 h. * $p < 0.05$

To investigate in more detail in which stage of the chlamydial development cycle the tested compound is able to exert a biological effect, we challenged infected cell monolayers with 64 μg/mL EOMS added at different stages separately (from the pretreatment to the post-incubation of 48 hours), then we determined the chlamydial infectivity as described above. The results shown in Figure 16 indicated that EOMS can affect chlamydial infectivity only if is present during the post-incubation of 48 hours, at least at these concentrations.

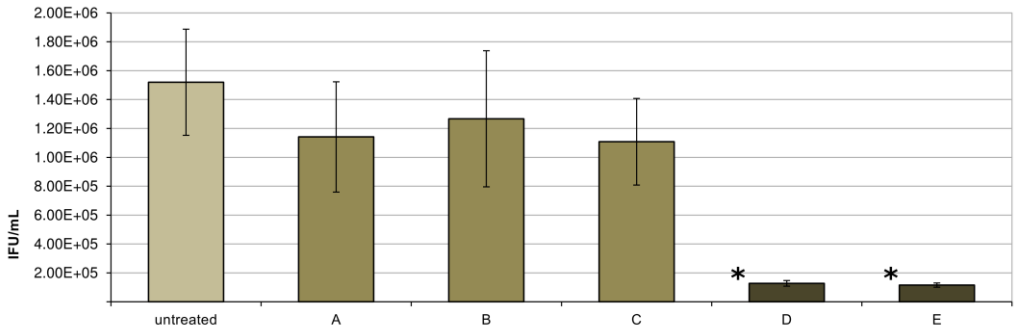

FIGURE 16. Infectivity yield of *C. trachomatis*-infected HeLa cell monolayers (MOI = 0.05) treated with 64 μg/mL EOMS. EOMS was added at different stages: (A) EBs suspension pretreatment for 2 h before *C. trachomatis* inoculation; (B) EBs suspension pretreatment for 2 h and *C. trachomatis* inoculation for 1 h; (C) *C. trachomatis* inoculation for 1 h; (D) *C. trachomatis* inoculation for 1 h and cells post-incubation for 48 h; (E) cells post-incubation for 48 h. * $p < 0.05$ vs untreated sample

By contrast, in a time killing experiment, as shown in Figure 17, we found that after 30 minutes of pretreatment with 2.0 mg/mL EOMS before the cell monolayers were inoculated, chlamydial EBs had completely lost their infectivity. We also observed a significant infectivity loss with 0.5 and 1.0 mg/mL EOMS ($p < 0.05$).

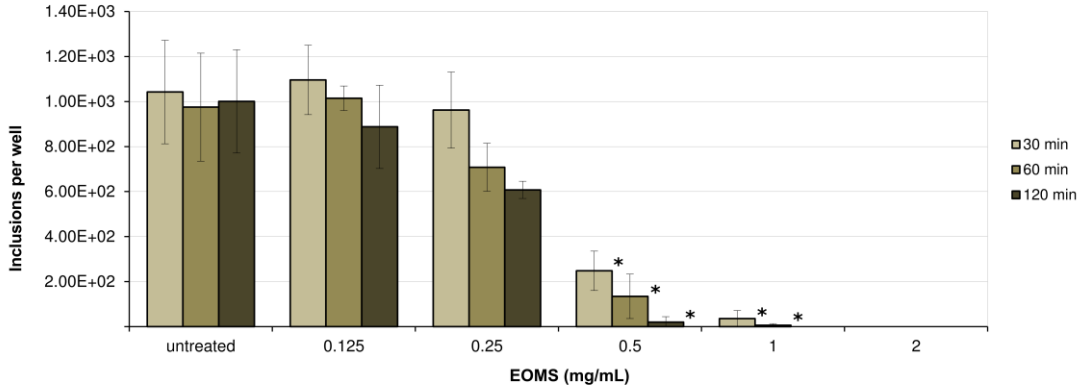

FIGURE 17. Time killing of EBs resuspended in the infection medium added with different concentrations of EOMS. After the incubation time (at 37 °C) the EBs were used to infect cell monolayers grown on glass coverslips. * $p < 0.05$ vs untreated sample

5 DISCUSSION

Chlamydiae, a family of obligate intracellular Gram-negative bacteria, cause a broad spectrum of diseases in humans. *C. pneumoniae* is a common agent of community-acquired pneumonia and other, generally mild, respiratory tract infections, while *C. trachomatis* is the leading cause of sexually transmitted diseases all over the world and of trachoma in developing countries. More importantly, these pathogens may lead to severe chronic sequelae. In fact, *C. pneumoniae* infection have been associated to atherosclerosis, whereas *C. trachomatis* infection may lead to ectopic pregnancy, obstructive infertility and reactive arthritis. These sequelae could result from the inflammatory state induced by persistent chlamydial infections.

In our research, we examined the atherogenic process by setting up a model of foam cells induction by means of macrophages infection with *C. pneumoniae*. In this model, we studied the production of IL-17A, a cytokine recently reported as proatherogenic (Chen *et al*., 2010), and the protective effects of resveratrol (Di Pietro *et al*., 2013d), a natural polyphenol.

Indeed, *C. pneumoniae* association with cardiovascular diseases as well as the involvement of this microorganism in the pathogenesis of atherosclerosis, has been suggested by numerous studies (Belland *et al*., 2004; Watson & Alp, 2008; Sessa *et al*., 2009). *C. pneumoniae* may promote the development and progression of the atherosclerotic process, whose first step consists in the foam cell formation. In more detail, *C. pneumoniae* is well known to play a causative role in the foam cell formation mainly through the stimulation of LDL oxidation as well as the uptake of oxidized LDL by macrophages.

Our results demonstrate for the first time that resveratrol counteracts the *C. pneumoniae*-induced intracellular lipid and cholesterol accumulation as well as the IL-17A production. A relevant decrease in the number of foam cells and IL-17A levels was observed in macrophages infected and treated with resveratrol, as compared to infected but untreated cells.

The decreased production of IL-17A and malondialdehyde observed in our study suggests that resveratrol prevents the inflammatory response and the LDL oxidation induced by *C. pneumoniae*. Several studies have suggested that resveratrol exhibited cardio-protective and anti-inflammatory properties, since this polyphenol has been shown to decrease the synthesis of several cytokines including IL-17A (Lanzilli *et al*., 2012; Li *et al*., 2012) as well as to efficiently contrast the LDL oxidation which contributes to the pathogenesis of atherosclerosis.

It is also well understood that cholesterol efflux through reverse cholesterol transporters (ABCA1 and ABCG1) directly contributes to macrophages transformation into foam cells (Pennings *et al*., 2006; Ye *et al*., 2011). More interestingly, it has been recently reported that *C. pneumoniae* infection down-regulates the ABCA1/ABCG1 expression via PPAR-γ (Mei *et al*., 2009). Our results reveal that the inhibition of PPAR-γ by a specific antagonist (GW9662), in presence of resveratrol and *C. pneumoniae,* enhanced intracellular lipid accumulation and the subsequent foam cell formation. As a matter of fact, GW9662 both simulates *C. pneumoniae* infection and prevents the positive effects of resveratrol by blocking the PPAR-γ pathway.

Particularly intriguing is the effect of resveratrol on IL-17A synthesis. This cytokine was first believed to be exclusively produced by a specialised subclass of T

cells, but it is now known to be secreted by other innate immune cell types including macrophages (Korn *et al*., 2009; Jha *et al*., 2011).

Our results demonstrates, for the first time, the *C. pneumoniae*-induced synthesis of IL-17A by macrophages and provides further evidence on its proatherogenic effect. In fact, in our study, it was observed a decrease in the production of IL-17A following resveratrol treatment of infected macrophages exposed to LDL, along with a lower foam cell formation. These findings are supported by *in vivo* studies, which demonstrated the proatherogenic effects of IL-17A since higher levels of IL-17A were associated with *C. pneumoniae*-induced atherosclerosis (Song & Schindler, 2004; Chen *et al*., 2010). In addition, concerning the proatherogenic properties of IL-17A, a recent study demonstrated that this cytokine activates vascular cells to secrete other cytokines that in turn enhance foam cell formation (Eid *et al*., 2009).

Another aspect of our research was to assess the antichlamydial properties of the essential oil extracted from *Mentha suaveolens* (De Santis *et al*., 2012), a well-known officinal plant widespread in the Mediterranean area. In fact, the emerging phenomenon of drug resistance in *C. trachomatis* as well as persistent chlamydial infections, have raised interest in substances of natural origin as a therapeutic alternative. Many natural phenols have been shown antichlamydial properties *in vitro*, leading to the hypothesis that polyphenols consumed on a daily basis may have prophylactic properties. Moreover, it is well documented the antibacterial activity of essential oils (EOs) from some medicinal plants against a broad range of pathogenic microorganisms (Reichling, 2009).

The essential oil of *Mentha suoveolens* (EOMS) contains a high percentage of oxides such as piperitenone oxide (PEO) and piperitone oxide (PO), terpenic alcohols

(phenol, p-cymen-8-ol, geraniol, terpineol and borneol) and terpenic ketones (pulegone and piperitenone) all of which account for 65% to 90% of the total essential oil.

We demonstrated that EOMS could completely abolish the infectivity of *C. trachomatis* elementary bodies (EBs) if used at concentrations higher than 1 mg/mL. This may lead to applications in topic products for prevention purposes, in this case we can think especially to *C. trachomatis* genital infections.

We also found out that EOMS could affect *C. trachomatis* development cycle, if added to the medium of cell-cultures during the post-incubation period, but without being able to completely eradicate the microorganism, at least if used at the concentrations that can be tolerated by human cells. However, we observed a dose-dependent decrease of the average size of the inclusions. Subsequent infectivity yield tests showed a proportional decrease in chlamydial progeny production.

Thus, we can also hypothesize a possible application of the EOMS as an adjuvant for the antibiotic treatments in order to reduce the risk of developing persistent infections, but this of course requires additional investigations.

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